

Astragaloside IV improves angiogenesis under hypoxic conditions by enhancing hypoxia-inducible factor-1 α SUMOylation

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Received January 27, 2020; Accepted October 8, 2020

DOI: 10.3892/mmr.2021.11883

Abstract. Improving angiogenic capacity under hypoxic conditions is essential for improving the survival of skin grafts, as they often lack the necessary blood supply. The stable expression levels of hypoxia-inducible factor-1 α (HIF-1 α) in the nucleus directly affect the downstream vascular endothelial growth factor (VEGF) signaling pathway and regulate angiogenesis in a hypoxic environment. Astragaloside IV (AS-IV), an active component isolated from *Astragalus membranaceus*, has multiple biological effects including antioxidant and anti-diabetic effects, and the ability to provide protection from cardiovascular damage. However, the mechanisms underlying these effects have not previously been elucidated. The present study investigated whether AS-IV promotes angiogenesis via affecting the balance between ubiquitination and small ubiquitin-related modifier (SUMO) modification of HIF-1 α . The results demonstrated that persistent hypoxia induces changes in expression levels of HIF-1 α protein and significantly increases the proportion of dysplastic blood vessels. Further western blotting experiments showed that rapid attenuation and delayed compensation of SUMO1 activity is one of the reasons for the initial increase then decrease in HIF-1 α levels. SUMO1 overexpression stabilized the presence of HIF-1 α in the nucleus and decreased the extent of abnormal blood vessel morphology observed following hypoxia. AS-IV induces vascular endothelial cells to continuously produce SUMO1, stabilizes the HIF-1 α /VEGF pathway and improves angiogenesis in hypoxic conditions. In summary, the present study confirmed that AS-IV stimulates vascular endothelial cells to continuously resupply

SUMO1, stabilizes the presence of HIF-1 α protein and improves angiogenesis in adverse hypoxic conditions, which may improve the success rate of flap graft surgery following trauma or burn.

Introduction

Skin flaps, also known as pedicled skin grafts, are organs with blood vessels and attached subcutaneous fat tissue, and can be transferred from one part of the body to another (1). Flap transplantation is widely used to repair large areas of skin defects caused by trauma or burns and to reconstruct organs (2,3). However, the choice of source and size of the flap, as well as the initial state of the area covered by the graft, such as presence of infection and diabetes, amongst others, can affect the survival of the flap (3). Among all factors that influence the survival of flap transplantation, up to 85% of the complications were caused by blood flow disruption, involving outflows and inflows (4). In severe cases, this can cause necrosis of large tissue grafts or complete failure of surgery (5). Therefore, the continuous improvement of surgical methods, and the search for novel alternative therapies, such as the development of new targeted drugs, or extraction of effective drug ingredients from traditional Chinese medicine, are required to improve the survival rate of skin grafts.

Astragalus mongholicus and *Astragalus membranaceus*, both known as Huangqi in China, are perennial herbs used in the treatment of a variety of diseases, such as dyspepsia, diarrhea, heart diseases, hepatitis and anemia (6). Astragaloside IV (AS-IV) is an active constituent extracted from the plant *A. membranaceus* which exhibits roles in improving diabetic nephropathy, inhibiting cardiac fibrosis, promoting functional recovery following spinal cord injury and inhibiting hepatic fibrosis, as well as serving anticancer functions (7-11). Results from *in vivo* studies demonstrated that AS-IV is able to prevent cognitive deficits induced by transient cerebral ischemia and reperfusion (12,13). In addition, AS-IV exerts protective effects against endothelial damage caused by hyperglycemia via inhibiting oxidative stress and calpain-1 activation (14). However, to the best of our knowledge, whether AS-IV also improves angiogenesis under hypoxia has not been reported.

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Key words: astragaloside IV, hypoxia, hypoxia inducible factor-1 α , angiogenesis, small ubiquitin-related modifier 1

Hypoxia-inducible factors (HIFs) are transcription factors that respond to changes in available oxygen in the cellular environment, specifically to a decrease in oxygen, or hypoxia (15). HIF-1 α regulates the transcription of >40 genes, including erythropoietin (16), glucose transporters (17), glycolytic enzymes (18), vascular endothelial growth factor (VEGF) (19) and other genes whose protein products increase oxygen delivery (20) or facilitate metabolic adaptation to hypoxia (21). In these complex pathophysiological mechanisms, the stable presence of HIF-1 α in the nucleus and the continued activation of the HIF-1 α /VEGF signaling pathway depend on the balance of ubiquitination and small ubiquitin-related modifier (SUMO) modification of HIF-1 α (22,23). At normal oxygen levels, HIF-1 α protein is degraded by ubiquitination-mediated proteolysis (24), whereas under hypoxic conditions HIF-1 α preferentially undergoes SUMO modification and the ubiquitination degradation pathway is inhibited (25). Through this molecular mechanism, HIF-1 α is continuously and stably expressed in the nucleus and promotes neovascularization via the VEGF pathway. The aim of the present study was to investigate whether AS-IV affects the balance of ubiquitination and SUMOylation of HIF-1 α and the regulation of its downstream VEGF pathway, promotes neovascularization and improves the survival rate of skin grafts, and therefore to provide novel ideas for the clinical treatment of large-scale skin defects.

