Astragaloside IV attenuates cardiac hypertrophy in rats born from mothers with intrauterine hypoxia through the PKCβII/Egr-1 pathway

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Abstract. Astragaloside IV (AS-IV) is a naturally occurring agent that confers several wide-ranging reported pharmacological effects, such as cardioprotective, antioxidative and pro-angiogenic activities. Although it was previously reported that AS-IV could attenuate neonatal rat myocardial ischemia-reperfusion injury, the possible effects of AS-IV on the development of cardiac hypertrophy associated with intrauterine hypoxia (IUH) remain unclear. The present study established a model of IHU by placing the pregnant rats in a plexiglass chamber with an oxygen supply of 10% before neonatal rat delivery. To investigate the *in vivo* effect of AS-IV on cardiac hypertrophy, neonatal rats with hypertension were randomly grouped to receive AS-IV (20 mg/kg), AS-IV (40 mg/kg), AS-IV (80 mg/kg) or vehicle for 12 weeks, followed by left ventricular (LV) hemodynamics and heart tissue histological analysis. Rats born from mothers with IHU displayed pathological features of cardiac hypertrophy. However, AS-IV 40 and 80 mg/kg significantly decreased the heart/body weight (BW), LV mass (LVM)/BW, heart mass/tibia length (TL) and LVM/TL ratios. H&E staining showed that 40 and 80 mg/kg AS-IV prevented the morphometric changes induced by IHU. According to data from LV hemodynamics measurements, AS-IV 80 mg/kg reversed the increased systolic blood pressure, diastolic blood pressure, LV systolic pressure, LV end-diastolic pressure, dP/dt maximum and heart rate induced by IHU. Mechanistically, ERK1/2 activation and early growth response 1 (Egr-1) protein expression were both upregulated by IHU induction, which was reversed by AS-IV treatment. In conclusion, these data suggested that AS-IV could attenuate cardiac hypertrophy in neonatal rats born from mothers with IHU through the protein kinase C β type isoform 2/Egr-1 pathway, but the underlying mechanism requires further investigation.

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Introduction

Intrauterine hypoxia is a relatively common complication that can occur during pregnancy (1). It can adversely impact cardiac myogenesis and increase the risk of heart disease in children (1). In addition, the health problems associated with intrauterine hypoxia (IUH) continue into adulthood (2). Cardiac hypertrophy is one such issue that can arise and increases the mortality rate (3). However, the underlying mechanism of cardiac hypertrophy development that is associated with IHU remains unclear.

Protein kinase C β type isoform 2 (PKCβII) belongs to the PKC family of serine- and threonine-specific protein kinases that can be activated by calcium and the second messenger diacylglycerol (4). PKCβII was shown to regulate several different cellular processes, including apoptosis induction, cell proliferation and metabolism, by phosphorylating a wide variety of different target proteins (5). Early growth response 1 (Egr-1) is one of these PKCβII downstream targets (6). Egr-1 is a member of the C2H2-type zinc-finger family of proteins and functions as a transcription regulator (7). It was demonstrated that hypoxia/reoxygenation could induce cardiomyocyte injury through the PKCβII/Egr-1 pathway (8). However, the potential effects of this pathway on the induction of cardiac hypertrophy associated with IHU remain unclear.

Astragaloside IV (AS-IV) is a bioactive, naturally occurring compound that can be extracted from the plant *Astragalus membranaceus* (8). It is applied as a traditional Chinese medicine for the treatment of viral and bacterial infections, inflammation and cancer (9). Previous studies showed that AS-IV could serve a potential role in protecting the heart against myocardial ischemia (10). The mechanism of action may involve antioxidative and nitric oxide-inducing properties, reduction of intracellular calcium levels and sarcoplasmic reticulum calcium load and decreased lipid peroxidation (11).

Furthermore, another previous study indicated that AS-IV could attenuate neonatal rat myocardial ischemia-reperfusion injury through the PKCβ/Egr-1 pathway (12).

The present study investigated the effects of AS-IV on the development of cardiac hypertrophy associated with IHU.

