Willed-movement training reduces middle cerebral artery occlusion-induced motor deficits and improves angiogenesis and survival of cerebral endothelial cells via upregulating hypoxia-inducible factor-1α

ZHIWEN ZHOU[1], XIANG REN[1], WENSHENG ZHOU[1] and LIJUN ZHENG[2]

Departments of [1]Neurology and [2]Rehabilitation, Hunan Provincial People's Hospital, The First-Affiliated Hospital of Hunan Normal University, Changsha, Hunan 410016, P.R. China

Received January 24, 2018; Accepted April 11, 2019

DOI: 10.3892/mmr.2019.10578

Abstract. Willed movement facilitates neurological rehabilitation in patients with stroke. Focal ischaemia is the hallmark of patients after stroke, though the detailed molecular mechanism by which willed movement affects neurological rehabilitation after stroke is not fully understood. The aim of the present study was to dissect the key factors of the hypoxia signaling pathway responsible for the willed movement-improved rehabilitation. Sprague-Dawley rats undergoing right middle cerebral artery occlusion (MCAO) surgery were randomly divided into four groups: MCAO alone, willed movement (WM), environmental modification (EM) and common rehabilitation (CR). The neurological behaviour score was assessed, and infarction areas were detected by TTC staining. In addition, angiogenesis-associated genes (vascular epithelial growth factor, angiogenin-1, matrix metalloproteinases-2 and -9) and hypoxia inducible factor (HIF)-1α expression was investigated in cells derived from MCAO, WM, EM and CR groups. Finally, the role of HIF-1α using HIF-1α knockdown in HUVECs under hypoxic conditions was evaluated. WM significantly improved neurological behaviour and rehabilitation by increasing the behaviour score and by decreasing the infarction area. In addition, CR, EM and WM raised the expression of angiogenesis-associated genes and HIF-1α, thereby promoting *in vitro* tube formation of primary endothelial cells. Knockdown of HIF-1α in HUVECs restored the increased expression of angiogenesis-associated genes to normal levels and inhibited *in vitro* tube formation of HUVECs. Willed movement most effectively improved the neurological rehabilitation of rats with focal ischaemia through upregulation of HIF-1α. The present findings provide insight into willed movement-facilitated rehabilitation and may help treat stroke-triggered motor deficit and improve angiogenesis of cerebral endothelial cells.

Correspondence to: Dr Lijun Zheng, Department of Rehabilitation, Hunan Provincial People's Hospital, The First-Affiliated Hospital of Hunan Normal University, 89 Guhan Road, Changsha, Hunan 410016, P.R. China
E-mail: zhenglijun171211@sina.com

Key words: willed movement, ischaemic stroke, angiogenesis, cerebral endothelial cells

Introduction

Stroke is a leading cause of mortality and adult disability worldwide (1). In an ischaemic stroke, blood supply to the brain is notably decreased, resulting in dysfunction of the brain (2). However, few advances in stroke therapy have been made to further improve patient outcomes. The post-stroke angiogenesis of brain endothelial cells is important for rehabilitation (3-5).

Motor skill training, including willed movement, can lead to structural and functional plasticity of neurons in the motor cortex, which will enhance brain health and motor function (6). Willed movement, a type of voluntary motor training, is characterized by serving attention to a goal and making efforts to achieve it (7). During willed movement therapy, food motivation is always utilized to induce activities of the body and limbs. Willed movement training is capable of inducing long-term depression in rats with focal cerebral ischaemia in a protein interacting with C kinase (PICK)1-dependent manner (8). In addition, willed movement training can reduce brain damage after stroke through upregulation of synaptic plasticity-associated genes (8-10).

Ischaemic stroke results from the lack of blood supply to the brain, which can lead to brain dysfunction (11). Hypoxia-inducible factor-1 (HIF-1) is essential for the adaptive response of cells under hypoxic conditions by transcriptionally activating its downstream target genes (such as vascular epithelial growth factor (VEGF), matrix metalloproteinase (MMP)s, Flt-1, angiogenin (Ang)-1), which are implicated in many diseases, including stroke (12,13). HIF-1 is a heterodimer comprised of O_2-regulated HIF-1α and constitutively expressing HIF-1β, and it binds to hypoxia-responsive elements upstream of hypoxia-regulated genes (14,15). The HIF-1 signaling pathway improves the blood supply to cerebral tissue and reduces hypoxia-induced ischaemic injury (16).

Notably, hypoxia can induce cell migration and endothelial tube formation of phenotypic processes in angiogenesis, which therefore facilitates the rehabilitation of patients with focal cerebral ischaemia (17,18). Thus, HIF-1α induction and accumulation is a highly promising therapeutic strategy for cerebral ischaemia.

In the present study, the effect of willed movement on the motor deficit, cell proliferation, and angiogenesis of rat brains with focal ischaemia was investigated. The present results revealed that HIF-1α transcriptional activity is essential for willed movement to alleviate hypoxia injury. In summary, the present study demonstrated that HIF-1α is required for willed movement to contribute to the cerebral rehabilitation of rats after ischaemic stroke and provides a novel strategy for treating patients after ischaemic stroke by accumulating HIF-1α.

Materials and methods

Animals. Male Sprague-Dawley rats (n=122, weight, 250-280 g; age, 60 days) were purchased from the Experimental Animal Centre of Central South University and were employed in the present study. These rats were housed in cages at room temperature and had free access to food and water with 12-h dark/light cycles. All experiments were performed according to guidelines for the care and use of animals and were approved by the animal ethics committees of Central South University.