Materials and methods

Cell lines and culture. Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC). The cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂. For hypoxia treatment, cells were cultured in an atmosphere of 3% O₂ for 7 days. Cells cultured in normoxic conditions (21% O₂) were used as controls, and referred to as the normoxic group.

Lentiviral plasmid and gene transfection. Lentiviral (pWPXLD-GFP-SUMO1) or control plasmids (pWPXLD-GFP) were synthesized by Biogot Technology, Co. Ltd. and were transfected into 293T cells (ATCC) for production of lentiviral particles. All constructs were verified via nucleic acid sequencing. HUVECs were cultured to 60-70% confluence in normoxic and aforementioned cell culture conditions, and 5 μ l viral suspension (1x10⁸ titer) was placed on the cell monolayers. Flasks were then incubated at 37°C and 5% CO₂ for 6 h, after which the viral suspension was removed and replaced with fresh medium. Gene transduction efficiency was verified via western blotting.

Drug preparation and treatment. AS-IV (\geq 98%) (CAS no. 84687-43-4; cat. no. CS-4272; \geq 99.15% purity) was purchased from Chengdu Biopurify Phytochemicals Ltd. AS-IV was dissolved in DMSO (<0.1%), then diluted with DMEM to a final concentration of 5 ng/ml. Subsequently, stable SUMO1 gene-transfected or control cells cultured under hypoxic or normal conditions were treated with AS-IV for 7 days. Untreated cells were considered the control group.

Western blotting. Total protein was extracted from cells using RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) with 1 mM PMSF and 20 mM N-ethylmaleimide, and protein concentration was determined using the BCA method (Thermo Fisher Scientific, Inc.). Total protein (100 μ g/lane) was separated by 4-12% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). Subsequently, membranes were blocked in 5% BSA (Sigma-Aldrich; Merck KGaA) at room temperature for 1 h and incubated with antibodies for specific target proteins overnight at 4°C. Antibodies used are listed in Table I. The membranes were then incubated for 1 h at room temperature with anti-rabbit IgG horse-radish peroxidase-conjugated secondary antibody (1:2,000; cat. no. Sc-516087; Santa Cruz Biotechnology, Inc.). β actin served as an internal control and was probed on the same membrane of the proteins of interest. A Super Signal protein detection kit (cat. no. 34095; Pierce; Thermo Fisher Scientific, Inc.) was used to detect protein signals according to the manufacturer's protocols. Data were evaluated using image analysis software (ImageJ; version 1.48; National Institutes of Health). All experiments were repeated three times.

Angiogenesis assays. A 2-mm-thick layer of semi-solid Matrigel (1:3; BD Biosciences) was pre-coated on the bottom of 96-well plates at 37°C overnight. Then, 0.25x10⁶ HUVECs transfected with SUMO1 or control plasmid were added to the surface of the gel in each well and inoculated with 0.1 ml DMEM supplemented with FBS as aforementioned in the presence or absence of AS-IV. The formation of blood vessels was observed under an inverted microscope (magnification, x200; Olympus cellSens Entry 1.16; Olympus Corporation) after 24 h.

Lactate dehydrogenase (LDH) activity detection. HUVECs (5x10³) transfected with SUMO1 or control plasmid were seeded into 96-well plates and incubated in the presence or absence of AS-IV for 7 days. The cells were then collected and sonicated at 5 MHz for 30 sec at 4°C, followed by centrifugation at 12,000 x g for 10 min at 4°C. The LDH content of the conditioned medium was measured via an ELISA-based LDH activity assay kit (cat. no. YM-ME0351; Shanghai Yuan Mu Biotechnology Co., Ltd.) in accordance with the manufacturer's instructions.

Statistical analysis. Data were analyzed using GraphPad Prism software (version 6; GraphPad Software, Inc.) and are presented as the mean \pm SD of \geq 3 independent experimental repeats. The statistical significance of differences in results were determined via one-way ANOVA or unpaired Student's t-test. Differences among multiple groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Sustained hypoxia leads to waveform expression levels of HIF-1 α protein and increases the proportion of abnormal blood vessels. In order to investigate the effect of sustained hypoxia on the expression levels of HIF-1 α protein, the levels of HIF-1 α protein in HUVECs cultured under hypoxic

Table I. Antibodies used for western blotting.

