



Astragaloside IV protects cardiomyocytes against hypoxia injury via *HIF-1 α* and the *JAK2/STAT3* pathway

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Background: Hypoxia is an important cause of myocardial injury due to the heart's high susceptibility to hypoxia. Astragaloside IV (AS-IV) is the main component of *Astragalus membranaceus* and could exert cardiac protective role. Here, the effect of AS-IV on hypoxia-injured H9c2 cardiomyocytes was elucidated.

Methods: First, H9c2 cells were exposed to hypoxia and/or AS-IV treatment. Cell apoptosis, death, and viability as well as *hypoxia-inducible factor 1 α* (*HIF-1 α*) expression and apoptotic proteins were analyzed. Next, transfection of si-*HIF-1 α* into H9c2 cells was carried out to test whether upregulation and stabilization of *HIF-1 α* influences the effect of AS-IV on hypoxia-treated H9c2 cells. Furthermore, the regulatory role of *Janus kinase 2/signal transducer and activator of transcription 3* (*JAK2/STAT3*) signaling on *HIF-1 α* levels was examined.

Results: Hypoxia suppressed viability and promoted the apoptosis and death of H9c2 cells. AS-IV eliminated hypoxia-induced H9c2 injury. Moreover, *HIF-1 α* signaling was further activated and stabilized by AS-IV in hypoxia-challenged H9c2 cells. Downregulation of *HIF-1 α* suppressed the function of AS-IV in hypoxia-challenged H9c2 cells. AS-IV promoted *JAK2/STAT3* signaling in hypoxia-induced injury. The beneficial functions of AS-IV in hypoxia-exposed H9c2 cells were linked to *HIF-1 α* upregulation and *JAK2/STAT3* signaling activation.

Conclusions: AS-IV relieved H9c2 cardiomyocyte injury after hypoxia, possibly by activating *JAK2/STAT3*-mediated *HIF-1 α* signaling.

Keywords: H9c2; hypoxia; astragaloside IV (AS-IV); *hypoxia-inducible factor 1 α* (*HIF-1 α*); *Janus kinase 2/signal transducer and activator of transcription 3* signaling (*JAK2/STAT3* signaling)

Submitted Jul 20, 2021. Accepted for publication Sep 18, 2021.

doi: 10.21037/atm-21-4080

View this article at: <https://dx.doi.org/10.21037/atm-21-4080>

Introduction

Cardiovascular disease (CVD) is a public health crisis that accounts for >30% of all deaths globally (1). Cardiovascular conditions induced by hypoxia, such as myocardial infarction, peripheral vascular disease,

stroke, and renal ischemia, contribute to high mortality and disability rates (2). In particular, apoptosis and death of cardiomyocytes are common in many forms of CVD (3). COVID-19 is an ongoing pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)

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infection (4,5). Myocardial injury has been described in many patients with COVID-19 (6,7). Hypoxia itself is one of the main mechanisms of COVID-19-related myocardial injury (8). Thus, experimental and clinical research is urgently needed to determine the molecular basis of hypoxia-induced cardiomyocyte injury and to identify effective treatments.

Astragaloside IV (AS-IV), 3-O-beta-d-xylopyranosyl-6-O-beta-d-glucopyranosyl-cycloastragenol (C₁₄H₆₈O₁₄), is extracted from the dried roots of *Astragalus membranaceus* (9) and was approved by the Chinese Pharmacopoeia 2010 (10). Previous studies have shown that AS-IV has anti-inflammatory (11), anti-viral (12), and anti-oxidative effects (13). In a rat aortic banding model, AS-IV prevented hypertrophy by increasing autophagy and inhibiting inflammation (14). In neonatal rat cardiomyocytes, AS-IV protected against hypoxia/reoxygenation (H/R)-induced injury by enhancing protein kinase A (PKA) and Ser(16) phosphorylated phospholamban [Ser(16)-PLN] expression (15). AS-IV pretreatment also attenuated anoxia/reoxygenation (A/R) injury by improving B cell lymphoma-2 (*Bcl-2*)-mediated mitochondrial function in both *in vitro* and *in vivo* experiments (16). Furthermore, the compatibility of tanshinone IIA and AS-IV restored H9c2 cell survival by inhibiting stress-responsive generic *mitogen-activated protein kinase* (MAPK) signaling and enhancing serine/threonine kinase 1 (*AKT*) phosphorylation (17). AS-IV might exert its treatment role via regulating calcium homeostasis and anti-apoptosis and anti-oxidation effect (18). AS-IV has calcium antagonistic role, inhibits calcium overload and restores myocardial calcium pumps activity (19). AS-IV could also promote angiogenesis via VEGF activation (20). Currently, the pathomechanism of AS-IV in hypoxia-triggered damage is not sufficiently understood.

Hypoxia-inducible factor-1 (*HIF-1*) comprises *HIF-1α* and *HIF-1β* that act as transcriptional mediators in response to hypoxia (21). The levels of *HIF-1* increase under decreased oxygen pressure. Its upregulation enhances erythropoiesis, modulation of vascular tone, glycolysis, and angiogenesis (22). Past studies have shown a significant increase in *HIF-1α* in tunnel positioning areas surrounding the infarct in human ischemic heart tissues and heart failure (HF) post-acute myocardial infarction (AMI) rats (22,23). There have also been reports showing that *HIF-1α* activation mediated through *prolyl 4-hydroxylase-2* (*PHD2*) gene silencing by siRNA treatment attenuates myocardial ischemia-reperfusion injury (IRI) (24). In a rabbit model

of IRI, stimulation of *HIF-1α* upregulated *heme oxygenase 1* (*HO-1*) levels, which attenuated proinflammatory cytokine production (25). Thus, stabilizing *HIF-1α* increases myocardial tissue tolerance to ischemia-induced injury.

Signal transducer and activator of transcription 3 (*STAT3*) is stimulated by *Janus kinase 2* (*JAK2*)-mediated phosphorylation and modulates cell growth and differentiation. *In vivo* experiments indicate that *STAT3* can subvert the negative effects associated with ischemia on reactive oxygen species and the electron transport chain in MLS-*STAT3E* mice (26). *In vitro* evidence has also shown that in glioma cells (U-87MG and U-138MG), *HIF-1α*, p-*STAT3*, and p-*JAK* are reduced by *EGF*, *latrophilin*, and seven *transmembrane domain containing 1* (*ELTD1*) knockdown. Further co-immunoprecipitation data showed that p-*STAT3* binds to *HIF-1α* (27). Previous studies have demonstrated that the *JAK2/STAT3/HIF-1α* axis is implicated in osteoclastogenesis (28), angiogenesis (29), neuroprotection (30), and anti-tumorigenesis (31).

There is limited data on the benefits of AS-IV in cardiovascular tissues and the effect on *JAK2/STAT3/HIF-1α* signaling in hypoxia. Here, we evaluated the impact of AS-IV on hypoxia-related damage to the rat cardiac muscle cell line H9c2, and its underlying mechanisms. We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-4080>).

Methods

Cell culture and hypoxia cell model

H9c2 cells were purchased from the National Collection of Authenticated Cell Cultures and passaged in DMEM-H medium (Gibco, New York, NY, USA) enriched with 10% FBS (Gibco), 2 mM glutamine (Gibco), 100 U/mL streptomycin (Beyotime, Shanghai, China), and 100 U/mL penicillin (Beyotime) in a humidified incubator at 37 °C, 5% CO₂. To induce injury, H9c2 cells were passaged in a hypoxic incubator with 1% O₂, 5% CO₂, and 94% N₂ for the indicated durations. Cells grown in normoxic conditions (21% O₂) were used as controls.