MCAO model. Focal cerebral ischaemia was induced by middle cerebral artery occlusion (MCAO) according to Hirayama and Koizumi (19). In brief, rats were anesthetized by intraperitoneal injection with 10% chloral hydrate (300 mg/kg body weight). A mid-line incision in the neck of the rat was made to expose the common and external carotid arteries and ligated them. Specifically, the internal carotid artery was closed by an artery clamp. Subsequently, a 6-0 silicon rubber-coated nylon monofilament (RWD Life Science Co., Ltd.) was inserted into the right internal carotid artery. Insertion was stopped after occlusion for 90 min. The animals were reanaesthetized and reperfused by withdrawal of the monofilament. After surgery, the rats were kept for ~2 h in a warm box heated by lamps to maintain body temperature. Finally, the common carotid artery was permanently ligated, and the neck wound was closed.

Training design. All rats with neurological deficits were randomly divided into the following four groups 24 h post-MCAO: i) MCAO, where rats were housed in standard cages after MCAO (n=6); ii) willed movement (WM), where rats were housed in a ladder-containing cage 24 h after MCAO surgery and had to climb the ladder or walls of the apparatus to reach food and water (n=6); iii) environmental modification (EM), where rats were housed in the same environment as the WM group but could get water and food without climbing the ladder (n=6); iv) common rehabilitation (CR), where rats were housed in the rolling-grid training cage 24 h after MCAO surgery and were trained twice a day (9:00 am and 4:00 pm) for 15 min each time (n=6).

Neurobehavioural assessment. Neurological evaluation was performed on days 0, 3, 7, 15 and 30 after excision using rats with a modified neurological deficit score (20). The rats were trained for 15 days before sacrifice, and those that died during training were excluded. Specifically, A total of 50 rats did not recover from anaesthesia or died after the MCAO operation. All rats were randomly allocated to groups for training, as aforementioned. Neurological scores were defined as follows: 0, no deficit; 1, flexion of the contralateral forelimb upon lifting the entire animal by the tail; 2, decrease in thrust towards the contralateral plane; and 3, circling to the contralateral side.

Triphenyltetrazolium chloride (TTC) staining. The cerebral infarction area was examined in six animals per group (n=24 for each replicate, the experiments were performed three times) for 15 days post-training. Rats were anaesthetized by intraperitoneal injection with 1% sodium pentobarbital (40 mg/kg) and decapitated. The whole brains were frozen at -20˚C for 20 min. The tissues were cut into 2-mm-thick coronal sections and then stained with 2% TTC solution at 37˚C for 30 min. The stained slides were immersed with 4% paraformaldehyde at 4˚C overnight. The infarct area on each TTC-stained section was measured using ImageTool 2.0 software (University of Texas Health Science Center) and calculated as the infarct area x thickness (2 mm).

Cell lines and primary cerebral endothelial cell isolation. Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection and grown in vascular cell basal medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 5 ng/ml VEGF (Sigma-Aldrich; Merck KGaA) at 37˚C, 5% CO_2. For primary cerebral endothelial cell isolation (3), brains from rats of each group 30 days post-training were removed and then stored in Dulbecco's Modified Eagle's Medium/F12 (Gibco; Thermo Fisher Scientific, Inc.) until further use. The tissues were washed with PBS several times and minced and incubated with Liberase Blendzyme 2 (Roche Diagnostics GmbH) at a concentration of 0.625 Wu/ml at 37˚C for 2 h. Subsequently, the cell suspension was washed with PBS and centrifuged at 228 x g for 5 min at 25˚C. Subsequently, the cells were plated in dishes coated with collagen type I (BD Biosciences) and grown in Endothelial Cell Growth Basal Medium (EBM) complete medium (Lonza Group Ltd). Cells were cultured at 37˚C, 5% CO_2. After 24 h, non-adherent cells were removed and seeded into new collagen type I-coated dish with EBM media. The cultured cells were passaged at a ratio of 1:4 every 14 days. To mimic focal ischemia *in vitro*, HUVEC were treated with 500 μM $CoCl_2$ (Shanghai Aladdin Biochemical Technology Co., Ltd.) treatment for 24 h.

Cell proliferation assay. The isolated primary cerebral endothelial cells from all four groups were examined via MTT analysis. The cells were counted and plated into 96-well plates for 24 h at 37˚C. Then 0.1 mg/ml MTT was added to cells at 37˚C for 3 h. The cells were lysed in DMSO at room temperature for 30 min. Finally, the absorbance was measured at 490 nm by a microplate reader (Bio-Tek China).

In vitro vascular tube formation assay. To examine the ability of endothelial tube formation *in vitro*, 24-well plates were coated with 10 μl of Matrigel (BD Biosciences) as previously described (21) and $2x10^4$ HUVECs or primary cerebral endothelial cell were seeded. The tube length was measured using

MetaMorph Microscopy Automation and Image Analysis Software (version 1.0; Molecular Devices, LLC). The images were taken and processed by an inverted microscope. In total, >5 randomly selected fields of view were analyzed.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol (Thermo Fisher Scientific, Inc.). Briefly, the cells were lysed with TRIzol buffer, and then 200 μl chloroform (100%) was added to the mixture, which was vortexed for 15 sec at 25˚C. The resulting solution was centrifuged at 17,366 x g for 15 min at 4˚C. The supernatant was harvested and mixed with an equivalent volume of isopropanol. The resultant mixture was centrifuged at 17,366 x g for 10 min at 4˚C. The supernatant was removed and 75% ethanol was added to wash the pellet and centrifuged at 17,366 x g for 10 min at 4˚C. Ethanol was discarded and the pellet was left to dry, then used 20-30 μl DEPC-treated H_2O (Invitrogen; Thermo Fisher Scientific, Inc.) to elute the RNA pellet.