Antibody	Dilution	Cat. no.	Supplier
Small ubiquitin-related modifier 1	1:1,000	ab11672	Abcam
Ubiquitin	1:1,000	3936	Cell Signaling Technology, Inc.
Hypoxia inducible factor-1 α	1:500	ab216842	Abcam
Vascular endothelial growth factor	1:500	ab2350	Abcam
β -actin	1:1,000	ab8227	Abcam

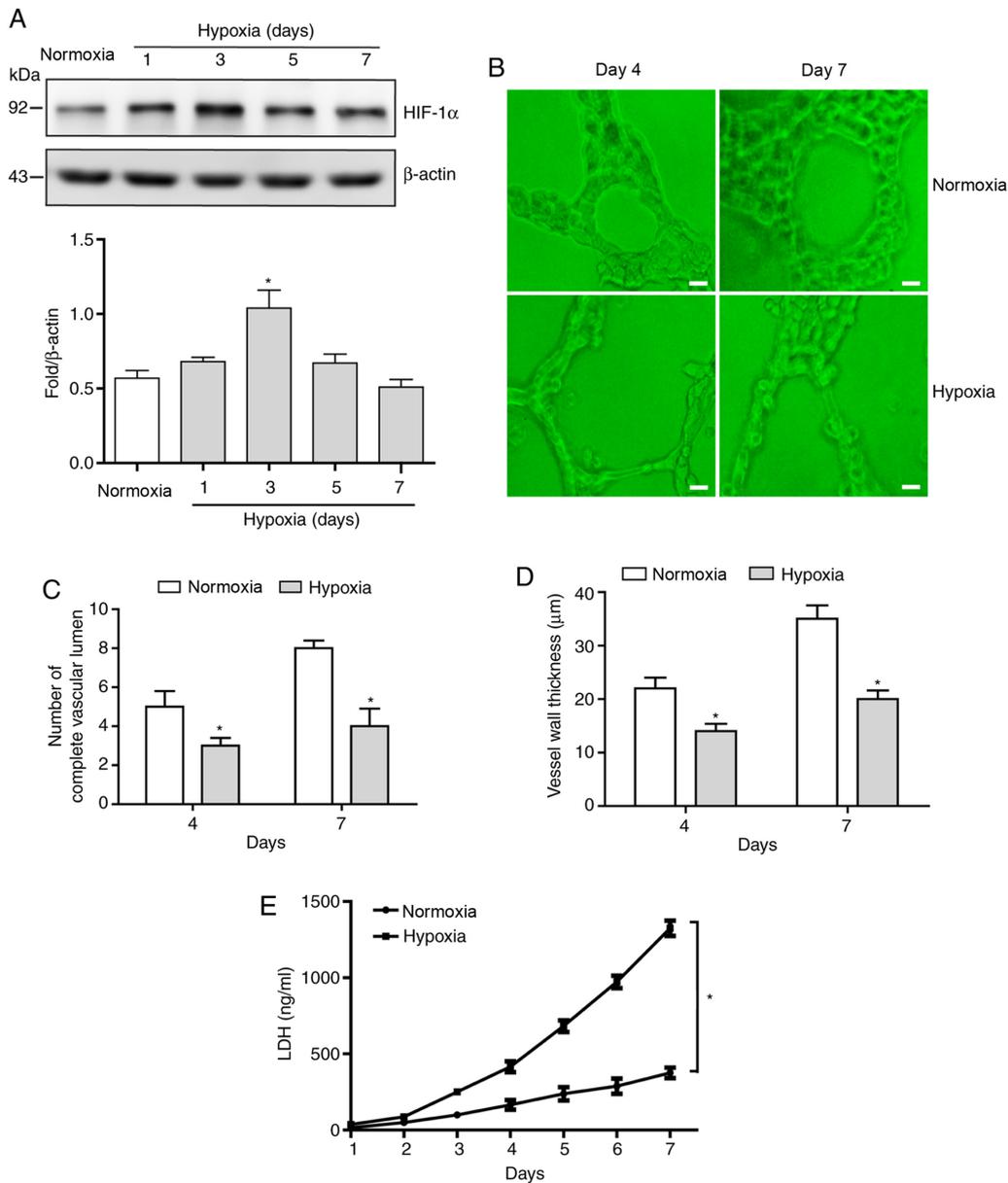


Figure 1. Sustained hypoxia leads to waveform expression levels of HIF-1 α protein and increases the proportion of abnormal blood vessels. (A) Levels of HIF-1 α protein in HUVECs cultured under hypoxic conditions for 7 days were examined via western blotting. (B) Angiogenic potential was examined using simulated angiogenesis experiments in HUVECs on days 4 and 7 of exposure to hypoxia (scale bar, 20 μ m). (C) The number of complete vascular lumens and (D) vessel wall thickness were measured. (E) LDH content was measured via ELISA following HUVEC exposure to hypoxia for 7 days. Data are presented as the mean \pm standard deviation. n=3. *P<0.05 vs. normoxia. HIF, hypoxia inducible factor; HUVEC, human umbilical vein endothelial cells; LDH, lactate dehydrogenase.