Cell transfection

Specific siRNAs targeting rat *HIF-1α* siRNA (si-*HIF-1α*) and negative control siRNA (si-NC) were obtained from RiboBio (Guangzhou, China) and transfected into

H9c2 cells at 100 nM using Exfect transfection reagent (Vazyme, Nanjing, China) according to the manufacturer's instructions. Briefly, H9c2 cells (2×10^5) were grown in 6-well plates for 24 h. Exfect transfection reagent was added to serum-free media followed by incubation for 5 min at room temperature (RT). The diluted siRNA was gently mixed with medium containing Exfect transfection reagent and allowed to incubate for 15 min at RT before adding the mixture to each well containing 80% confluent cells and medium. The transfected cells were grown under 37 °C and CO₂ conditions for 24 h prior to downstream experiments.

AS-IV and AG490 treatment

AS-IV (Shyuanye, Shanghai, China) and AG490 (MedChemExpress, Shanghai, China) were diluted in DMSO. H9c2 cells were challenged with AS-IV (12.5 μM) and AG490 (5 μM) individually or in combination under normoxic/hypoxic conditions for the indicated durations.

Cell survival analysis

H9c2 cell survival was assessed using the Cell Counting Kit-8 assay (CCK-8, Dojindo, Shanghai, China). H9c2 cells (100 μL) were grown at 2×10^4 cells/mL in 96-well plates overnight. After treatment, 100 μL of media enriched with 10% CCK-8 solution was added to the cells through media exchange modes. After 1 h of incubation, absorbance was measured at 450 nm on a microplate reader (Molecular Devices, San Jose, CA, USA). Each experiment was performed was repeated at least 6 times. The cell survival rate was expressed as a percentage of the control.

Calcein-AM/propidium iodide (PI) staining

After different treatments, the cells were rinsed twice using 1× buffer. Then, each group of cells were reconstituted in 1× assay buffer and stained with 2 μM calcein-AM (Dojindo, Shanghai, China) and 5 μM PI (Dojindo, Shanghai, China) per well and incubated at 37 °C for 30 min protected from light. Representative images were then captured on the IX73 fluorescence microscope (Olympus, Tokyo, Japan).

Immunocytochemistry analysis

For immunocytochemistry, 4% PFA (Boster, Wuhan, China) was used to fix cells at 4 °C for 30 min followed by permeabilization with 0.5% Triton X-100 (Beyotime) for

15 min. Subsequently, non-specific blocking was performed with 2% BSA (Beyotime) at 4 °C for 1 h, followed by incubation with anti-*HIF-1α* (ab179483, Abcam, Cambridge, UK) at 1:500 overnight at 4 °C. Next, the cells were subjected to another incubation with DyLightFluor® 555-conjugated donkey anti-rabbit IgG secondary antibody (A0453, Beyotime) at 1:500 for 1 h at RT and counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) at 10 ng/mL in the dark for 15 min. Finally, imaging was performed immediately on an Olympus IX73 microscope.

Assessment of cell apoptosis

An Annexin V-FITC/PI apoptosis kit (Keygentec, Nanjing, China) was used for cell staining and flow cytometry following the manufacturer's instructions. Briefly, cells from each group were washed twice with PBS and 2×10^5 cells were reconstituted in 500 μL of binding buffer. Immediately after, 5 μL of Annexin V-FITC was added and mixed well. This was followed by the addition of 10 μL PI and incubation for 5 min at RT in the dark in preparation for apoptosis analysis on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

Isolation of total RNA and real-time PCR

The TRIzol reagent (Vazyme) was utilized for RNA extraction followed by cDNA synthesis with a reverse transcription kit (Vazyme). The reverse transcription PCR (RT-PCR) assay was then performed on a CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA) with the following parameters: denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and extension at 72 °C for 25 s. The 2^{-ΔΔCT} method was used to determine mRNA levels. The PCR was based on the indicated primers: *HIF-1α* (forward: 5'-atgtaccctaacaagccggg-3', reverse: 5'-acaatcagcaccagcagc-3'), *β-actin* (forward: 5'-cccatctatgagggttagc-3', reverse: 5'-ttaaagtgcagcagcatttc-3'). All the primers were obtained from by Genaray Bioengineering (Shanghai, China).

Protein extraction and western blot assay

Briefly, we used RIPA buffer (Beyotime) to lyse cells for protein extraction following the manufacturer's instructions. After separation with 10% SDS-PAGE and transfer to

PVDF membranes (Millipore, Billerica, MA, USA), the membranes were then blocked using 5% BSA (Beyotime) for 1 h and incubated with the indicated antibodies at 4 °C overnight: *HIF-1 α* (ab179483, 1:1,000), *Bcl-2* (ab196495, 1:1,000), *Bax* (ab32503, 1:5,000) (all from Abcam), caspase-3 (14220, 1:1,000), *JAK2* (3230, 1:1,000), *p-JAK2* (3776, 1:1,000), *STAT3* (9139, 1:1,000), *p-STAT3* (9145, 1:2,000) (all from Cell Signalling Technology, Boston, MA, USA), and *β -actin* (AF0003, 1:2,000, Beyotime). After washing with TBST thrice (10 min each), the membrane was probed with HRP-conjugated anti-rabbit IgG (A0208, Beyotime) or anti-mouse IgG (A0216, Beyotime) secondary antibodies at RT for 1 h and washed thrice with TBST. The signal was then developed using ECL (Beyotime) and imaged on a chemiluminescence detection system (Bio-Rad Laboratories). Band intensities were analyzed by Image J.

Statistical analysis

All the experiment were repeated three times. All data are shown as mean \pm SD and were analyzed with Graphpad Prism 5.0. Comparisons between groups were assessed using Student's *t*-test. $P < 0.05$ indicated statistical significance.

Results

Hypoxia triggers injury in H9c2 cells

Cell survival, cell death, apoptosis, and western blot assays were conducted to elucidate the impact of hypoxia. Sustained hypoxia treatment significantly decreased the cell survival rate, as the survival was only $31.6\% \pm 7.1\%$ after 72 h (Figure 1A). The representative images of calcein-AM/PI staining showed that hypoxia treatment markedly enhanced cell death in H9c2 cells (Figure 1B). Additionally, hypoxia treatment significantly induced H9c2 cell apoptosis compared with the normoxia-treated group (Figure 1C). The expression levels of apoptosis-related proteins also changed markedly, with the *Bcl-2/Bax* ratio reduced and cleaved caspase-3 levels elevated by hypoxia (Figure 1D). These effects of hypoxia on cells were time-dependent.

Hypoxia alters HIF-1 α protein level in H9c2 cells

HIF-1 α modulates responses to hypoxia (32). Compared with the normoxic group, 6 h of hypoxia treatment significantly increased *HIF-1 α* levels. However, *HIF-1 α* levels did not consistently increase after hypoxia treatment

for 12–48 h, but gradually fell. Immunocytochemistry analysis results were consistent with the western blot findings (Figure 2A,2B).