Total RNA (1 μg) was used to perform reverse transcription according to the manufacturer's protocol (PrimeScript Kit; Takara Bio, Inc.). For quantitative PCR, SYBR Green (QuantiFast SYBR Green PCR Kit; Qiagen GmbH) was used as the probe dye and the signal was detected according to the manufacturer's protocol. GAPDH was used as the internal control. The following primers were used: HIF-1α-Forward, CGCAAGTCCTCAAAGCACAG and HIF-1α-Reverse, TCT GTTTGGTGAGGCTGTCC; VEGF-Forward, TTTCTG CTGTCTTGGGTGCA and VEGF-Reverse, CCAGGG TCTCGATTGGATGG; Ang-1-Forward, GCCTGATCT TACACGGTGCT and Ang-1-Reverse, GCCTGATCTTAC ACGGTGCT; MMP2-Forward, GATCTACTCAGCCAG CACCC and MMP2-Reverse, ACGACGGCATCCAGG TTATC; MMP9-Forward, TACTCGACCTGTACCAGC GA and MMP9-Reverse, ATGCCATTCACGTCGTCCTT; GAPDH-Forward, GAGTCAACGGATTTGGTCGT and GAPDH-Reverse, TTGATTTTGGAGGGATCTCG. The thermocycling conditions were the following: Initial PCR activation step at 95˚C for 15 min, followed by 40 cycles of denaturation at 94˚C for 15 sec, annealing at 53˚C for 30 sec, and elongation at 72˚C for 30 sec. Cq values were used for quantification using the $2^{-\Delta\Delta Cq}$ method (22).

Western blotting. In total, $1x10^6$ cells of each group were harvested and washed with 1xPBS once. Subsequently, 2xSDS loading buffer (Dalian Meilun Biotech Co., Ltd.) was used to lyse cells. The lysates were boiled at 95˚C for 10 min and centrifuged at 16,172 x g for 1 min at 4˚C. Approximately 50 μg of total protein was loaded onto a 10% SDS-polyacrylamide gel and resolved at 120 V for 0.5-1 h at 25˚C. Subsequently, proteins were transferred onto polyvinylidene fluoride membranes at 300 mA for 2-3 h at 4˚C. The membrane was blocked with 5% non-fat milk in 1xTBS with 0.1% Tween (TBST) for 1 h at 25˚C, then incubated with the corresponding primary antibodies at 4˚C overnight. The following day, the membrane was washed with 1xTBST 3 times and incubated with secondary antibodies at room temperature for 1 h. Finally, the membrane was incubated with enhanced chemiluminescence reagent (7Sea Biotech) and exposed using the Bio-Rad ChemiDoc Touch Imaging System. The following antibodies were used in the present study: Anti-HIF-1α (1:1,000; cat. no. ab51608; Abcam), anti-VEGF (1:1,000; cat. no. ab52917; Abcam), anti-Ang-1 (1:1,000; cat. no. ab95230; Abcam), anti-MMP-2 (1:1,000; cat. no. 87809; Cell Signaling Technology), anti-MMP-9 (1:1,000; cat. no. 3852; Cell Signaling Technology Inc.). The secondary antibody horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (cat. no. A0208; 1:5,000; Beyotime Institute of Biotechnology) was incubated with the membranes at room temperature for 1 h.

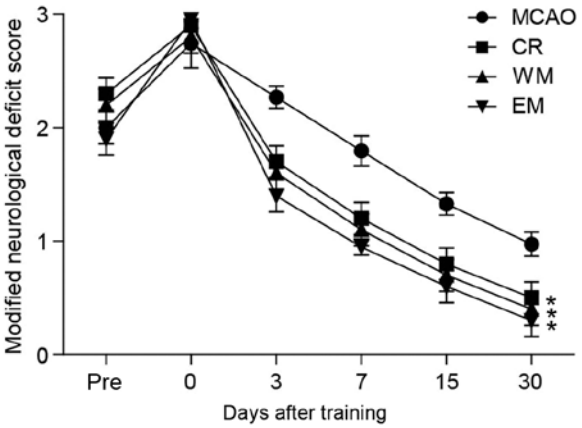

Figure 1. Willed movement reduces motor deficits. Neurological defect scores were examined in MCAO and exercise groups prior to training and following 30 days of exercise. Statistically significant differences were observed in all exercise groups compared with the MCAO group. *P<0.05 vs. MCAO group. No statistically significant differences between the three exercise groups were found. CR, common rehabilitation; EM, environmental modification; MCAO, middle cerebral artery occlusion; Pre, 2 h following MCAO surgery; WM, willed movement.

Small interference (si)RNA transfection. SiRNAs (scrambled and siHIF-1α) were synthesized by GenePharma and diluted to 20 μM as stock. To transfect cells, siRNAs were diluted to 50 nM and were mixed with RNAiMAX reagent (Thermo Fisher Scientific, Inc.) for 20 min at 25˚C, and the mixture was added to cultured cells. The sequence of siHIF-1α was GCT CCCAATGTCGGAGTTT. Cells were harvested 48 h after transfection.