Materials and methods

Animals. AS-IV was purchased from Sigma-Aldrich (Merck KGaA) and was dissolved in distilled water. The animal study protocol was approved by the Medical Ethics Committee of Zhejiang Provincial People's Hospital (approval no. 2020022). Experiments were performed on 8-week-old male and female rats (body weight, 220-250 g) obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Charles River Laboratories, Inc.). All of the animals were kept in an environment with controlled temperature (22-25˚C) and humidity (50-65%) and a 12-h light/dark cycle with free access to food and water.

The adult parent rats were sacrificed by inhalation of 4% isoflurane followed by cervical dislocation after delivering the neonatal rats. Neonatal rats were sacrificed by inhalation of 4% isoflurane followed by cervical dislocation at the end of the experiment.

The humane endpoints of the present study were: i) Abnormal physical appearance, including abnormal posture, rough coat, head tucked into the abdomen, exudate surrounding eyes and/or nose, skin lesions and abnormal breathing; ii) ≥20% body weight loss (as compared with the original body weight of the animal); and iii) body condition score <2.0.

Experimental protocol. For the present study, 3-month-old male and female rats were mated in one cage at a ratio of one male to two females. The female rats were adjudged to be pregnant by checking if there was a vaginal plug or if there was sperm in the vaginal secretion smear of the female rat obtained the following morning. If a rat was found to be pregnant at this stage, that was considered as day 0 of pregnancy.

In the hypoxia group, 10 pregnant rats were then placed in a plexiglass chamber from day 15-21 of pregnancy with an oxygen supply of 10% for 4 h every day. In the control group, 2 pregnant rats were placed in an atmosphere with a normal air composition. The percentage of oxygen in the plexiglass chamber (XBS-03; AIPU Laboratory; https://www.cn-aipu.com/laboratory.html) was monitored using a continuous infusion of a mixture of nitrogen gas and air with an oxygen analyzer (2KY-4F; AIPU Laboratory). During hypoxia, arterial blood was collected every 60 min, which was immediately used to measure oxygen partial pressure, blood oxygen saturation and pH using a detection chip (ABL-9 blood gas analyzer; Radiometer Medical ApS). The arterial partial pressure of oxygen in the pregnant rats was maintained at 50-55 mmHg and the blood oxygen saturation was maintained at 80-85% (13,14). Subsequently, 100 μl arterial blood was collected from the tail artery and the rats did not need to be anesthetized before this step. By contrast, the pregnant rats in the control group were kept at room temperature with an oxygen concentration of 21% until natural delivery.

After birth, neonatal rats from the two treatment groups were reared with their mothers and housed under room air conditions. Neonatal rats born from the pregnant rats in the control group constituted the control (Ctrl) group for the subsequent experiments performed on neonatal rats.

After 4 weeks, the blood pressure of the rats was measured and the 42 neonatal rats with hypertension were randomly divided into four groups: i) IUH; ii) IUH + AS-IV (20 mg/kg); iii) IUH + AS-IV (40 mg/kg); and iv) IUH + AS-IV (80 mg/kg). Rats in the Ctrl and IUH groups were administered with distilled water (1 ml/kg/day), whilst the treatment groups received AS-IV 20, 40 or 80 mg/kg/day (oral gavage) for 5 days a week (administering AS-IV continuously for 5 days, then suspended for 2 day) for 12 weeks (15). All rats from different groups were kept in the same environment with controlled temperature (22-25˚C) and humidity (50-65%) and a 12-h light/dark cycle and free access to food and water.

