Astragaloside IV Stimulates Angiogenesis and Increases Hypoxia-Inducible Factor-1α Accumulation via Phosphatidylinositol 3-Kinase/Akt Pathway

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ABSTRACT

Astragaloside IV is the major active constituent of Astragalus membranaceus, which has been widely used for the treatment of cardiovascular diseases in China. The aim of this study was to determine the angiogenic effect of astragaloside IV and its underlying mechanism. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay, Western blotting, real-time polymerase chain reaction, and immunofluorescence to detect the effect of astragaloside IV on proliferation of human umbilical vein endothelial cells (HUVECs), the phospho-Akt protein level, hypoxia-inducible factor-1α (HIF-1α) accumulation, vascular endothelial growth factor mRNA expression, and applied cell migration, tube formation, and chick chorioallantoic membrane assays to study the angiogenic effect of astragaloside IV. Results indicate that astragaloside IV promoted cell proliferation and stimulated HIF-1α accumulation during hypoxia. Mechanism studies revealed that astragaloside IV did not affect the degradation of HIF-1α protein or the level of HIF-1α mRNA. In contrast, astragaloside IV apparently activated the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which regulates HIF-1α protein synthesis. Moreover, astragaloside IV also stimulated cell migration, increased tube formation, and promoted angiogenesis in the chick chorioallantoic membrane assay. All angiogenic effects of astragaloside IV were reversed by the PI3K inhibitor. Taken together, our data collectively reveal that astragaloside IV is a novel regulator of HIF-1α and angiogenesis through the PI3K/Akt pathway in HUVECs that are exposed to hypoxia.

Introduction

In the clinical situation, myocardial ischemia is the most common cause of cardiac hypoxia. Pressure overload increases myocardial oxygen demand when oxygen delivery is inadequate to meet myocardial metabolic requirements of the heart. To meet this demand, short-term survival strategies, such as transactivating genes that increase glucose uptake, glycolysis, red blood cell production, and vasodilatation, are activated to respond to low tissue O2 levels. However, these responses do not resolve the fundamental problem of O2 deficiency. This deficiency can be overcome by angiogenesis, which increases the overall cross-sectional area of the coronary microvasculature (Arsham et al., 2002; Dorn, 2007; Shohet and Garcia, 2007). In general, angiogenesis is dependent on the accumulation of hypoxia-inducible factors (HIFs), which are heterodimeric transcription factors constituted of α and β subunits. HIF-1 is unstable in normal cells in which it is degraded by hydroxylation with prolyl hydroxylases but is stabilized in hypoxic cells wherein there is inhibition of hydroxylation, and HIF-1 then acts as a key transcriptional regulator of angiogenic factors, including the vascular endothelial growth factor (VEGF) (Fong, 2008).

Astragaloside IV is the major active constituent of Astragalus membranaceus, a widely used herb for the treatment of cardiovascular diseases in China (Miller, 1998). The Chinese
Expression of interferon-γ activities against Coxsackievirus B3 and by increasing the potency of antiviral treatment for viral myocarditis by its potent antiviral effect in a focal cerebral ischemia-reperfusion rat model. Astragaloside IV was also demonstrated to be a potential therapeutic agent for hypoxia control (HC) and prevention of vessel contraction was attributed to blockage of calcium influx (Zhang et al., 2006a, 2007). In hypoxia and reoxygenation-induced injury in vitro, astragaloside IV demonstrated a protective effect by normalization of the sarcoplasmic reticulum Ca²⁺ pump expression and prevention of depression in Ca²⁺ handling by the sarcoplasmic reticulum (Xu et al., 2008b). The improvement by astragaloside IV of sarcoplasmic reticulum Ca²⁺ pump function was also confirmed by its action in isoproterenol-induced myocardial injury in vivo, for which the result indicated that astragaloside IV improved cardiac function by preventing changes of Ca²⁺-ATPase activity and Ser16-phosphorylated phospholamban protein expression (Xu et al., 2007). Qu et al. (2009) reported that astragaloside IV stimulates HIF-1α accumulation and VEGF expression by arresting the PI3K/Akt pathways under hypoxic conditions; thus, it promotes angiogenesis and protects against cardiac hypoxia during myocardial ischemia.