Statistical analysis. GraphPad Prism (version 6.0; GraphPad Software, Inc.) was used for statistical analysis. Each experiment was performed three times, and all values are presented as the mean ± standard deviation. Comparison of two groups was performed using the two-tailed unpaired Student's t-test. Comparison of multiple groups was performed using one-way analysis of variance followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

CR, WM and EM reduce motor deficit. The neurological defect score in the MCAO, CR, WM and EM groups was measured first (n=6 per group). The results showed that rats in the CR, WM and EM groups exhibited a significantly lower score compared with the MCAO group. However, the groups CR, WM, and EM did not display significant differences from each other (Fig. 1).

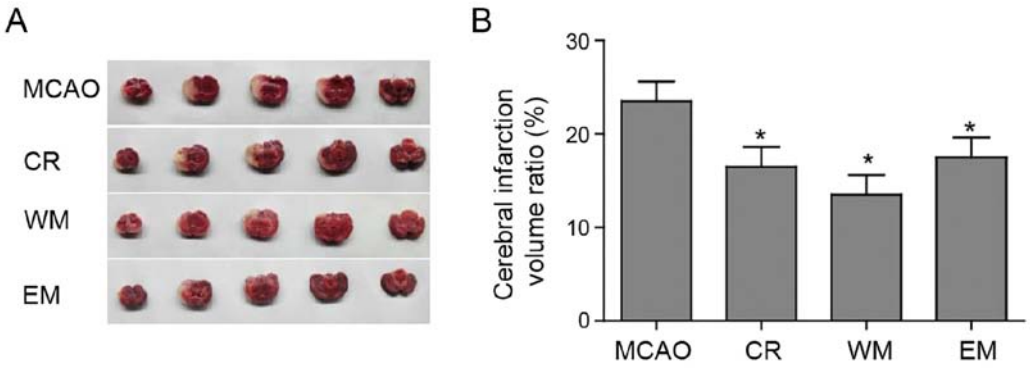

Figure 2. Willed movement improves neurological rehabilitation of brains with ischaemia. (A) TTC staining of ischaemic brains in four groups of rats. Representative different sections per group are shown. Infarcted areas are shown in white, and normal tissue in red. (B) Statistical analysis of the relative infarct volume in the four groups. The data are presented as the mean ± standard deviation *P<0.05 vs. MCAO group. CR, common rehabilitation; EM, environmental modification; MCAO, middle cerebral artery occlusion; WM, willed movement.

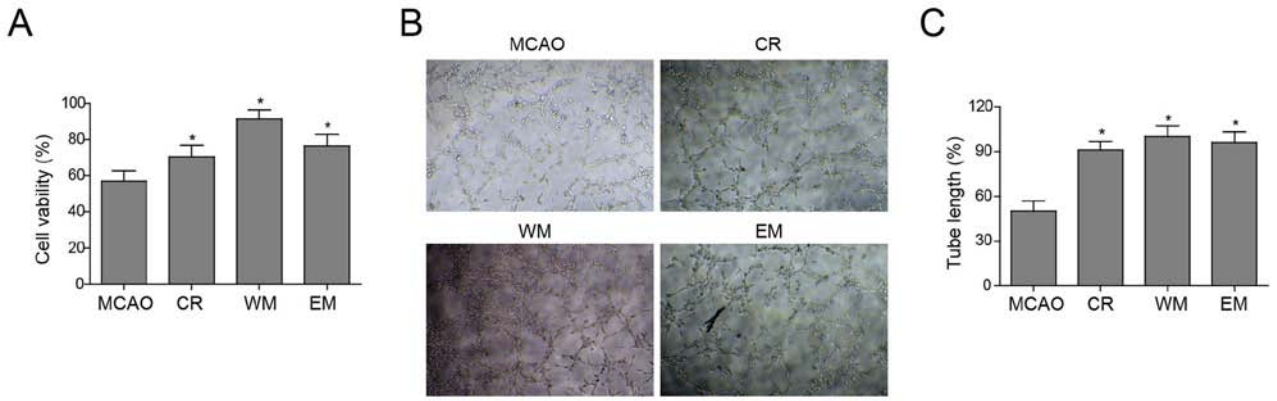

Figure 3. Willed movement promotes cell survival and angiogenesis of ischaemic endothelial cells. (A) Cell viability of primary cerebral vascular endothelial cells isolated from all four groups of rats was examined by MTT assay. (B) *In vitro* vascular tube formation assay of primary cerebral vascular endothelial cells isolated from all four groups of rats. (C) Quantification of tube length formed by all four groups of primary cerebral vascular endothelial cells. *P<0.05 vs. MCAO group. CR, common rehabilitation; EM, environmental modification; MCAO, middle cerebral artery occlusion; WM, willed movement.

Willed movement improves neurological rehabilitation of brains with ischaemia. To investigate whether willed movement therapy decreased the focal ischaemia damage, TTC staining of cerebral tissue was performed to assess the infarctions. Representative tissues correspond to different coronary sections per group as presented in Fig. 2A. The unstained areas in Fig. 2A are infarcted tissues, and the red areas are normal tissues (Fig. 2A). In the four groups, different volumes of infarcted tissue areas were observed. Compared with the MCAO group, the CR, WM and EM groups displayed statistically significant decreases in the volume of cerebral infarcted tissue (Fig. 2B). The volume of cerebral infarction tissue in the WM group was not significantly lower than the other two training groups (Fig. 2B). In addition, there was no significant difference between CR and EM group.

Willed movement promotes cell survival and angiogenesis of ischaemic endothelial cells. To investigate the molecular mechanism by which the three forms of exercise improved neurological rehabilitation, primary cerebral endothelial cells were isolated from rats subjected to CR, WM and EM and cultured. Subsequently, the MTT method was performed to examine cell viability in the MCAO, CR, WM and EM groups. The endothelial cells of the MCAO group showed the lowest viability (Fig. 3A).