AS-IV suppresses hypoxia-induced injury and upregulates HIF-1 α levels in H9c2 cells in a sustained way

Hypoxia triggers H9c2 cell injury (33). The CCK-8 assay showed that treatment with AS-IV (12.5 μ M) time-dependently improved the survival rate of hypoxia-exposed H9c2 cells (Figure 3A). Next, we assessed how AS-IV affects *HIF-1 α* expression in H9c2 cells challenged with hypoxia for 6 and 48 h. We observed that relative to normoxic controls, *HIF-1 α* levels were sharply elevated upon 6 h of hypoxic injury and were markedly reduced after 48 h. Interestingly, after treatment of H9c2 cells subjected to hypoxia for 6 or 48 h with AS-IV (12.5 μ M), *HIF-1 α* levels remained elevated relative to the normoxic and hypoxia-only groups. Immunocytochemistry results were consistent with the western blot findings (Figure 3B,3C).

Downregulation of HIF-1 α represses AS-IV-mediated protection against hypoxic damage

To examine whether *HIF-1 α* has an essential role in AS-IV-mediated protection of hypoxic injury, *HIF-1 α* was inhibited using siRNA in H9c2 cells. It was confirmed that siRNA2 efficiently suppressed *HIF-1 α* expression using quantitative RT-PCR (RT-qPCR) and western blot analyses. Thus, we chose *HIF-1 α* siRNA2 for subsequent cytology experiments (Figure 4A,4B). At 24 h after siRNA transfection, AS-IV (12.5 μ M) treatment was simultaneously performed when H9c2 cells were placed under hypoxic conditions at specified time points (6 and 48 h). Western blot and immunocytochemistry results revealed that the *HIF-1 α* level in the NC + hypoxia + AS-IV group was significantly higher than in the si-*HIF-1 α* + hypoxia + AS-IV group (Figure 4C,4D). Similarly, there were corresponding findings in the next series of experiments. The cell survival rate was apparently lower, while cell death and apoptosis were discernibly higher in the si-*HIF-1 α* + hypoxia + AS-IV group compared to the NC + hypoxia + AS-IV group (Figure 4E-4G). To confirm this result, we assessed the expression levels of apoptosis-related proteins in all samples. The western blot results showed that the level of cleaved caspase-3 was visibly increased, while the ratio of *Bcl-2/Bax* was significantly decreased in the si-*HIF-1 α* + hypoxia + AS-IV group compared to the NC + hypoxia + AS-IV

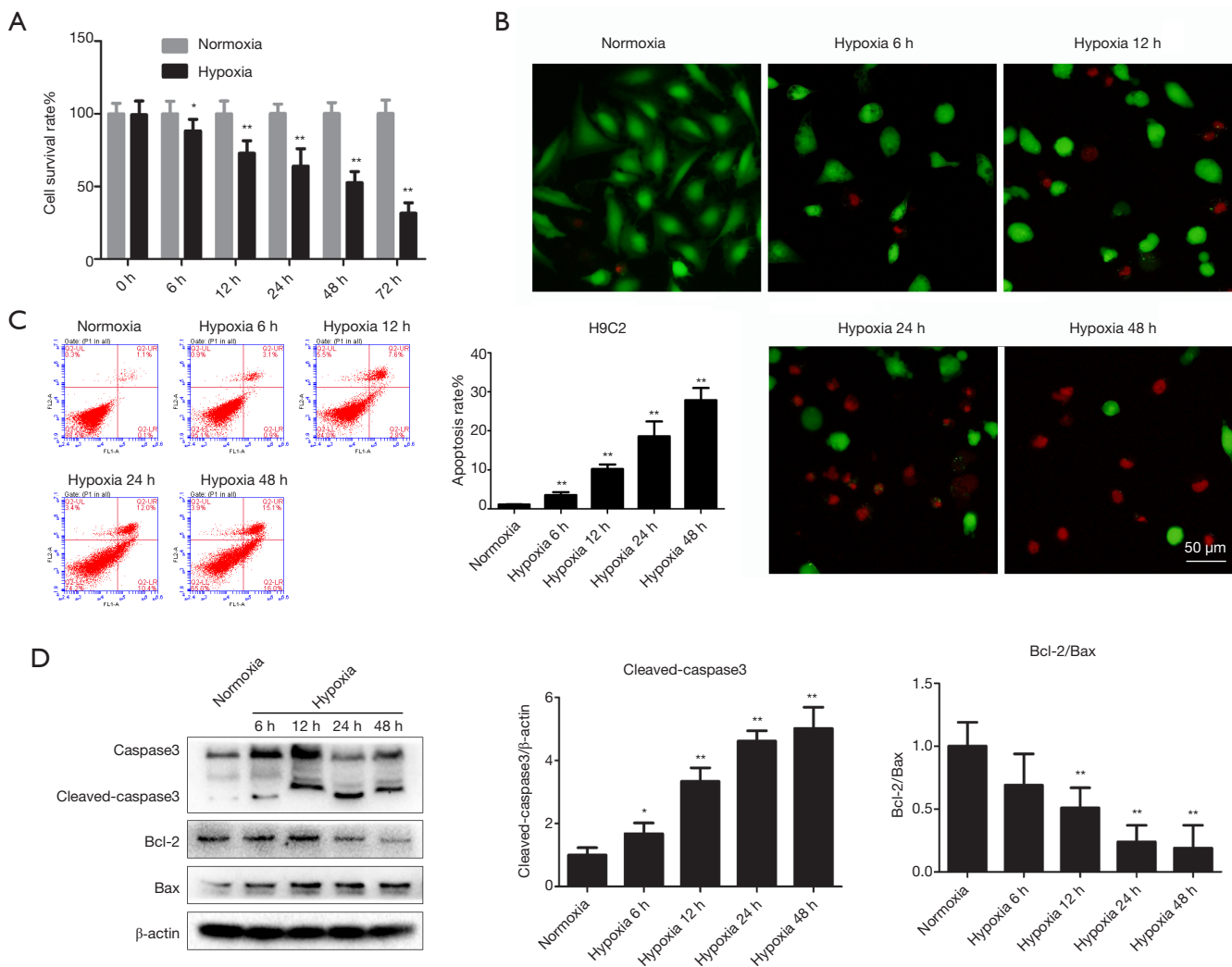


Figure 1 Hypoxia causes hypoxic injury in H9c2 cells. The effects of hypoxia on H9c2 cells were examined by assaying cell survival, apoptosis, calcein-AM/PI staining, and apoptosis-related proteins. (A) The H9c2 cell survival rate was determined after exposure to hypoxia for the indicated periods. (B) Cell death was detected using calcein-AM (green) and PI (red) staining. (C) Apoptosis was evaluated using flow cytometry. (D) Western blot analysis of the levels of the apoptotic proteins cleaved caspase-3, *Bcl-2*, and *Bax*. β -actin was used as the loading control. Results are shown as mean \pm SD of three independent experiments performed in duplicate. * $P < 0.05$ or ** $P < 0.01$ vs. normoxia group. PI, propidium iodide.

group (Figure 4H). The results described above indicate that *HIF-1 α* silencing blocks AS-IV-mediated protection under hypoxic conditions.