conditions for 7 days was evaluated. The expression levels of HIF-1 α protein exhibited a notable pattern of an initial increase and then a decrease, with peak expression levels appearing on

day 4 of hypoxia treatment (Fig. 1A). Observation of simulated blood vessel morphology on days 4 and 7 using angiogenesis assays demonstrated that, compared with the normoxic group,

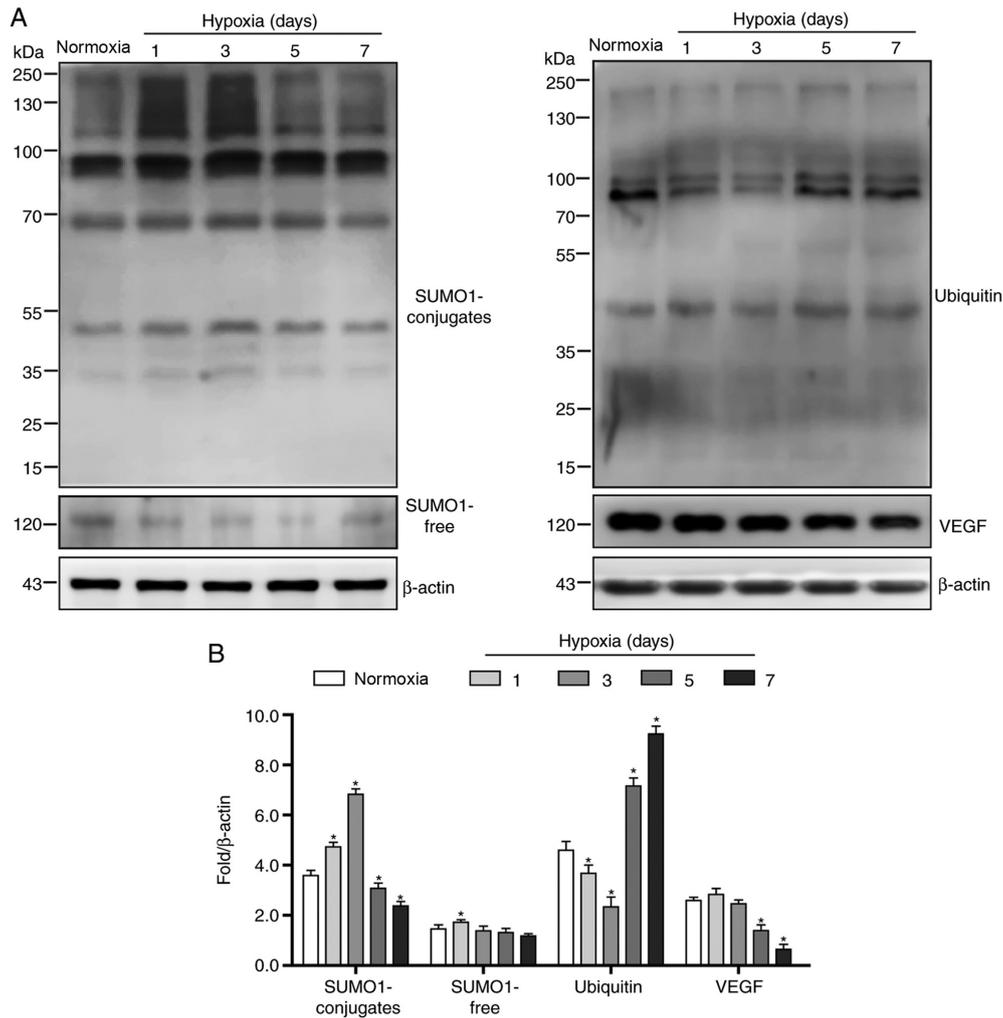


Figure 2. Rapid depletion and slow compensation of SUMO1 protein are key factors for HIF-1 α protein degradation. (A) SUMO1, ubiquitin and VEGF protein expression levels were investigated via western blotting. (B) Protein expression levels were normalized to those of β -actin. Data are presented as the mean \pm standard deviation. n=3. *P<0.05 vs. normoxia. SUMO1, small ubiquitin-related modifier 1; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor.

significantly fewer intact blood vessels formed in the hypoxic group (Fig. 1B and C) and their walls were significantly thinner (Fig. 1B and D) at both time points.

Since LDH content in the medium directly reflects the degree of cell damage, LDH levels in the conditioned medium were assessed following culturing of HUVECs under hypoxic or normoxic conditions. The results demonstrated that LDH levels in the normoxic group remained low and did not change significantly over time (Fig. 1E); however, gradually increasing levels of LDH were detected in the conditioned medium of the hypoxia group (Fig. 1E), which indicated that the degree of cell damage increases with the prolongation of hypoxia time.

Rapid depletion and slow compensation of SUMO1 protein are key factors for HIF-1 α protein degradation.