To investigate whether these therapies improved tube formation of cerebral endothelial cells, cells of the CR, WM and EM groups possessed a stronger ability for tube formation (evaluated by total tube length) *in vitro* compared with the MCAO group. Cells of the CR, WM and EM groups showed no significant differences from each other (Fig. 3B). The tube length of vessels formed in the MCAO group was half of that in the other three groups, while no length disparity appeared between the WM, EM and CR groups (Fig. 3C). Taken together, these results demonstrated that CR, EM, and WM increased cell viability and enhanced angiogenesis of cerebral endothelial cells compared with the MCAO group.

As angiogenesis has been associated with some classical genes (12), and angiogenesis-associated gene expression levels in the MCAO, CR, WM and EM groups were investigated next. RT-qPCR was employed to detect the mRNA expression levels of VEGF, Ang-1, MMP-2 and MMP-9 in cerebral endothelial cells of all four groups. The CR, EM and WM groups showed elevated mRNA expression levels of all four genes compared with MCAO (Fig. 4A). Additionally, the protein expression levels of these four angiogenesis genes were also investigated.

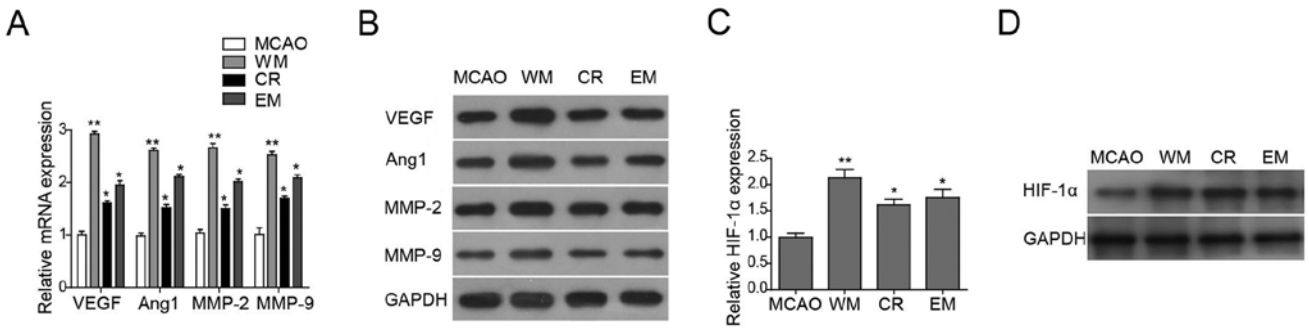

Figure 4. Willed movement upregulates HIF-1α and angiogenesis-associated genes. (A) RT-qPCR analysis and (B) western blot analysis of angiogenesis-associated genes in primary cerebral vascular endothelial cells isolated from all four groups of rats. (C) RT-qPCR analysis of HIF-1α and (D) western blot analysis of HIF-1α in primary cerebral vascular endothelial cells isolated from all four groups of rats. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. MCAO. Ang, angiogenin; CR, common rehabilitation; EM, environmental modification; HIF, hypoxia-inducible factor; MCAO, middle cerebral artery occlusion; MMP, matrix metalloproteinase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; VEGF, vascular endothelial growth factor; WM, willed movement.

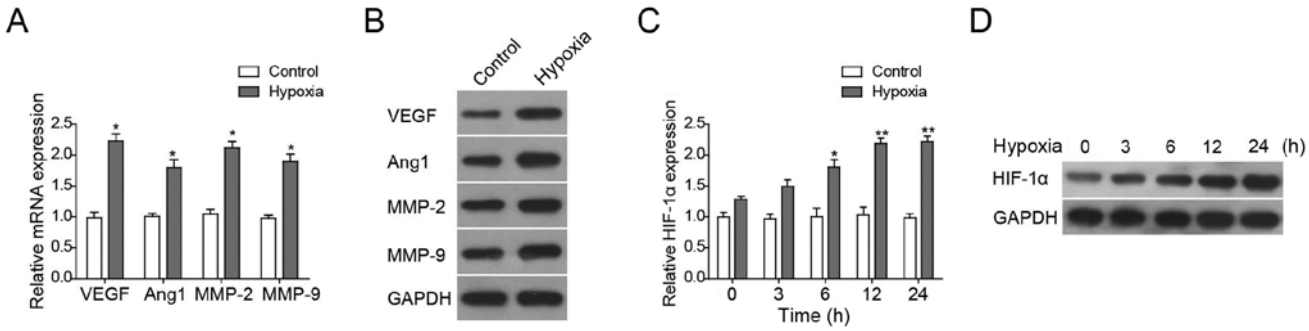

Figure 5. Hypoxia induces angiogenesis-associated genes and HIF-1α. (A) RT-qPCR analysis and (B) western blot analysis of angiogenesis-associated molecules in HUVECs under normoxic and hypoxic conditions. (C) RT-qPCR analysis and (D) western blot analysis of HIF-1α in HUVECs under normoxic and hypoxic conditions. All data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. MCAO. Ang, angiogenin; CR, common rehabilitation; EM, environmental modification; HIF, hypoxia-inducible factor; MCAO, middle cerebral artery occlusion; MMP, matrix metalloproteinase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; VEGF, vascular endothelial growth factor; WM, willed movement.

Notably, the protein expression levels of these genes only increased in the WM group compared with the MCAO group, whereas the CR and EM groups showed no difference in their expression levels (Fig. 4B), suggesting that the protein expression level was regulated at the post-transcriptional level.