Materials and Methods

Drugs. Astragaloside IV was purchased from the National Institute for the Control of the Pharmaceutical and Biological Products (Beijing, China), and its purity is greater than 98%. The compound was dissolved in dimethyl sulfoxide (DMSO), and the final DMSO concentration did not exceed 0.1% (v/v).

Cell Culture. Primary HUVECs were isolated, grown, and identified, as described earlier (Hermenegildo et al., 2005). HUVECs, at passages between 4 and 8, were cultured in gelatin-coated flasks on medium 199 (M199) (Invitrogen, Carlsbad, CA) that was supplemented with 12.5% fetal bovine serum (FBS) (Invitrogen), 50 U/ml penicillin (Northern China Pharmaceutical Co., Shijiazhuang, Hebei, China) and streptomycin (Shanghai 1 Biochemical Pharmaceutical Co., Shanghai, China), 30 μg/ml endothelial cell growth supplement (BD, Franklin Lakes, NJ), 10 ng/ml epidermal growth factor (BD), and 100 μM penicillin [Zhongguo Pharmaceutical (Shijiazhuang) Co., Hebei, China] with streptomycin (Northern China Pharmaceutical Co., Shijiazhuang, Hebei, China). This study conforms to the principles outlined in the Declaration of Helsinki. The Ethics Committee of the Medical College of the Zhejiang University approved all study procedures.

Cell Proliferation Assay during Hypoxia. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Pozzolini et al., 2003). In brief, HUVECs were cultured on collagen-coated 96-well plates at a density of 2 × 10⁴ cells/well. After the medium was replaced with 0.1 ml of M199 containing 2% FBS, the cells were treated with astragaloside IV at various concentrations (0.1, 0.25, 0.5, 1, and 5 μM) or DMSO (hypoxia control [HC]) and placed in a controlled-environment chamber (Thermo Fisher Scientific, Waltham, MA) that was flushed with 1% O₂/5% CO₂/94% N₂ for 24 h. The normal control (NC) group was exposed to normal oxygen levels under standard culture conditions in a humidified incubator maintained at 5% CO₂/95% room air and...
37°C for 24 h. MTT (5 mg/ml MTT in PBS; Sigma-Aldrich, St. Louis, MO) was added to the culture to obtain the final concentration of 0.5 mg/ml, and the culture was incubated for 4 h. The medium was removed, and formazan crystals were dissolved in 150 μl of DMSO. Each well was measured at 550 nm with a Universal Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Cell proliferation of 0.25 μM astragaloside IV under hypoxic conditions was also assayed at different time points. The cell proliferation rate was calculated for each well as (A150 treated cells – A150 hypoxia control cells)/A150 hypoxia control cells × 100%.