AS-IV prevents hypoxic injury of H9c2 cells by regulating JAK2/STAT3/HIF-1 α signaling

JAK2 phosphorylates *STAT3* and activates the *JAK2/STAT3* pathway (34). To test whether *JAK2/STAT3* signaling

mediates hypoxia-induced injury in cardiomyocytes, the phosphorylation levels of *JAK2* and *STAT3* were measured. This analysis revealed that p-*STAT3* and p-*JAK2* levels consistently fell after hypoxia treatment for 6 and 48 h, while AS-IV (12.5 μ M) restored and even increased their levels (Figure 5A). To confirm these findings, we suppressed the *JAK2/STAT3* pathway using AG490 (5 μ M), a *JAK2* inhibitor (35), and assessed p-*STAT3*, *STAT3*, p-*JAK2*, *JAK2*, and *HIF-1 α* expression by western

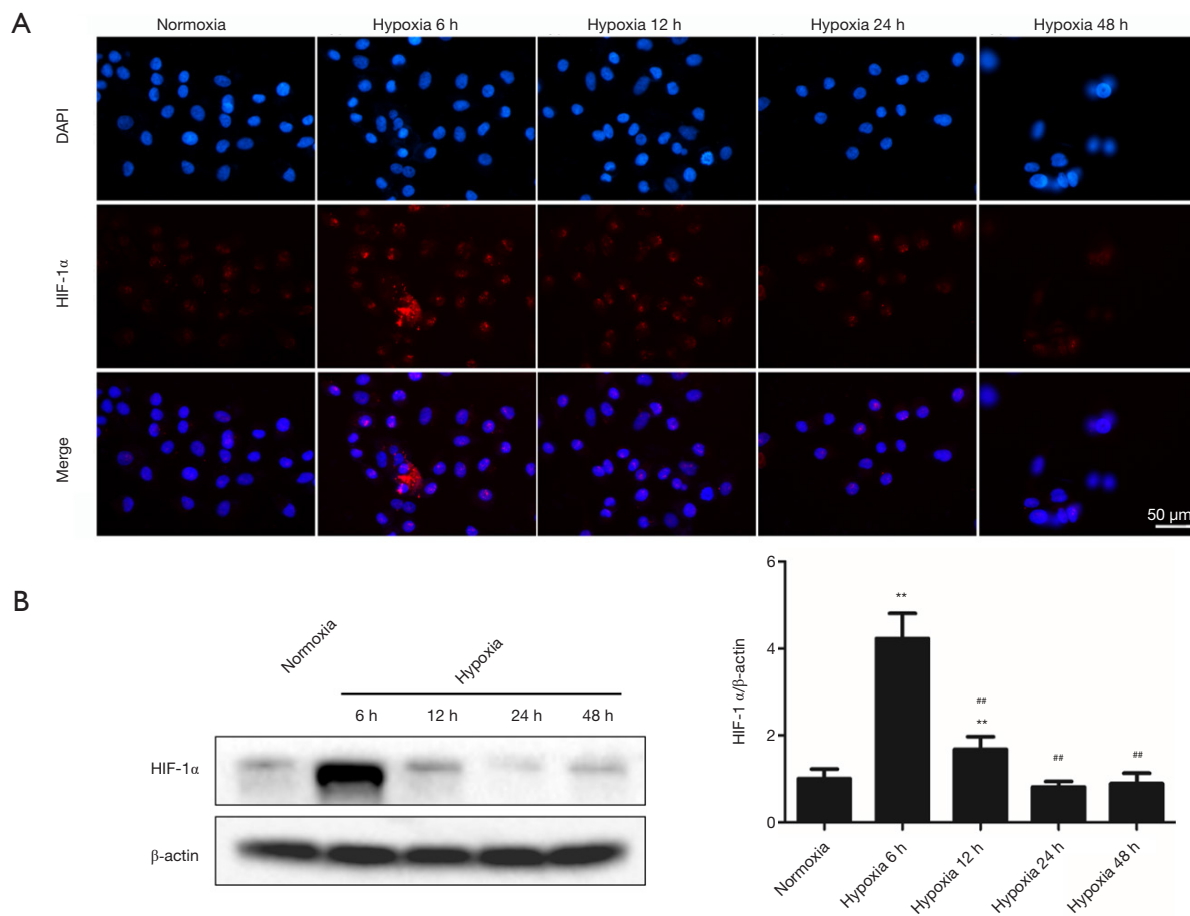


Figure 2 *HIF-1α* protein levels in response to hypoxia induction. (A,B) *HIF-1α* levels were measured using immunocytochemistry and western blot after exposure to hypoxia for the indicated periods. Results are mean \pm SD of three independent experiments performed in duplicate. ** $P < 0.01$ vs. normoxia group; ## $P < 0.01$ vs. 12 h hypoxia group. *HIF-1α*, hypoxia-inducible factor 1 α .

blot. The western blot analysis revealed a marked reduction of p-*JAK2* and p-*STAT3* expression in the hypoxia + AS-IV + AG490 group relative to the hypoxia + AS-IV group (Figure 5B). Moreover, *HIF-1α* protein levels followed the same trend (Figure 5B). The corresponding immunocytochemistry observation of *HIF-1α* expression strengthened the western blot results (Figure 5C). These findings suggested that AS-IV promotes *HIF-1α* expression and stability by regulating upstream factors like p-*JAK2* and p-*STAT3*. The CCK-8 assay revealed that the cell survival rate in the hypoxia + AS-IV group was obviously increased relative to the hypoxia + AS-IV + AG490 group at both 6 and 48 h (Figure 5D). The degree of cell death and apoptosis and the expression of apoptosis-related proteins in the hypoxia + AS-IV group were much lower than the

hypoxia + AS-IV + AG490 group (Figure 5E-5G). Together, these data demonstrate that AG490 suppresses the benefits of AS-IV on hypoxic damage by blocking the *JAK2/STAT3/HIF-1α* signaling pathway.

Discussion

CVD claims many lives worldwide. Oxygen is crucial to the metabolism and survival of aerobic cells. Oxygen tension influences numerous cellular processes, including proliferation, cell differentiation, glycolysis, apoptosis, angiogenesis, and gene expression. Severe hypoxia may cause apoptosis and even necrosis. Previous study found angiogenesis is critical in the recovery of myocardial ischemia. AS-IV could regulate angiogenesis and *HIF-1α*