In order to investigate whether persistent hypoxia affects the HIF-1 α /VEGF pathway via the regulation of HIF-1 α SUMOylation and thereby alters angiogenesis, the expression levels of SUMO1 and VEGF were assessed. The results demonstrated that HUVECs expressed basal levels of SUMO1 (Fig. 2A and B). At the initial stage of exposure to

hypoxic conditions, the levels of covalently modified SUMO1 significantly increased, accompanied by a rapid decline of free SUMO1 (Fig. 2A and B). With the prolongation of hypoxia time, levels of both covalently modified SUMO1 and free SUMO1 decreased significantly until day 7 (Fig. 2A and B). Correspondingly, VEGF protein levels began to decline between days 3 and 7 (Fig. 2A and B). However, ubiquitin conjugates showed the opposite trend to SUMO1; ubiquitin levels initially decreased and then increased rapidly between days 3 and 7 (Fig. 2A and B).

Overexpression of SUMO1 stabilizes HIF-1 α protein and partially restores vascular morphology. In order to further investigate the effect of SUMOylation on HIF-1 α protein expression levels and angiogenesis in continuous hypoxic conditions, lentiviral-mediated gene transfection was used to achieve SUMO1 overexpression in HUVECs. Western blotting results confirmed that SUMO1 was overexpressed in HUVECs following gene transfection (Fig. 3A and B). Further results showed that overexpression of SUMO1 increased the covalent binding of SUMO1 to HIF-1 α protein, antagonized