LV hemodynamics experiments. Rats were anaesthetized using isoflurane, 4% for induction and 1.5% for maintenance of anesthesia for ~15-30 min by following the guidelines of Animal Care Committee of The University of British Columbia (16). A 2-F microtip pressure-volume catheter (SPR-838; Millar, Inc.) was then inserted into the right carotid artery and advanced into the ascending aorta. After a 5-min stabilization period, the arterial blood pressure was recorded before the catheter was advanced into the left ventricle under pressure control. Using a special pressure-volume analysis machine (cat. no. BL422I; Chengdu Techman Co., Ltd.), heart rate, systolic and diastolic blood pressures (SBP and DBP, respectively), mean arterial pressure, LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), stroke volume, ejection fraction, cardiac output, the maximal slope of the systolic pressure increment (dP/dt maximum), the maximal slope of the diastolic pressure decrement (dP/dt minimum) and systemic vascular resistance were all computed and calculated. To exclude the influence of body weight differences, cardiac output and stroke volume were normalized to body weight, yielding the cardiac and stroke volume indices. Ventricular relaxation was assessed using the time constant of LV pressure decay (τ), calculated using the Glantz method (τ-g; regression of dP/dt vs. pressure) (17). LV pressure-volume relations were assessed by transiently compressing the inferior vena cava. The slope E_{max} of the LV end-systolic pressure-volume relationship, pre-load recruitable stroke work and the slope of dP/dt maximum/end-diastolic volume relationship were calculated as load-independent indices of LV contractility. The slope of the end-diastolic pressure-volume relationship was calculated as an index of LV stiffness (18). Subsequently, the heart was removed and weighed.

Histological analysis. For histological analyses, heart tissues were fixed in 4% paraformaldehyde for 24-48 h at room temperature. The tissues were subsequently dehydrated in a graded ethanol series, cleared in toluene, embedded in paraffin and sliced into 5-μm sections. They were then stained with hematoxylin and eosin (H&E; C0105S; Beyotime Institute of Biotechnology) and images were captured to assess the overall cardiac morphology using an optical microscope (BX53; Olympus Corporation).

Dr Baomei He (co-author) and the technicians evaluated the histological preparations.

Western blot analysis. The heart tissues were dissected on ice and transferred into round-bottomed microcentrifuge tubes, where they were snap-frozen by immersing in liquid nitrogen. Ice-cold lysis buffer (1 mg tissue/50 μl; RIPA; Beyotime Institute of Biotechnology) was then added and the tissues were homogenized using an electric homogenizer. An additional 50 μl lysis buffer was added during homogenization. The lysates were then agitated at 4˚C for 2 h and centrifuged at 16,000 x g for 20 min at 4˚C before the supernatant was collected in a fresh tube and placed on ice. Total protein was quantified using a Bradford assay kit (Beyotime Institute of Biotechnology) before equivalent amounts of protein per lane (50 mg) were separated using SDS-PAGE on a 10% gel. The separated proteins were transferred onto PVDF membranes, which were blocked in TBS-Tween 20 (0.1%) containing 5% (w/v) skimmed milk (Beyotime Institute of Biotechnology) at 37˚C for 1 h. The membranes were incubated overnight at 4˚C with primary rabbit antibodies against Egr-1 (1:1,000; cat. no. sc-515830; Santa Cruz Biotechnology, Inc.), ERK1/2 (1:1,000; cat. no. 9102; Cell Signaling Technology, Inc.), phosphorylated (p-)-p44/42 ERK1/2 (1:1,000; cat. no. 9101; Cell Signaling Technology, Inc.), anti-MyHC polyclonal antibody (1:1,000; cat. no. K107673P; Solarbio Science & Technology Co., Ltd) and α-tubulin (1:1,000; H-300, cat. no. sc-5546; Santa Cruz Biotechnology, Inc.). After washing, the samples were incubated with HRP-labeled goat anti-rabbit IgG (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) or HRP-labeled goat anti-mouse IgG (1:1,000; cat. no. A0216; Beyotime Institute of Biotechnology) secondary antibodies at room temperature for 1 h. The immunoreactive proteins were then developed using an ultrasensitive ECL luminescent solution (Proteintech Group, Inc.) and captured using the Amersham Imager 680 (GE Healthcare). α-tubulin was used as the loading control.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from the heart tissue samples using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Total RNA was reverse transcribed into cDNA using a reverse transcription kit (PrimeScript™ RT Reagent Kit; cat. no. RR037A; Takara Biotechnology Co., Ltd.). The synthetized cDNA was amplified by RT-qPCR using the following primers: Egr-1 forward, 5'-AACAACCCT ACGAGCACCTG-3' and reverse, 5'-AAAGGGGTTCAG GCCACAAA-3'; and GAPDH forward, 5'-GCATCTTCTTGT GCAGTGCC-3' and reverse, 5'-GATGGTGATGGGTTTCCC GT-3'. The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec, 95˚C for 15 sec and 60˚C for 60 sec, before finishing with 95˚C for 15 sec. The target gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene GAPDH (19).