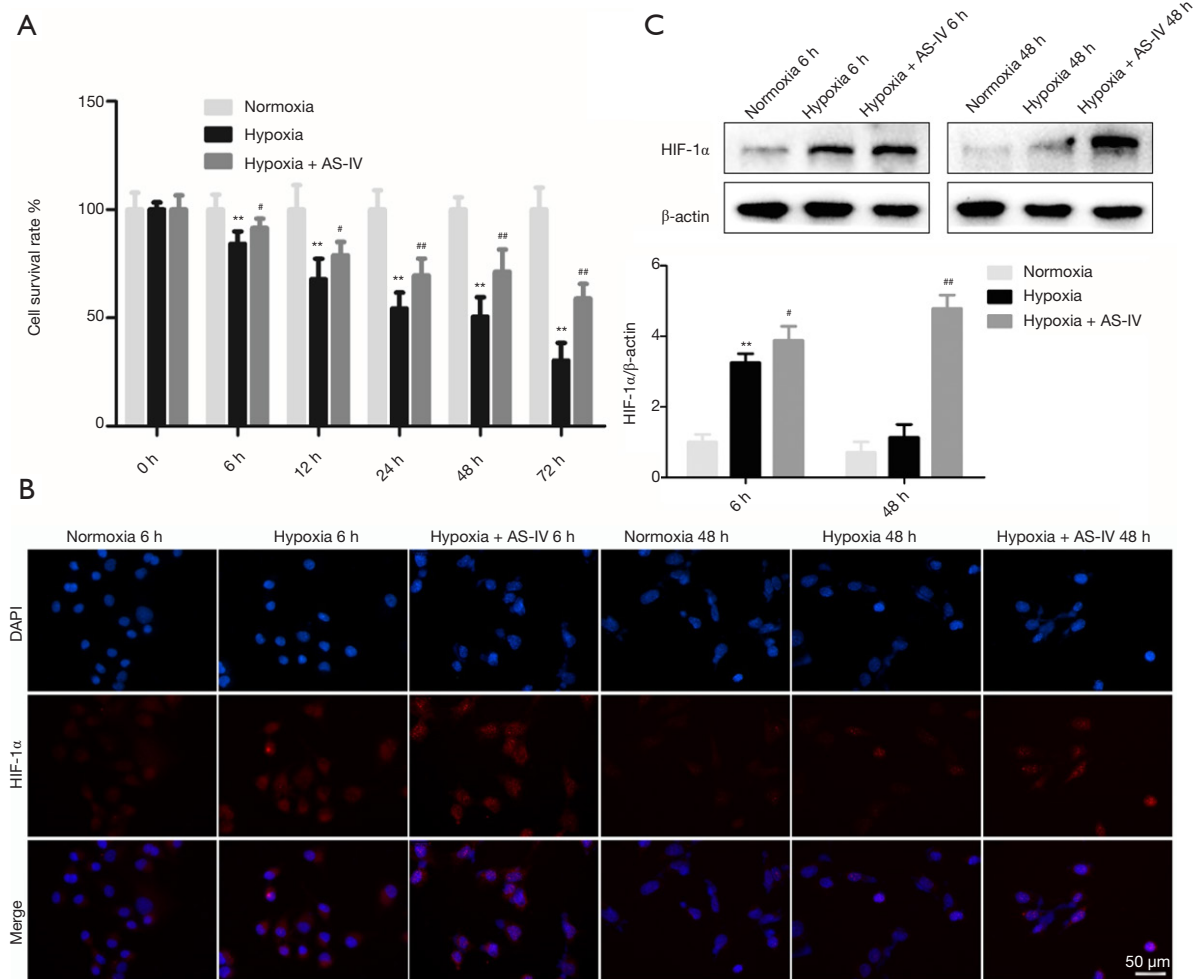
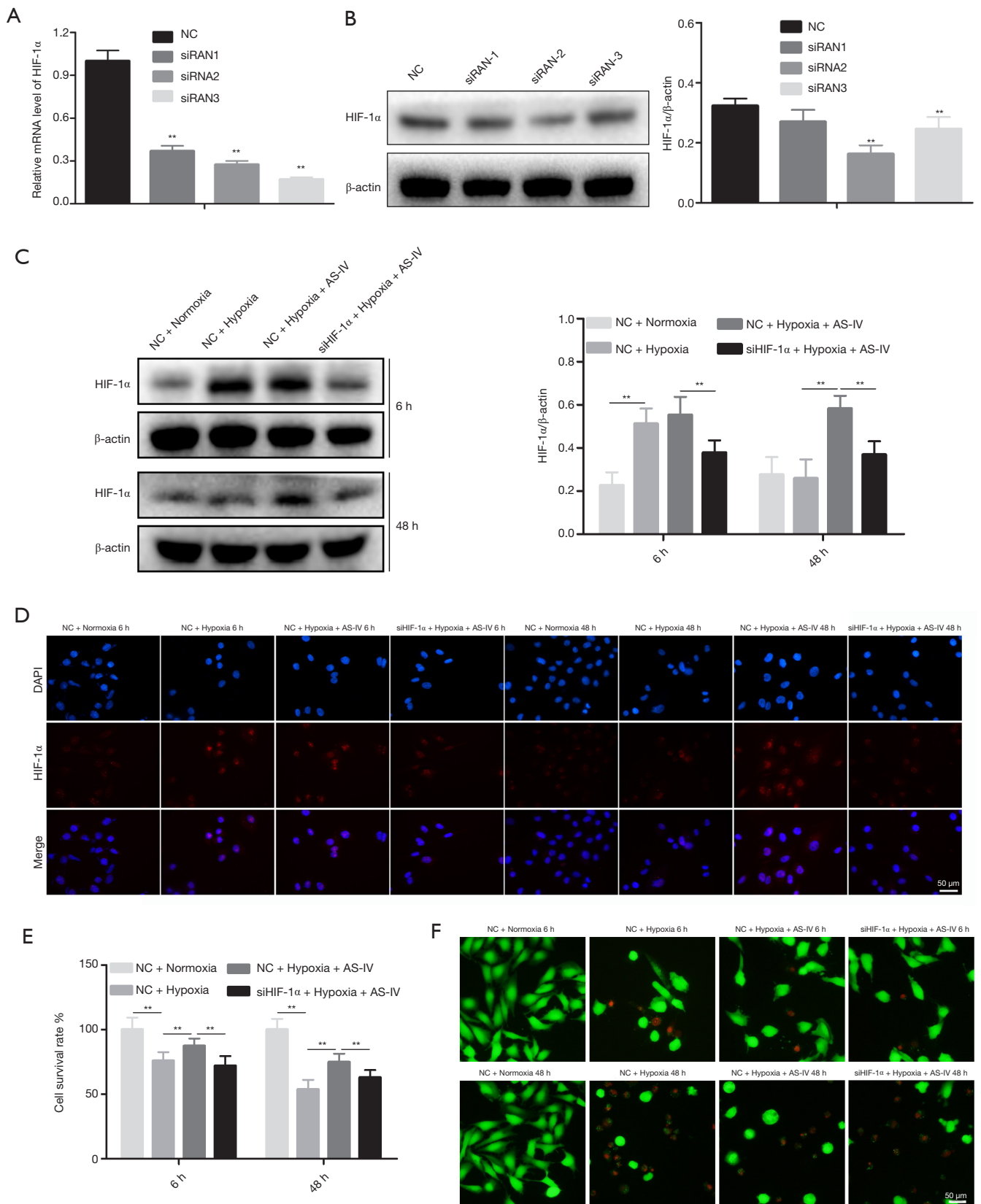


Figure 3 The effects of AS-IV treatment on hypoxic injury and *HIF-1α* protein expression. (A) The H9c2 cell survival rate was determined after exposure to hypoxia and AS-IV treatment for the indicated durations. (B,C) *HIF-1α* protein levels were determined by immunocytochemistry and western blot after exposure to hypoxia and AS-IV treatment for the indicated durations. Results are mean \pm SD of three independent experiments performed in duplicate. ** $P < 0.01$, hypoxia group vs. normoxia group; # $P < 0.01$ or ## $P < 0.01$, hypoxia + AS-IV group vs. hypoxia group. AS-IV, astragaloside IV; *HIF-1α*, hypoxia-inducible factor 1 α .

level in human umbilical vein endothelial cells via PI3K/Akt pathway (36). Hypoxia induces *HIF-1α* expression, and its expression level in the nucleus depends on the balance between its ubiquitination and small ubiquitin-related modifier (SUMO) modification (37). The expression of SUMO1 could stabilize the HIF-1 α . AS-IV could promote the expression of SUMO1. Thus, the treatment of AS-IV might promote angiogenesis via promoting the level of SUMO1 and the stabilization of *HIF-1α* (38). Cardiomyocytes are the main functional cells in pumping blood. Cardiomyocyte injury by hypoxia is a cause of several ischemic heart disorders (39). Here, we examined

how AS-IV affects hypoxia-induced damage of H9c2 cells. Our data show that AS-IV stimulated and stabilized *HIF-1α* expression in H9c2 cells challenged with hypoxia. *HIF-1α* upregulation contributed to the effects of AS-IV on enhancing viability and reducing the apoptosis of H9c2 cells under hypoxic conditions. Our data also indicate that AS-IV enhanced the activation of *JAK2/STAT3* signaling, which upregulated *HIF-1α* in H9c2 cells.