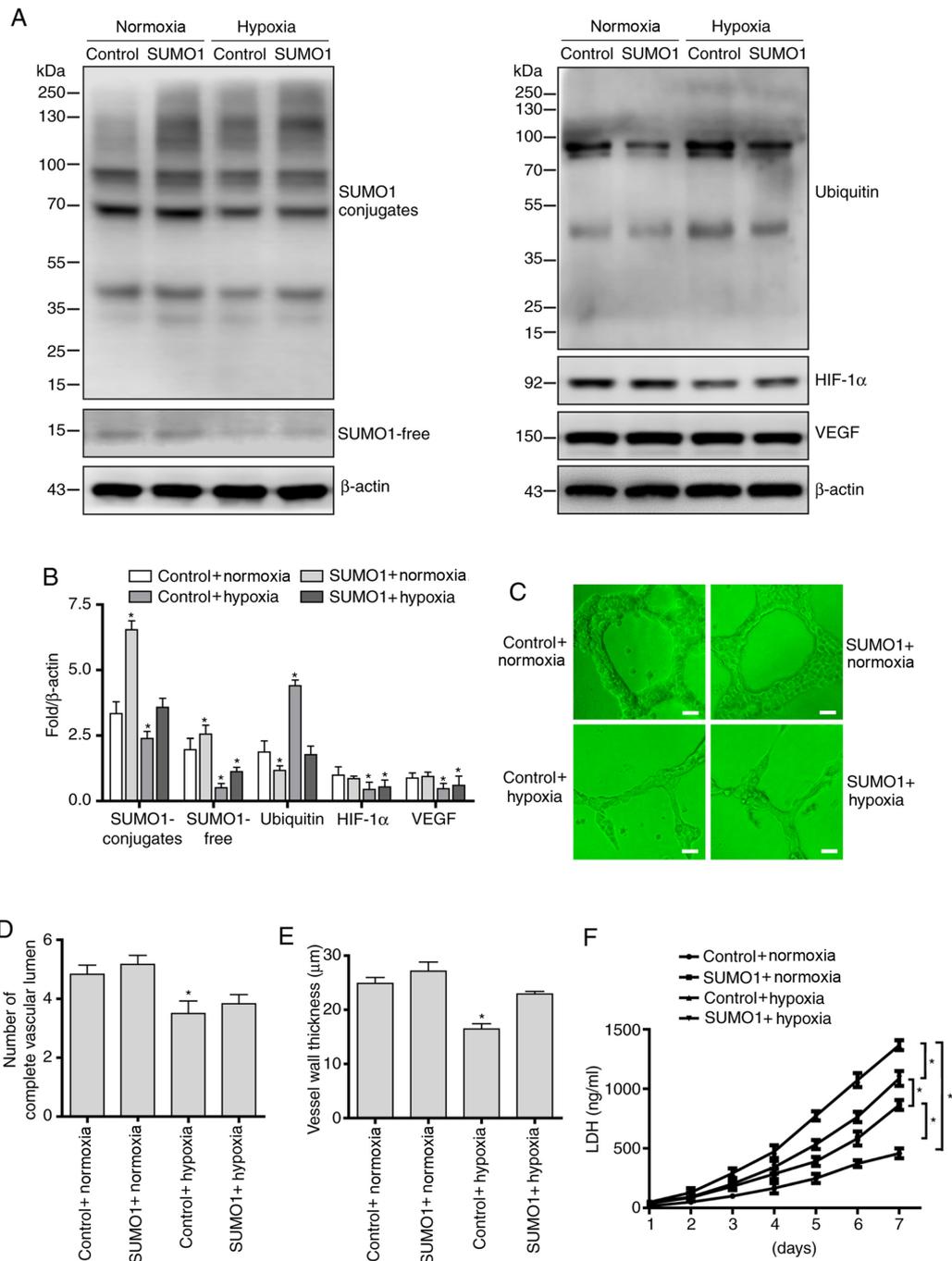


Figure 3. SUMO1 overexpression stabilizes HIF-1 α protein and partially restores vascular morphology. SUMO1, ubiquitin, HIF-1 α and VEGF protein expression levels were (A) investigated by western blotting and (B) normalized to those of β -actin following exposure of HUVECs transfected with SUMO1 gene or empty plasmid (control) transfection to normoxia or hypoxia for 7 days. (C) Angiogenic potential was examined via simulated angiogenesis experiments (scale bar, 20 μ m). (D) Number of complete vascular lumens and (E) vessel wall thickness were measured. (F) LDH content was measured via ELISA following exposure of SUMO1- and empty control-transfected HUVECs to normoxia or hypoxia for 7 days. Data are presented as the mean \pm standard deviation. n=3. *P<0.05 vs. control. SUMO1, small ubiquitin-related modifier 1; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cells; LDH, lactate dehydrogenase.

the degradation of HIF-1 α protein induced by ubiquitination modification, and further regulated downstream function via the HIF-1 α /VEGF pathway under hypoxic conditions (Fig. 3A and B).

Next, the effects of SUMO1 overexpression on angiogenesis and cell damage under hypoxic conditions were evaluated. The results demonstrated that SUMO1 overexpression significantly improved the angiogenic potential of HUVECs under hypoxic

conditions, with cells overexpressing SUMO1 showing a more complete vessel lumen structure (Fig. 3C and D) and thicker vessel walls (Fig. 3C and E) than the empty control-transfected cells. Finally, cytotoxicity was assessed via testing LDH levels. The results showed that overexpression of SUMO1 decreased LDH levels in conditioned medium in both normoxic and hypoxic cells compared with empty control-transfected cells (Fig. 3F).