**Quantitative Real-Time PCR**. After treatment of HUVECs (5 × 10^5 cells/well) with 0.25 μM astragaloside IV or DMSO at hypoxic or normal oxygenation conditions for 1, 3, or 6 h, HIF-1α mRNA was measured by real-time PCR using TRIZol and a SuperScript II Reverse Transcriptase Kit (Invitrogen). For the VEGF mRNA assay, HUVECs (5 × 10^5 cells/well) were treated with 0.25 μM astragaloside IV, 10 μM 2-(4-morpholinyl)-8-phenyl-4H-1-benzo[4-1-one (LY294002; Tocris Bioscience, Ellisville, MO) (incubated for 30 min before astragaloside IV stimulation), or DMSO at hypoxic or normal oxygenation conditions for 6 h. The primers of HIF-1α, VEGF, and β-actin were as follows: sense, 5′-TCACCAAGAGCACTACAGGATGCC-3′ and anti-sense, 5′-CCGCAAGTTAAAGGACTCGTTCC-3′ for HIF-1α; sense, 5′-AGGGGCGGACATCATCAGC-3′ and anti-sense, 5′-AAGGCCCCAGGGTTTCT-3′ for VEGF; and sense, 5′-CGGTGCACTCAGGAAAGAG-3′ and anti-sense, 5′-GGAAAGATTGCAGGAGGCC-3′ for β-actin (Shanghai Sangon, Shanghai, China). All reactions were performed using a realplex W217215 machine (Eppendorf, Hamburg, Germany) and a Quantitect SYBR Green PCR Kit (Applied Biosystems, Warrington, UK) according to the manufacturer’s protocol. Samples were incubated for initial denaturation at 95°C for 15 min, followed by 40 cycles. For HIF-1α, each cycle consisted of 95°C for 15 s, 62°C for 30 s, and 72°C for 30 s; for VEGF, each cycle consisted of 95°C for 15 s, 66°C for 30 s, and 72°C for 30 s. The relative quantities of different mRNAs were calculated according to a standard calibration curve constructed for each amplicon. Data were normalized to β-actin and are expressed relative to the mean control value. Amplification of specific transcripts was confirmed by melting curve profiles generated at the end of each run.

**Western Blotting**. Each well, with 5 × 10^5 cells/well, was treated with 0.25 μM astragaloside IV, 10 μM LY294002, or DMSO for the indicated duration under hypoxic or normal oxygenated conditions. The cells were collected and lysed in 2.5× SDS gel loading buffer (30 mM Tris-HCl (pH 6.8), 1% SDS, 0.05% bromophenol blue, 12.5% glycerol, and 2.5% β-mercaptoethanol) and then boiled for 30 min. The same amounts of cell lysates were resolved on 10 or 12% SDS-polyacrylamide gels, and the proteins were electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The blots were incubated with the indicated primary antibodies, HIF-1α (1:500; R&D Systems, Minneapolis, MN), phospho-Akt (1:1000; Millipore Corporation, Billerica, MA), and Actin (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and then were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was visualized using Immobilon Western substrate (Millipore Corporation).

**Immunofluorescence**. HUVECs were cultured on collagen-coated 96-well plates at a density of 2 × 10^4 cells/well and treated with 0.25 μM astragaloside IV, 10 μM LY294002, or DMSO under hypoxic or normal oxygenated conditions for 4 h. These cells were then fixed with methanol at −20°C for 10 min. After blocking with 3% bovine serum albumin in PBS for 1 h, cells were incubated with a mouse monoclonal anti-HIF-1α antibody (Thermo Fisher Scientific) diluted 1:20 in 3% bovine serum albumin in PBS overnight at 4°C and washed with PBS, following which the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (1:100) for 1 h at room temperature. After a further wash, cells were stained with 4,6-diamidino-2-phenylindole (5 μg/ml; Beyotime, Haimen, China) for 15 min at room temperature. The cells were then imaged using fluorescence microscopy (DMI6000 B microscope; Leica, Wetzlar, Germany).

**Transwell Migration Assay during Hypoxia**. HUVEC migration was performed in a Transwell chemotaxis chamber (24-well culture plates; Corning Glassworks, Corning, NY) as described previously (Redmond et al., 1999). In brief, cells were seeded (1 × 10^5 cells/well) into the upper chamber containing 2% FBS growth media, and the lower reservoir was supplemented with 2% FBS growth media treated with astragaloside IV (0.25 μM), LY294002 (10 μM), or DMSO. HUVECs were subsequently allowed to migrate across a fibronectin-coated-polyextrufiber filter (8-μm pore) under hypoxic condition for 24 h. Nonmigrating cells were removed from the top side of the filter by washing three times with PBS and gentle scraping. The cells that migrated and were present on the lower aspect of the filter were subsequently fixed with 10% formaldehyde for 30 min and then stained with 0.1% crystal violet for 20 min. Five fields were counted for each well.