Myocardial tissue has very limited innate regenerative capacity, and there are no myocardial tissue regeneration strategies available. Thus, myocardial injury is irreversible (40). Cardiomyocyte injury is mainly caused by cardiomyocyte



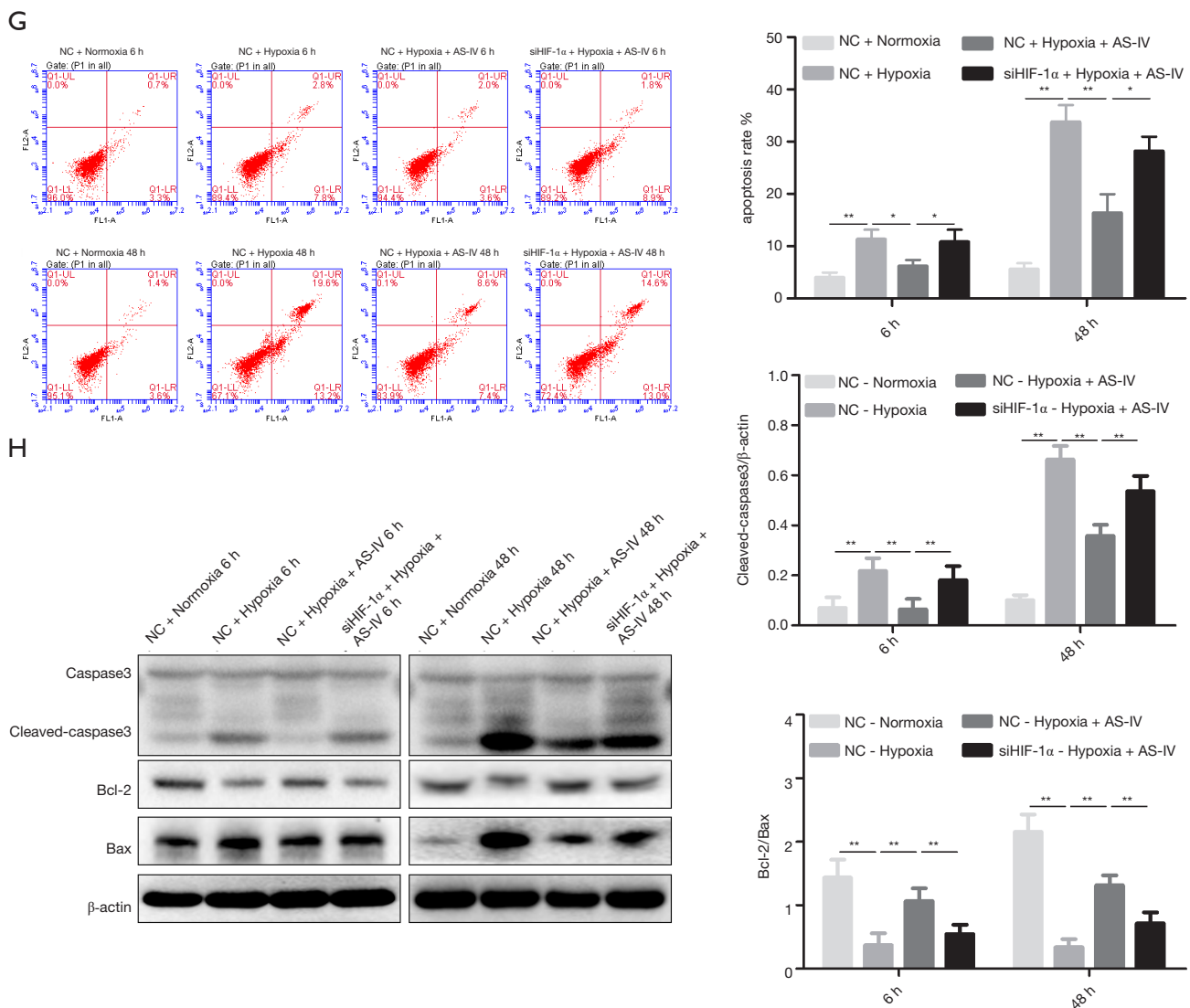


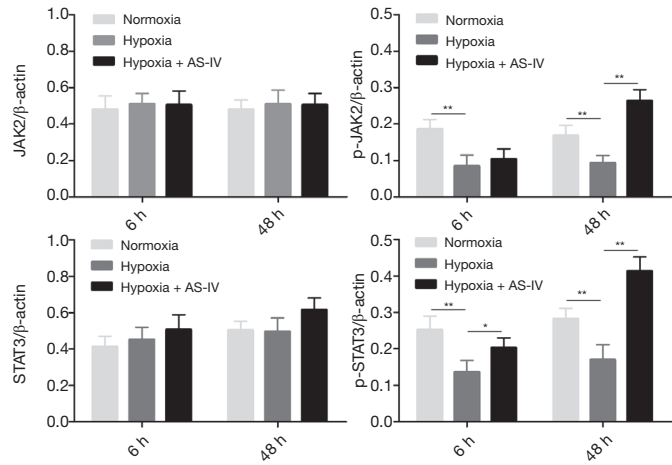
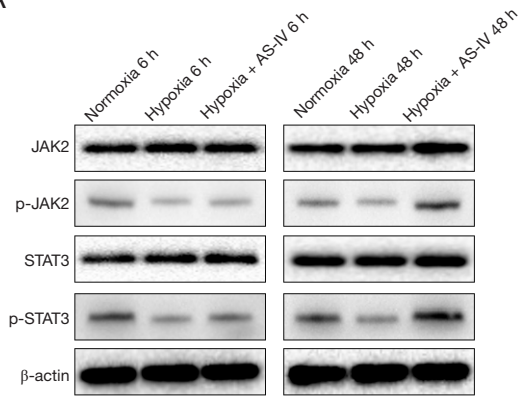
Figure 4 The protective effects of AS-IV on hypoxic injury were suppressed by *HIF-1α* downregulation. (A,B) RT-qPCR and western blot analysis of *HIF-1α* expression in H9c2 cells transfected with si-*HIF-1α* or control si-NC. (C,D) *HIF-1α* protein levels after siRNA transfection, and the effects of hypoxia and AS-IV treatment were determined by immunofluorescence and western blot analyses. (E) Cell survival was measured using the CCK-8 assay. (F) Cell death was assayed by calcein-AM/PI staining. (G) Apoptosis was assayed by flow cytometry. (H) Western blot analysis of the levels of the apoptotic proteins cleaved caspase-3, *Bcl-2*, and *Bax*. β -actin was used as the loading control. Results are mean \pm SD of three independent experiments performed in duplicate. * $P < 0.05$ or ** $P < 0.01$ vs. corresponding group. AS-IV, astragaloside IV; *HIF-1α*, hypoxia-inducible factor 1 α ; RT-qPCR, quantitative reverse transcription PCR; si-*HIF-1α*, *HIF-1α* siRNA; si-NC, negative control siRNA; CCK-8, Cell Counting Kit-8; PI, propidium iodide.

apoptosis. Here, we exposed H9c2 cells to hypoxia to establish an *in vitro* model of myocardial injury and tested cell viability, apoptosis, and death. Our data show that hypoxia stimulates cell damage and apoptosis, and transiently upregulates *HIF-1α*.