Willed movement upregulates HIF-1α. HIF-1α has an important role in angiogenesis and endothelial cell survival (18). The expression levels of HIF-1α in endothelial cells of the MCAO, CR, WM and EM groups were investigated. RT-qPCR and western blot analyses were performed to determine the HIF-1α mRNA and protein expression levels, respectively. HIF-1α mRNA and protein expression levels were higher in endothelial cells of the CR, WM and EM groups compared with the MCAO (Fig. 4C and D). In conclusion, the present study suggested that CR, WM and EM may promote cell proliferation and angiogenesis and upregulation of angiogenesis-associated genes and HIF-1α. However, the direct effects of CR, WM and EM on angiogenesis and cell proliferation require further investigation.

Hypoxia induces angiogenesis-associated genes and HIF-1α expression. To mimic focal ischaemia *in vitro*, we generated a HUVEC-based hypoxia model using $CoCl_2$ treatment in culture. First, whether the expression of VEGF, Ang-1, MMP-2 and MMP-9 was affected in hypoxic cells was investigated by RT-qPCR and western blot analysis. It was found that these genes were induced by hypoxia (Fig. 5A and B). HIF-1α was gradually upregulated when cells were treated by hypoxia and peaked at 12 h after hypoxia treatment (Fig. 5C and D). Taken together, the present results suggested that hypoxia upregulated the expression levels of HIF-1α and its downstream genes.

HIF-1α is necessary for hypoxia-induced angiogenesis of HUVECs. To confirm the role of HIF-1α in angiogenesis of cerebral endothelial cells, HIF-1α-knockdown HUVECs using siRNA against HIF-1α was constructed. RT-qPCR analysis showed the mRNA expression levels of HIF-1α was notably reduced in HIF-1α-knockdown HUVECs compared with the negative control (Fig. 6A). Subsequently, the effect of HIF-1α on hypoxia-induced angiogenesis was investigated. The results demonstrated that angiogenesis was potentiated in cells under hypoxic conditions. However, HIF-1α depletion reduced the *in vitro* tube formation in cells under hypoxia and significantly shortened the tube length (Fig. 6B). Together, these data demonstrated that HIF-1α is essential for hypoxia-enhanced angiogenesis of cerebral endothelial cells. Finally, whether HIF-1α depletion impacted on angiogenesis-associated gene expression levels under hypoxic conditions was investigated. The results showed that hypoxia induced the expression of

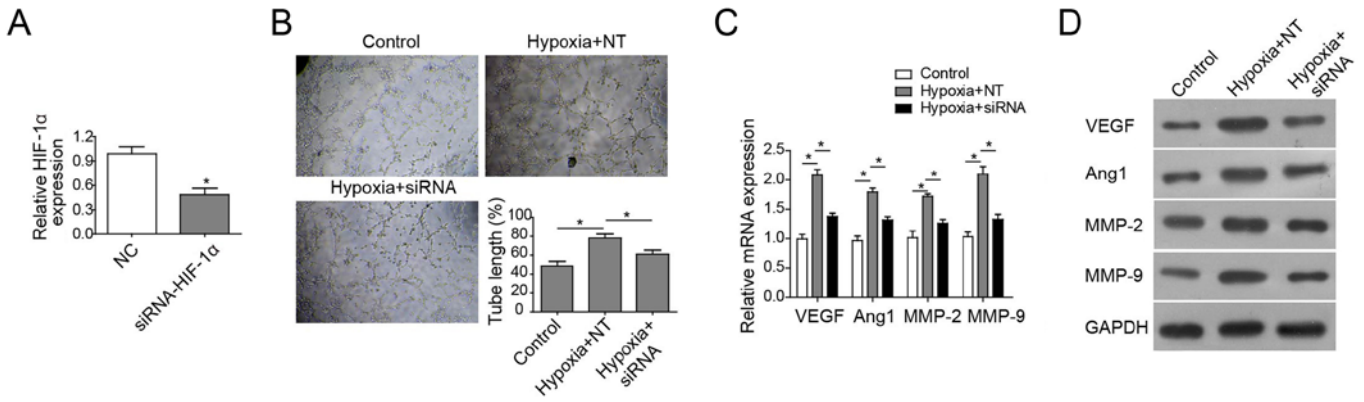

Figure 6. HIF-1α is necessary for hypoxia-induced angiogenesis in HUVECs. (A) RT-qPCR analysis of HIF-1α in HUVECs transfected with scrambled siRNA or siRNA against HIF-1α. (B) *In vitro* vascular tube formation assay of HUVECs under normoxia and hypoxia. Cells were transfected with scrambled siRNA or siRNA against HIF-1α. (C) RT-qPCR analysis and (D) western blot analysis of angiogenesis-associated genes in HUVECs transfected with scrambled or siRNA against HIF-1α. All data are presented as the mean ± standard deviation. *P<0.05 vs. control. Ang, angiogenin; CR, common rehabilitation; EM, environmental modification; HIF, hypoxia-inducible factor; MCAO, middle cerebral artery occlusion; MMP, matrix metalloproteinase; NC, normal control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si, small interference; VEGF, vascular endothelial growth factor; WM, willed movement; NT, negative control siRNA; siRNA, small interfering RNA.

VEGF, Ang-1, MMP-2 and MMP-9 at the mRNA and protein levels, whereas HIF-1α knockdown reversed the increased expression of these genes (Fig. 6C and D). Collectively, these findings demonstrated that HIF-1α serves a key role in *in vitro* tube formation of endothelial cells under hypoxic conditions, but this needs to be verified with future studies.