**Tube Formation during Hypoxia**. A Matrigel tube-formation assay was also performed to assess in vitro angiogenesis (Xu et al., 2008a). Growth factor-reduced Matrigel (BD) was placed in 96-well culture plates (50 μl/well) and allowed to set at 37°C for 1 h. Then 1 × 10^5 HUVECs were added to each well and incubated in 2% FBS M199 basic medium with 0.25 μM astragaloside IV, 10 μM LY294002, or DMSO under hypoxic conditions for 7 h. Five fields were counted for each well. The length of the tube was measured by Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD).

**Chick Chorioallantoic Membrane Assay**. The CAM assay was performed according to the method described by Ma et al. (2007). Fertilized chicken eggs were incubated in a humidified 70% atmosphere at 37°C for 7 days. The eggs were then cleaned with 70% ethanol, and a round window (diameter 1.2 cm) was made on the air sac to expose the CAM. The external egg membrane was removed from the CAM completely using a drop of sterilized physiological saline. CAMs were treated with DMSO, astragaloside IV (0.25 μM), and HIF-1α mRNA and HIF-1α protein degradation. A, cells were treated with 0.25 μM astragaloside IV at hypoxia for the indicated times. Then the total RNA was extracted, and real-time PCR was used to detect the HIF-1α mRNA level. Data are expressed as the mean ± S.E.M. and are representative of three independent experiments. B, cells were treated with astragaloside IV together with MG132 under the indicated conditions. Then the cells were collected, and Western blotting was performed to detect protein levels of HIF-1α and β-actin.
LY294002 (10 μM), and astragaloside IV (0.25 μM) + LY294002 (10 μM), in which sterile filter paper was soaked (0.25 × 0.25 cm). The windows were covered with tape, and the eggs were returned to the incubator for another 2 days. Then CAMs were fixed with acetone-methanol (1:1, v/v) for at least 30 min, cut out from the eggs, and photographed under a stereomicroscope.

**Data Analysis.** All values are expressed as means ± S.E.M. Statistical significance was estimated using the one-way analysis of variance. Values with $P < 0.05$ were considered significant. Statistical analyses were performed using Minitab 14 (Mintab Inc., State College, PA).

**Results**

**Astragaloside IV Stimulates Cell Proliferation.** Astragaloside IV significantly stimulated cell proliferation in the concentration range of 0.1 to 1 μM under hypoxic conditions, whereas 0.25 μM astragaloside IV demonstrated the optimal effect. We then applied a cell-proliferation assay of 0.25 μM astragaloside IV at different time points. The results indicated that cell viability was significantly inhibited under hypoxic conditions compared with normal conditions, from 12 to 72 h, whereas astragaloside IV notably stimulated cell proliferation from 12 to 72 h, compared with the hypoxia control (Fig. 1, B and C).

**Astragaloside IV Did Not Affect the Degradation of HIF-1α Protein or the Level of HIF-1α mRNA.** The effect of astragaloside IV activation on regulation of the HIF-1α protein level could be mediated by alterations in HIF-1α mRNA or protein stability. There was no significant change in the HIF-1α mRNA level with astragaloside IV (Fig. 2A). Therefore, the effect of astragaloside IV activation on HIF-1α protein degradation was evaluated with the selective proteasome inhibitor N-benzyloxycarbonyl (Z)-Leu-Leu-leucinal (MG132). The inhibitor increased the accumulation of HIF-1α protein under hypoxic conditions, whereas astragaloside IV enhanced the HIF-1α protein level higher after treatment with MG132, indicating that astragaloside IV did not affect proteasome-mediated degradation (Fig. 2B). The results show that astragaloside IV does not reduce the level...