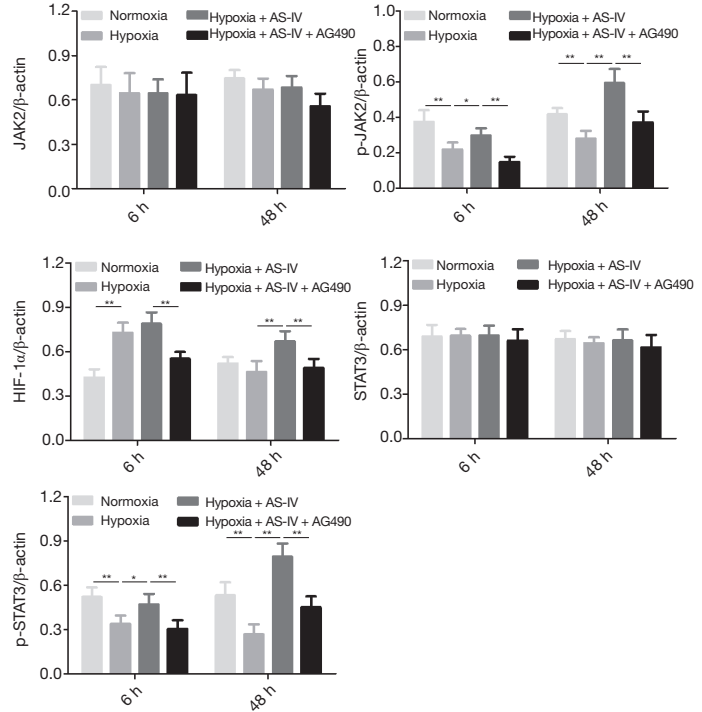
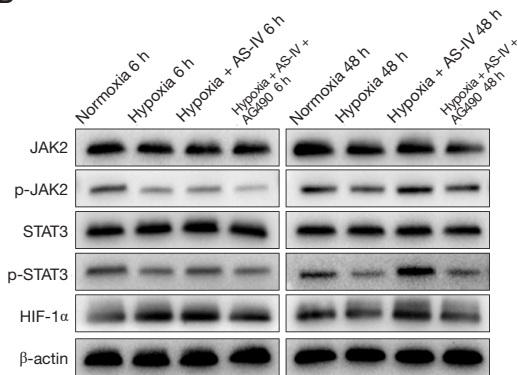
As-iv is one of the main active components of Astragalus

membranaceus. AS-IV can reduce the apoptosis of neurons after intracerebral hemorrhage (41) and reduce the area of myocardial infarction (42). AS-IV has been widely used to treat cardiovascular, liver, kidney, and skin diseases in China (43). AS-IV is a therapeutic agent with a variety of activities and a potential therapeutic role in myocardial

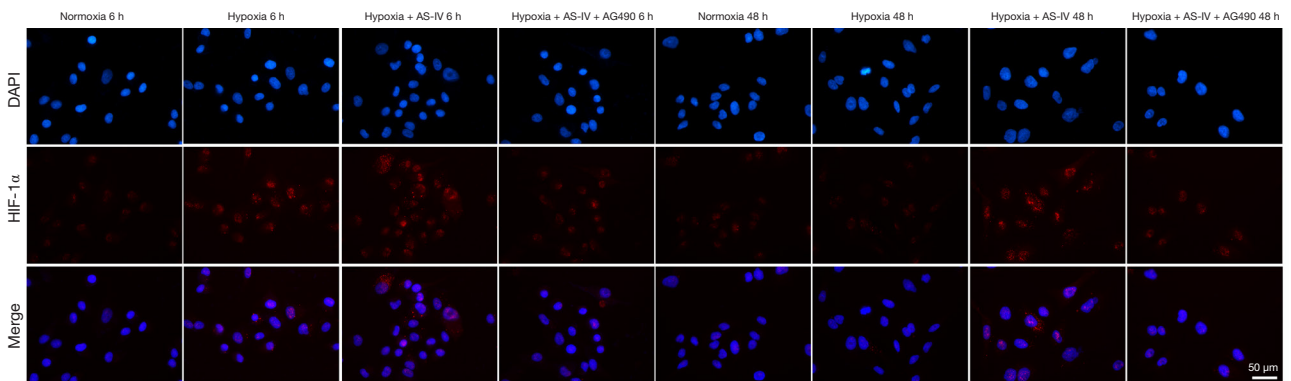
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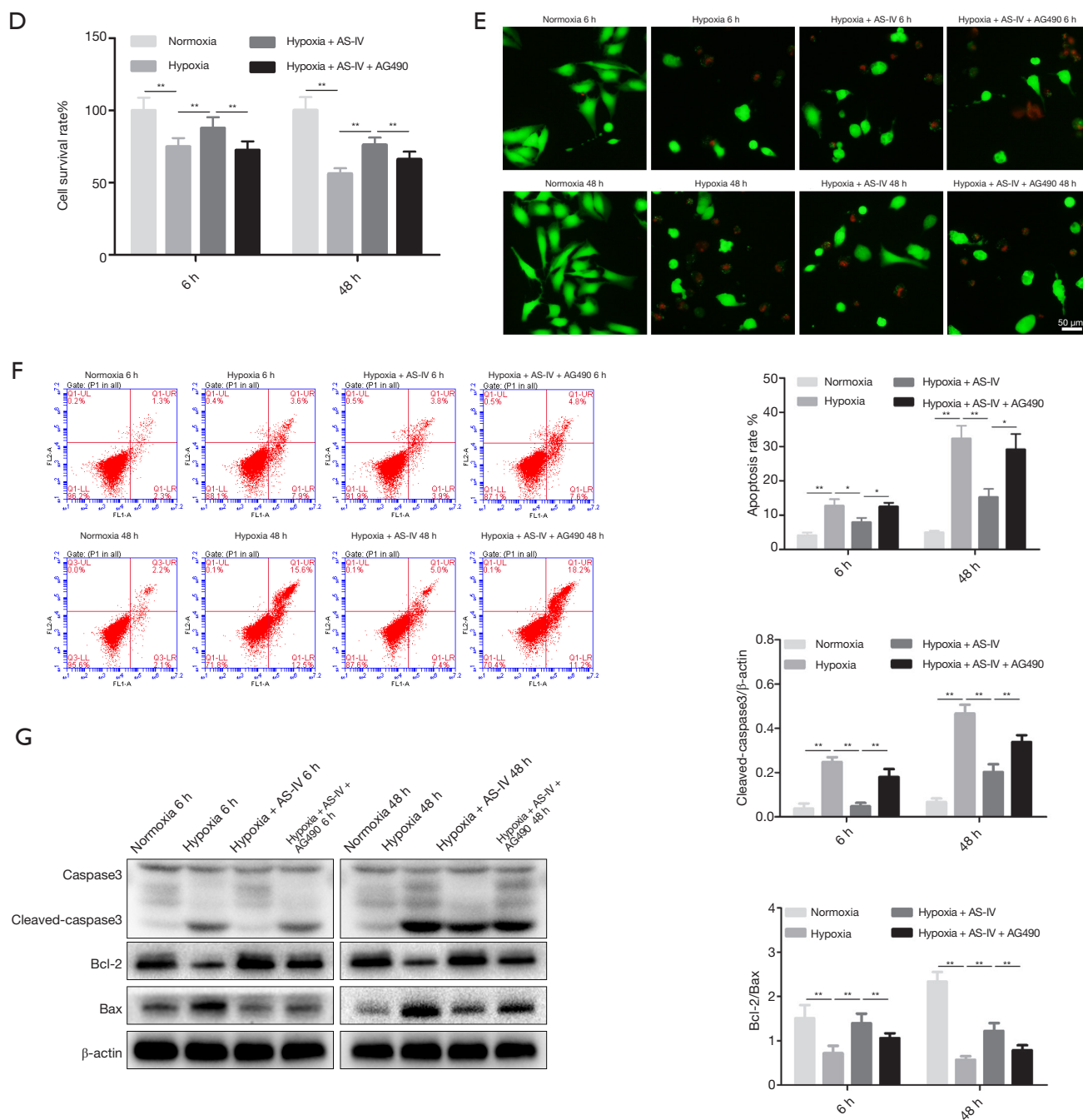