Discussion

In the present study, exercise therapies, particularly willed movement, were protective interventions in a rat model of ischaemic stroke. Specifically, neurological behaviour assessment and TTC staining showed that willed movement significantly attenuated the injury caused by focal cerebral ischaemia and reperfusion. Furthermore, the three therapies used in the present study, promoted proliferation and *in vitro* tube formation of brain vascular endothelial cells via upregulation of HIF-1α and its downstream genes, including VEGF, Ang-1, MMP-2 and MMP-9. In human umbilical vein endothelial cells (HUVECs), it was demonstrated that HIF-1α induced the expression of VEGF, Ang-1, MMP-2 and MMP-9 under hypoxic conditions. The present findings suggest that willed movement may contribute to angiogenesis of endothelial cells by upregulating HIF-1α, but this needs to be verified in the future.

In stroke research, the key goal is to prevent neuronal cell death and improve recovery (23). However, few therapeutic targets have emerged. HIF-1α serves an important role in neurological outcomes after ischaemic stroke via regulation of its downstream genes that promote cell survival and angiogenesis (24). Previous studies have shown that HIF-1α is induced in ischaemic brains (25). HIF-1α prevents apoptosis via blockage of cytochrome c release, PARP cleavage and caspase activation (24,26). Furthermore, HIF-1α can suppress p53 activation and thus maintain cell survival (27). However, Baranova *et al* (28) and Helton *et al* (29) reported that HIF-1α exerts distinct roles in neuronal injuries following ischaemia. Baranova *et al* (28) found that HIF-1α knockdown increased tissue damage and decreased the survival rate of MCAO rats, which indicated HIF-1α was neuroprotective. By contrast, Helton *et al* (29) observed HIF-1α knockout alleviated ischaemic damage, indicating HIF-1α may induce tissue damage in the ischaemic brain. One explanation for this discrepancy could be that Baranova used a mild ischaemic model (30 min ischaemia with unilateral common carotid artery occlusion), whereas Helton used a severe ischaemic model (75 min ischaemia with bilateral occlusion). Together, the two studies support the idea that HIF-1α may lead to cell death in severe ischaemia and promote cell survival after mild ischaemic insults.

Willed movement training has been suggested to upregulate some genes (such as, synaptophysin, AMPA and PICK1) that are involved in synaptic plasticity and transmission in the brain of rats undergoing MCAO (2,8,12,13). However, the detailed molecular mechanism of how willed movement training induces the expression of these genes remains unclear. In the present study, WM upregulated HIF-1α and induced its target angiogenesis-associated genes. Additionally, the mechanism of willed movement-activated HIF-1α expression also requires further investigation. To address this question, whether intracellular signaling pathways are activated by willed movement and thus activate the transcription of HIF-1α need to be investigated. To systemically identify targets essential for HIF-1α-affected rehabilitation of patients after ischaemic stroke is another issue, which can be addressed by ChIP-seq and RNA-seq approaches.

In summary, the present findings emphasized the role of willed movement in promoting HIF-1α expression, which in turn enhanced angiogenesis and improved neurological rehabilitation of brains with focal ischaemia. The present findings provide novel insights into the mechanism of the effect of hypoxia on ischaemic stroke and suggest HIF-1α accumulation as a strategy for treating patients following stroke. However, all experiments were performed *in vitro* and in rat cerebral tissue, therefore whether similar results can be obtained using WM to patients remains to be investigated.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ conceived the study, and performed data and statistical analysis. LZ and ZZ conceived and designed the study. XR performed the literature search, experimental studies and clinical studies. WZ performed data acquisition and edited the manuscript. LZ drafted the manuscript. All authors read and approved the final manuscript, revising it critically for important intellectual content.

Ethics approval and consent to participate

All animal experiments were performed according to guidelines for the care and use of animals and were approved by the animal ethics committees of Central South University.

Patient consent for publication

Not applicable.

Competing interests

Authors declare that they have no competing interests.