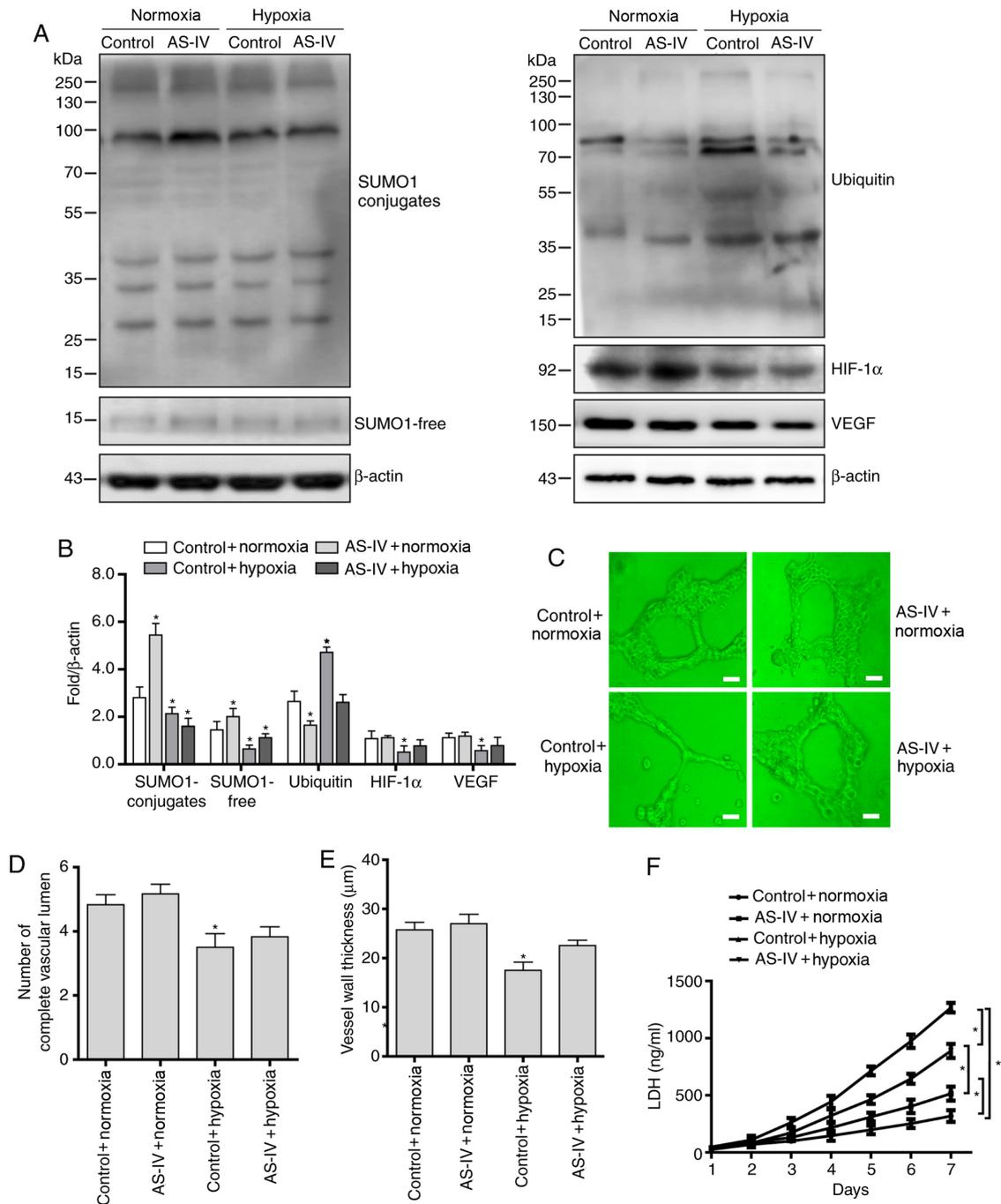


Figure 4. AS-IV activates HVUECs to continuously express SUMO1 and improves angiogenesis in a hypoxic environment. SUMO1, ubiquitin, HIF-1 α and VEGF protein expression levels were (A) examined via western blotting and (B) normalized to those of β -actin following exposure of HUVECs in the presence or absence of AS-IV treatment to normoxic or hypoxic conditions. (C) Angiogenic potential was measured in simulated angiogenesis experiments in AS-IV-treated (5 ng/ml) and untreated cells in a normoxic or hypoxic environment for 5 days (scale bar, 20 μ m). (D) The number of complete vascular lumens and (E) vessel wall thickness were measured. (F) LDH content was measured via ELISA following exposure of HUVECs in the presence or absence of AS-IV treatment to normoxic or hypoxic conditions for 7 days. Data are presented as the mean \pm standard deviation. $n=3$. * $P<0.05$ vs. control. AS-IV, astragaloside IV; HUVEC, human umbilical vein endothelial cells; SUMO1, small ubiquitin-related modifier 1; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; LDH, lactate dehydrogenase.

AS-IV activates HVUECs to continuously express SUMO1 and improves angiogenesis in a hypoxic environment. In order to determine whether AS-IV exerts its antagonistic effect on hypoxia-induced endothelial cell injury via affecting the SUMO1 modification of HIF-1 α and the HIF-1 α /VEGF pathway, HUVECs were treated with AS-IV under hypoxic conditions. The present results demonstrated that AS-IV

continuously induced SUMO1 expression levels, increased SUMOylation of HIF-1 α protein, decreased its ubiquitination modification and stabilized HIF-1 α protein levels, leading to activation of the HIF-1 α /VEGF signaling pathway (Fig. 4A and B).

Next, the effect of AS-IV on the angiogenesis potential and cytotoxicity of HUVECs under hypoxic conditions was

evaluated. The results demonstrated that AS-IV significantly improved the angiogenic potential of HUVECs under hypoxic conditions, with AS-IV-treated cells exhibiting a relatively intact vascular lumen (Fig. 4C and D) and thicker vessel walls (Fig. 4C and E) than those of the untreated cells. Finally, the results of the cytotoxicity assay showed that AS-IV decreased the LDH content of HUVECs under hypoxic conditions (Fig. 4F).

Discussion

In orthopedics and plastic surgery, flap transplantation is a common method for repairing large-scale skin wounds (26,27). Factors affecting the quality of flap survival include arterial blood supply (28), venous return (29), tissue damage (30) and ischemia-reperfusion injury (31). Among these, ischemic injury is the key factor leading to flap necrosis following surgery (32). Certain drugs targeting ischemic injury have been proven effective, such as L-arginine (33) and estrogen (34) to relax micro-vessels, recombinant connective tissue growth factor (35), erythropoietin (36) and VEGF (37) to promote vascular regeneration, and others to control inflammation and scavenge oxygen free radicals. However, the safety and effectiveness of these treatments in humans need to be further verified by clinical studies.

Traditional Chinese medicine and herbal medicine have proven effective through thousands of years of practice, although active ingredients and underlying mechanisms of action have not been fully elucidated. Among these, AS-IV, the primary active component of *A. membranaceus*, has been reported to exhibit numerous effects including improvement in the function of endothelial cells (38) and neovascularization (39), anti-inflammatory (40) and antioxidant effects (41), regulation of energy metabolism (42), protection of the nervous system (43) and anticancer effects (44). However, the specific mechanism underlying the promotion of vascular regeneration by AS-IV, particularly under hypoxic conditions, remains unclear.