Figure 5 Influence of $JAK2$ - $STAT3$ signaling on the protective effects of AS-IV against hypoxic injury. (A) Western blot analysis of $JAK2$ /p- $JAK2$ and $STAT3$ /p- $STAT3$ expression after exposure to hypoxia and AS-IV treatment for the indicated durations. (B) Western blot analysis of $JAK2$ /p- $JAK2$, $STAT3$ /p- $STAT3$, and $HIF-1\alpha$ expression after exposure to hypoxia, AG490, and AS-IV for the indicated durations. (C) After the corresponding treatment, $HIF-1\alpha$ expression changes were detected by immunocytochemistry. (D) Cell survival was evaluated using the CCK-8 assay. (E) Cell death was detected using calcein-AM/PI staining. (F) Apoptosis was evaluated by flow cytometry. (G) Western blot analysis of the apoptotic proteins cleaved caspase-3, $Bcl-2$, and Bax. β -actin was used as the loading control. Results are mean \pm SD of three independent experiments performed in duplicate. * $P < 0.05$ or ** $P < 0.01$ vs. corresponding group. $JAK2$, Janus kinase 2; $STAT3$, signal transducer and activator of transcription 3; AS-IV, astragaloside IV; $HIF-1\alpha$, hypoxia-inducible factor 1 α ; CCK-8, Cell Counting Kit-8; PI, propidium iodide.

protection. Besides AS-IV, there are other drugs could protect cardiomyocytes against hypoxia damage, like Dimethyl malonate (44), chloramphenicol succinate (45), mitochondrial division inhibitor-1 (46), miR-499 (47) and rapamycin (48). Astragalus membranaceus is a traditional drug that widely used in China for thousand years. AS-IV is the main component of Astragalus membranaceus. AS-IV has great treatment potential and is relatively easy to transfer to clinical application.

Past findings show that AS-IV relieves H9c2 cardiomyocyte damage under hypoxic conditions by modulating miRNAs (49), lncRNAs (50), Ca^{2+} homeostasis (51), and *PI3K/AKT/HO-1* signaling (52). Evidence has also showed AS-IV can exert myocardial protective role via *PI3K/AKT/GSK-3 β* pathway (53). The angiogenesis could also be regulated by AS-IV via *PTEN/PI3K/Akt* pathway (54). Here, we found that *HIF-1 α* was increased and hypoxia-induced injury were decreased hypoxia model with treatment of 12.5 μ M AS-IV.

HIF-1 α is a key transcription factor that controls adaptive responses to hypoxia by regulating downstream target genes (55). *HIF-1 α* could induce the expression of iNOS. The level of iNOS is upregulated in cardiomyocytes after hypoxia treatment. iNOS pathway could be activated by AS-IV induced *HIF-1 α* activation (56). Normally, *HIF-1 α* activity depends on the amount of *HIF-1 α* protein, which is markedly upregulated by hypoxia. However, the *HIF-1 β* protein is constitutively expressed in cells regardless of oxygen tension (57). *HIF-1 α* protein levels are determined by a balance between protein synthesis and degradation. *HIF-1 α* protects against oxygen-glucose deprivation/reperfusion (OGD/R) injury by upregulating the expression of *BCL2*/adenovirus E1B 19 kDa protein-interacting protein 3 (*Bnip3*) in H9c2 cells (58). *Ubiquitin-conjugating enzyme E2S (UBE2S)* protected against apoptosis after myocardial ischemia/reperfusion (MI/R) injury, which might have contributed to the activation of *HIF-1 α* (59). In the present study, *HIF-1 α* was shown to be upregulated in the presence of AS-IV treatment.

JAK2/STAT3 is an important signaling pathway involved in hypoxia/ischemia and oxidative stress in various tissues and organs, as well as having protective roles in hypoxic and ischemic injury (60). Past studies show that *STAT3* acts upstream of *HIF-1 α* , and elevated *HIF-1 α* levels correspond with elevated *STAT3* phosphorylation (61,62). A mouse model of myocardial ischemia revealed that myocardial cell apoptosis and the area of myocardial infarction were

significantly increased by IRI after treatment with AG490. To confirm whether *JAK2/STAT3/HIF-1 α* signaling mediated hypoxia-induced injury in cardiomyocytes, we silenced *HIF-1 α* using siRNA and inhibited *JAK2/STAT3* phosphorylation with AG490 and found that AS-IV protection was weakened in the hypoxia model.

In conclusion, our data show that *HIF-1 α* expression is upregulated by short exposure to hypoxia. In contrast, this change was reduced by prolonged modeling time and aggravated cell injury. However, upon treatment with AS-IV under hypoxic conditions, *HIF-1 α* expression was upregulated and stabilized. Furthermore, analysis of *JAK2/STAT3* signaling activation suggested that AS-IV positively regulates *HIF-1 α* by enhancing *JAK2/STAT3* phosphorylation. In contrast, genetic silencing of *HIF-1 α* and blocking of *JAK2/STAT3* signaling by AG490 resulted in the inhibition of the protective effect of AS-IV. Together, these findings indicate that AS-IV protects H9c2 cells from hypoxic injury by modulating *JAK2/STAT3/HIF-1 α* signaling. Our findings may provide initial theoretical support for the study of the regulatory mechanism of AS-IV in hypoxia-induced cardiomyocyte injury, and suggest innovative therapeutic strategies for the treatment of CVDs.

Acknowledgments

Funding: The work was financially supported by Science and Technology Research Project of Jiangxi Provincial Department of Education (Project No. GJJ201541).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://dx.doi.org/10.21037/atm-21-4080>

Data Sharing Statement: Available at <https://dx.doi.org/10.21037/atm-21-4080>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/atm-21-4080>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are 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.

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- (English Language Editor: C. Betlzar)

Cite this article as: Li B, Yu J, Liu P, Zeng T, Zeng X. Astragaloside IV protects cardiomyocytes against hypoxia injury via *HIF-1 α* and the *JAK2/STAT3* pathway. *Ann Transl Med* 2021;9(18):1435. doi: 10.21037/atm-21-4080