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**Fig. 3.** Effects of astragaloside IV on phospho-Akt (p-Akt) and HIF-1α protein expression. Cells were treated with astragaloside IV together with LY294002 at hypoxia for the indicated times. Then the cells were collected, and Western blotting was performed to detect p-Akt and Akt (A) and HIF-1α and β-actin (B). p-Akt/Act and HIF-1α/β-actin ratios were expressed relative to the mean value of NC. Data are expressed as the mean ± S.E.M. ($n = 3$ for each experimental group). #, $P < 0.01$ versus NC; *, $P < 0.05$ versus HC.
of HIF-1α protein by interference with HIF-1α protein degradation or the transcription of HIF-1α mRNA.

**Astragaloside IV Increases the Cellular Accumulation of HIF-1α Protein through the PI3K/Akt Pathway and Affects the Nuclear Translocation of HIF-1α.** The PI3K/Akt pathway has been shown to control the translation of HIF-1α (Mottet et al., 2003; Fang et al., 2007). To address whether the PI3K/Akt pathway involves astragaloside IV-driven events, we introduced the PI3K inhibitor LY294002 into HUVECs. The introduction of LY294002 reduced phospho-Akt protein levels after hypoxia (Fig. 3A). Astragaloside IV significantly increased phospho-Akt protein levels in HUVECs after hypoxia for 3 and 6 h (Fig. 3A). Treatment with astragaloside IV increased HIF-1α protein levels after hypoxia for 6 and 12 h and showed a significant increase in HIF-1α protein levels after hypoxia for 6 h. The effect of astragaloside IV could be abrogated by LY294002 (Fig. 3B). These results indicate that astragaloside IV can activate the PI3K/Akt pathway and may thus promote translation of the HIF-1α protein.

Under hypoxic conditions, the HIF-1α protein accumulates and translocates to the nucleus, where it forms an active complex with HIF-1β and activates transcription of target genes (Xia et al., 2009). We next studied the intracellular localization of HIF-1α and determined whether astragaloside IV affects the nuclear translocation of hypoxia-induced HIF-1α protein. As shown in Fig. 4, astragaloside IV treatment under hypoxic conditions resulted in an increased nuclear accumulation of HIF-1α compared with DMSO treatment. Based on these results, we hypothesize that astragaloside IV increases the cellular accumulation of HIF-1α protein and affects nuclear translocation of HIF-1α, and the PI3K/Akt signaling pathway may be involved in this process.

**Astragaloside IV Increases the mRNA Level of VEGF.** To detect whether the accumulation of HIF-1α protein by astragaloside IV changes the expression of the HIF-1α-targeted VEGF gene, real-time PCR was used to measure VEGF mRNA in cells treated with astragaloside IV together with LY294002 under hypoxic conditions for 6 h. It was shown that the VEGF mRNA level increased nearly 3-fold in the hypoxia control group compared with the normal oxygenation condition control; treatment with astragaloside IV raised VEGF mRNA expression 2.8-fold compared with that in the hypoxia control. The effect of astragaloside IV was abrogated by LY294002 (Fig. 5).

**Astragaloside IV Promotes Angiogenesis Both In Vitro and Ex Vivo.** To investigate whether astragaloside IV has angiogenic effects, we used a series of standard angiogenesis models. In the Transwell migration assay, 0.25 μM astragaloside IV obviously enhanced cell migration, and this effect was neutralized by LY294002 (Fig. 6A). In terms of tube formation, 0.25 μM astragaloside IV potently formed elongated capillary-like tubes and complete vascular net-
works, whereas the control culture developed small amounts of capillary-like structures and incomplete networks; this effect was also abolished by LY294002 (Fig. 6B). An ex vivo model further demonstrated that 0.25 μM astragaloside IV strongly stimulated neovascularization of microvessels in CAM and this effect was abolished by LY294002 (Fig. 6C).

Discussion

The major findings of this study are that astragaloside IV is a novel regulator of HIF-1α and angiogenesis. In this study, astragaloside IV stimulated cell proliferation and promoted angiogenesis under hypoxic conditions. It was further noted that the compound stimulated HIF-1α protein accumulation and nuclear transcription under hypoxic conditions. Moreover, the angiogenic effect and HIF-1α protein accumulation of astragaloside IV may have been based on activation of the PI3K/Akt pathway.