HIF-1 is a key transcription factor for the cellular adaptive response to hypoxia (45). Structurally, HIFs are heterodimers composed of two different subunits, HIF- α and HIF- β . The HIF-1 α subunits accumulate in the cytoplasm and translocate to the nucleus to form heterodimers with a β subunit. Then, the heterodimers associate with co-activators and bind to hypoxia response elements in gene promoters to initiate gene transcription (46). Hypoxia induces epithelial-to-mesenchymal transition and metastasis, mediated by various signaling pathways such as TGF- β , PI3K/AKT, Wnt and Jagged/Notch. Concomitantly, the hypoxic environment stimulates vessel growth via the HIF-1/VEGF axis and other secondary factors, including angiopoietin 2, fibroblast growth factor and hepatocyte growth factor (47). The transcriptional activity, protein stabilization, protein-protein interactions and cellular localization of HIF-1 α , the oxygen-sensitive subunit of HIF-1, are modulated by various post-translational modifications, including phosphorylation, acetylation, methylation, and alkylation, as well as the covalent linkage of fatty acids, saccharides or small proteins such as ubiquitin and SUMO (48,49). A recent study demonstrated that SUMOylation, the covalent attachment of SUMOs to proteins, is involved in activation of the hypoxic

response and the ensuing signaling cascade (50). The stable presence of HIF-1 α in the nucleus under hypoxic conditions depends on the balance between its SUMOylation and ubiquitination modification (51). The present study investigated how sustained hypoxic treatment (7 days) affects the protein presence of HIF-1 α and angiogenesis. The results showed that the expression levels of SUMO1 protein gradually decreased with the extension of hypoxia time, and its compensation cycle was slow, and the SUMOylation state of HIF-1 α protein was not maintained. The relative deficiency of HIF-1 α was insufficient to activate its downstream VEGF pathway, leading to abnormal vascular morphology.

In order to determine the effect of SUMO1 on maintaining the stability of HIF-1 α protein, gene transfection was used to achieve overexpression of SUMO1 in HUVECs. As expected, overexpression of SUMO1 inhibited the degradation of HIF-1 α by ubiquitinase, maintained HIF-1 α at a high level, stimulated the HIF-1 α /VEGF signaling pathway and improved angiogenesis in hypoxic environments. These results indicated that inducing HUVECs to continuously express SUMO1 protein via the administration of drugs may improve angiogenesis under hypoxia, and that this strategy could be used to treat conditions that result in vascular abnormalities due to hypoxia.

A number of traditional Chinese medicine monomer components were screened in preliminary experiments, which demonstrated that AS-IV induced HUVECs to continuously produce SUMO1 (data not shown). Therefore, it was hypothesized that AS-IV may improve angiogenesis via promoting the stability of HIF-1 α and its downstream VEGF signaling pathway. The present results validated this hypothesis. Under sustained exposure to AS-IV, HUVECs overexpressed SUMO1, continuously activated the HIF-1 α /VEGF pathway and promoted angiogenesis. Nie *et al* (52) found that administration of AS-IV significantly improved endothelial dysfunction associated with diabetes in diabetic rats by decreasing oxidative stress and levels of calpain-1. Leng *et al* (53) demonstrated that AS-IV improved vascular endothelial dysfunction induced by hyperglycemia, and that the protective effect of AS-IV may be achieved via the toll-like receptor4/NF- κ B signaling pathway. Zhang *et al* (54) confirmed that AS-IV exhibited vessel dilatation properties via the endothelium-dependent NO-cGMP pathway in normal and hypertensive rats; this blocks extracellular calcium influx and results in vessel relaxation, partly via phenylephrine and angiotensin II inhibition, when perivascular fat is present (54). These results provide understanding of the potential molecular mechanisms underlying the protective effect of AS-IV on vascular endothelial cells.

In conclusion, the present study identified a novel mechanism by which AS-IV drugs improve angiogenesis under hypoxic conditions, which may be useful to develop future treatments of numerous diseases associated with flap transplantation. More detailed assessments of the molecular mechanisms, as well as further animal experiments, should be performed in future investigations.

Acknowledgements

The authors would like to thank Dr Melony Black (Liwen Bianji, Edanz Editing China), for his language editing of this manuscript.

Funding

The present study was supported by grants from the Tianjin Natural Science Foundation of China (grant nos. 18JCQNJC12800 and 19JCZDJC35200), Tianjin Special Project of New Generation Artificial Intelligence Technology (grant no. 18ZXZNSY00260) and Binhai Health and Family Planning Commission Science and Technology Projects (grant no. 2014BWKZ008 and 2019BWKQ030).

Availability of data and materials

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

Authors' contributions

CH designed the experiments. BW, CZ, DC, XM, TY and XL performed the experiments and collected and analysed the data. BW and XL drafted the manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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