References

1. Zhang JH, Li JK, Ma LL and Lou JY: RNA interference-mediated silencing of S100B improves nerve function recovery and inhibits hippocampal cell apoptosis in rat models of ischemic stroke. J Cell Biochem 119: 8095-8111, 2018.
2. Nie J, Yang X, Tang Q, Shen Q and Li S: Willed-movement training reduces brain damage and enhances synaptic plasticity related proteins synthesis after focal ischemia. Brain Res Bull 120: 90-96, 2016.
3. Navone SE, Marfia G, Nava S, Invernici G, Cristini S, Balbi S, Sangiorgi S, Ciusani E, Bosutti A, Alessandri G, *et al*: Human and mouse brain-derived endothelial cells require high levels of growth factors medium for their isolation, in vitro maintenance and survival. Vasc Cell 5: 10, 2013.
4. Yu QJ, Tao H, Wang X and Li MC: Targeting brain microvascular endothelial cells: A therapeutic approach to neuroprotection against stroke. Neural Regen Res 10: 1882-1891, 2015.
5. Guell K and Bix GJ: Brain endothelial cell specific integrins and ischemic stroke. Exp Rev Neurother 14: 1287-1292, 2014.
6. Waterland JC: The supportive framework for willed movement. Am J Phys Med 46: 266-279, 1967.
7. Tang Q, Yang Q, Hu Z, Liu B, Shuai J, Wang G, Liu Z, Xia J and Shen X: The effects of willed movement therapy on AMPA receptor properties for adult rat following focal cerebral ischemia. Behav Brain Res 181: 254-261, 2007.
8. Tang Q, Tan L, Yang X, Shen Q, Huang X, Wang G, Chen H, Nie J, Li S and Wu L: Willed-movement training reduces motor deficits and induces a PICK1-dependent LTD in rats subjected to focal cerebral ischemia. Behav Brain Res 256: 481-487, 2013.
9. Volk L, Kim CH, Takamiya K, Yu Y and Huganir RL: Developmental regulation of protein interacting with C kinase 1 (PICK1) function in hippocampal synaptic plasticity and learning. Proc Natl Acad Sci USA 107: 21784-21789, 2010.
10. Clem RL, Anggono V and Huganir RL: PICK1 regulates incorporation of calcium-permeable AMPA receptors during cortical synaptic strengthening. J Neurosci 30: 6360-6366, 2010.
11. Moretti A, Ferrari F and Villa RF: Pharmacological therapy of acute ischaemic stroke: Achievements and problems. Pharmacol Ther 153: 79-89, 2015.
12. Thirusangu P, Vigneshwaran V, Ranganatha VL, Vijay Avin BR, Khanum SA, Mahmood R, Jayashree K and Prabhakar BT: A tumoural angiogenic gateway blocker, Benzophenone-1B represses the HIF-1α nuclear translocation and its target gene activation against neoplastic progression. Biochem Pharmacol 125: 26-40, 2017.
13. Lu Y, Wang B, Shi Q, Wang X, Wang D and Zhu L: Brusatol inhibits HIF-1 signaling pathway and suppresses glucose uptake under hypoxic conditions in HCT116 cells. Sci Rep 6: 39123, 2016.
14. Sharp FR and Bernaudin M: HIF1 and oxygen sensing in the brain. Nat Rev Neurosci 5: 437-448, 2004.
15. Hirota K and Semenza GL: Regulation of angiogenesis by hypoxia-inducible factor 1. Crit Rev Oncol Hematol 59: 15-26, 2006.
16. Sun Y, He W and Geng L: Neuroprotective mechanism of HIF-1α overexpression in the early stage of acute cerebral infarction in rats. Exp Ther Med 12: 391-395, 2016.
17. Phillips PG, Birnby LM and Narendran A: Hypoxia induces capillary network formation in cultured bovine pulmonary microvessel endothelial cells. Am J Physiol 268: L789-L800, 1995.
18. Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, Iyer N, LaRusch J, Pak B, Taghavi P and Semenza GL: Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res 63: 1138-1143, 2003.
19. Hirayama Y and Koizumi S: Hypoxia-independent mechanisms of HIF-1α expression in astrocytes after ischemic preconditioning. Glia 65: 523-530, 2017.
20. Li Y, Xu L, Zeng K, Xu Z, Suo D, Peng L, Ren T, Sun Z, Yang W, Jin X and Yang L: Propane-2-sulfonic acid octadec-9-enyl-amide, a novel PPARα/γ dual agonist, protects against ischemia-induced brain damage in mice by inhibiting inflammatory responses. Brain, Behav Immun 66: 289-301, 2017.
21. Kuo CH, Chen PK, Chang BI, Sung MC, Shi CS, Lee JS, Chang CF, Shi GY and Wu HL: The recombinant lectin-like domain of thrombomodulin inhibits angiogenesis through interaction with Lewis Y antigen. Blood 119: 1302-1313, 2012.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
23. Chassagnon IR, McCarthy CA, Chin YK, Pineda SS, Keramidas A, Mobli M, Pham V, De Silva TM, Lynch JW and Widdop RE *et al*: Potent neuroprotection after stroke afforded by a double-knot spider-venom peptide that inhibits acid-sensing ion channel 1a. Proc Natl Acad Sci USA 114: 3750-3755, 2017.
24. Piret JP, Lecocq C, Toffoli S, Ninane N, Raes M and Michiels C: Hypoxia and CoCl2 protect HepG2 cells against serum deprivation- and t-BHP-induced apoptosis: A possible anti-apoptotic role for HIF-1. Exp Cell Res 295: 340-349, 2004.
25. Zhang L, Luo X, Chen F, Yuan W, Xiao X, Zhang X, Dong Y, Zhang Y and Liu Y: LncRNA SNHG1 regulates cerebrovascular pathologies as a competing endogenous RNA through HIF-1α/VEGF signaling in ischemic stroke. J Cell Biochem 119: 5460-5472, 2018.
26. Sasabe E, Tatemoto Y, Li D, Yamamoto T and Osaki T: Mechanism of HIF-1alpha-dependent suppression of hypoxia-induced apoptosis in squamous cell carcinoma cells. Cancer Sci 96: 394-402, 2005.
27. Li J, Zhang X, Sejas DP, Bagby GC and Pang Q: Hypoxia-induced nucleophosmin protects cell death through inhibition of p53. J Biol Chem 279: 41275-41279, 2004.
28. Baranova O, Miranda LF, Pichiule P, Dragatsis I, Johnson RS and Chavez JC: Neuron-specific inactivation of the hypoxia inducible factor 1 alpha increases brain injury in a mouse model of transient focal cerebral ischemia. J Neurosci 27: 6320-6332, 2007.
29. Helton R, Cui J, Scheel JR, Ellison JA, Ames C, Gibson C, Blouw B, Ouyang L, Dragatsis I, Zeitlin S, *et al*: Brain-specific knock-out of hypoxia-inducible factor-1alpha reduces rather than increases hypoxic-ischemic damage. J Neurosci 25: 4099-4107, 2005.