Hypoxia, widely occurring in ischemia and tumor, increases HIF-1α accumulation mainly by blocking O2-dependent and proteasome-executed degradation. Indeed, HIF-1α function is regulated by changes in transcription and post-translational mechanisms, such as hydroxylation and phosphorylation. HIF-1α is continuously synthesized but degraded via ubiquitination and proteasome activity under normal oxygenation conditions, whereas it accumulates rapidly after exposure to hypoxia (Pi et al., 2005; Dai et al., 2010). In our study, astragaloside IV increased HIF-1α protein accumulation after the use of the selective proteasome inhibitor MG132, which is suggestive of the relationship with proteasome-mediated degradation. At the same time, transcription of HIF-1α mRNA was not interfered with by astragaloside IV. These results indicate that astragaloside IV may increase synthesis of HIF-1α protein.

The PI3K/Akt pathway is activated during ischemia and angiogenesis (Yang et al., 2009). It regulates a variety of cellular processes and plays a critical role in controlling the balance between cell survival and apoptosis. The rate of HIF-1α protein synthesis can be increased by the PI3K/Akt/mammalian target of rapamycin pathways (Choi et al., 2010). The pathway phosphorylates and activates translational factors, including p70s6k and 4EBP1 and increases the synthesis of multiple proteins, including HIF-1α. However, the activation of the PI3K/Akt signaling pathway by hypoxia is more likely to be cell type-specific (Arsham et al., 2002; Shafee et al., 2009). In our study, a direct correlation between increased PI3K/Akt signaling and HIF-1α protein synthesis was demonstrated, and astragaloside IV activated the PI3K-Akt pathway, thus promoting translation of HIF-1α protein.

Stabilized HIF-1α translocates to the nucleus and increases the transcription of the VEGF gene by binding to the hypoxia response element in the VEGF promoter; increased VEGF expression under hypoxic conditions leads to angiogenesis, ATP synthesis, oxygen supply, and cell survival.
In this study, VEGF mRNA transcripts accumulated in endothelial cells exposed to hypoxia, and the effect was significantly enhanced by astragaloside IV. Astragaloside IV significantly increased VEGF mRNA levels during hypoxia, and the enhanced effect was induced by the PI3K/Akt pathway. Furthermore, an angiogenic effect of astragaloside IV was observed in endothelial cells. All these data indicate that astragaloside IV stimulates cell migration, increases tube formation, and promotes angiogenesis in CAM. The angiogenic effect of astragaloside IV was reversed by LY294002. Thus, we hypothesized that astragaloside IV increases HIF-1α protein synthesis and translocates to the nucleus, thereby increasing the transcription of the VEGF gene and, consequently, promoting angiogenesis under hypoxic conditions, thus leading to a solution for the fundamental problem of O2 deficiency. In summary, our present study showed that angiogenesis is significantly stimulated as a result of astragaloside IV treatment, accompanied by increasing HIF-1α protein accumulation and VEGF gene transcription. HIF-1α protein synthesis and VEGF mRNA expression were inhibited by inhibition of PI3K using a specific inhibitor, denoting the involvement of the PI3K/Akt pathway in the synthesis of HIF-1α protein that was induced by astragaloside IV. Our present work provides new insight into the angiogenic effect and mechanism of action of astragaloside IV and provides evidence that the PI3K/Akt pathway acts as a regulator in HUVECs exposed to hypoxia.

Authorship Contributions

Participated in research design: Zhang, Liu, Zhao, Gao, and Wang. Conducted experiments: Zhang, Liu, and Lu. Performed data analysis: Zhang, Liu, and Lu. Wrote or contributed to the writing of the manuscript: Zhang, Zhao, Gao, and Wang.

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