Statistical analysis. All statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software; Dotmatics). Statistical significance was analyzed using ANOVA followed by Tukey's post hoc test. Data are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

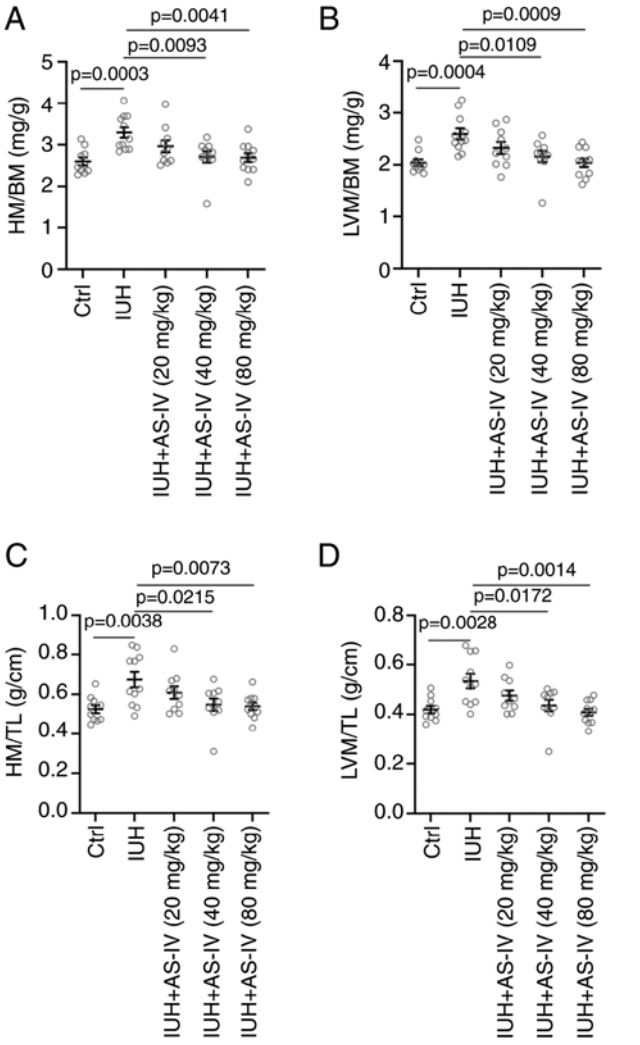

Figure 1. Astragaloside IV prevents the increase in the heart indices of rats born from mothers with intrauterine hypoxia. Quantitative analysis of (A) HM/BW, (B) LVM/BW, (C) HM/TL and (D) LVM/TL in control and treatment groups. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. IUH, intrauterine hypoxia; Ctrl, control group; AS-IV, astragaloside IV; BW, body weight; HM, heart mass; LVM, left ventricular mass; TL, tibia length.

Results

AS-IV prevents the increases in heart mass (HM)/body weight (BW), LVM/BW, HM/TL and LVM/TL ratios in rats born from mothers with IHU. Following the experimental procedure, the neonatal rats were anaesthetized at 12 weeks after birth for the subsequent tests. As shown in Fig. 1, compared with those in the control group (where their mothers were maintained at room temperature with an oxygen concentration of 21% until natural delivery) rats born from mothers with IHU exhibited the characteristics of cardiac hypertrophy, with higher HW/BW (Fig. 1A), LVM/BM (Fig. 1B), HM/TL (Fig. 1C) and LVM/TL (Fig. 1D) ratios. However, AS-IV 40 and 80 mg/kg attenuated these characteristics of cardiac hypertrophy and significantly decreased the

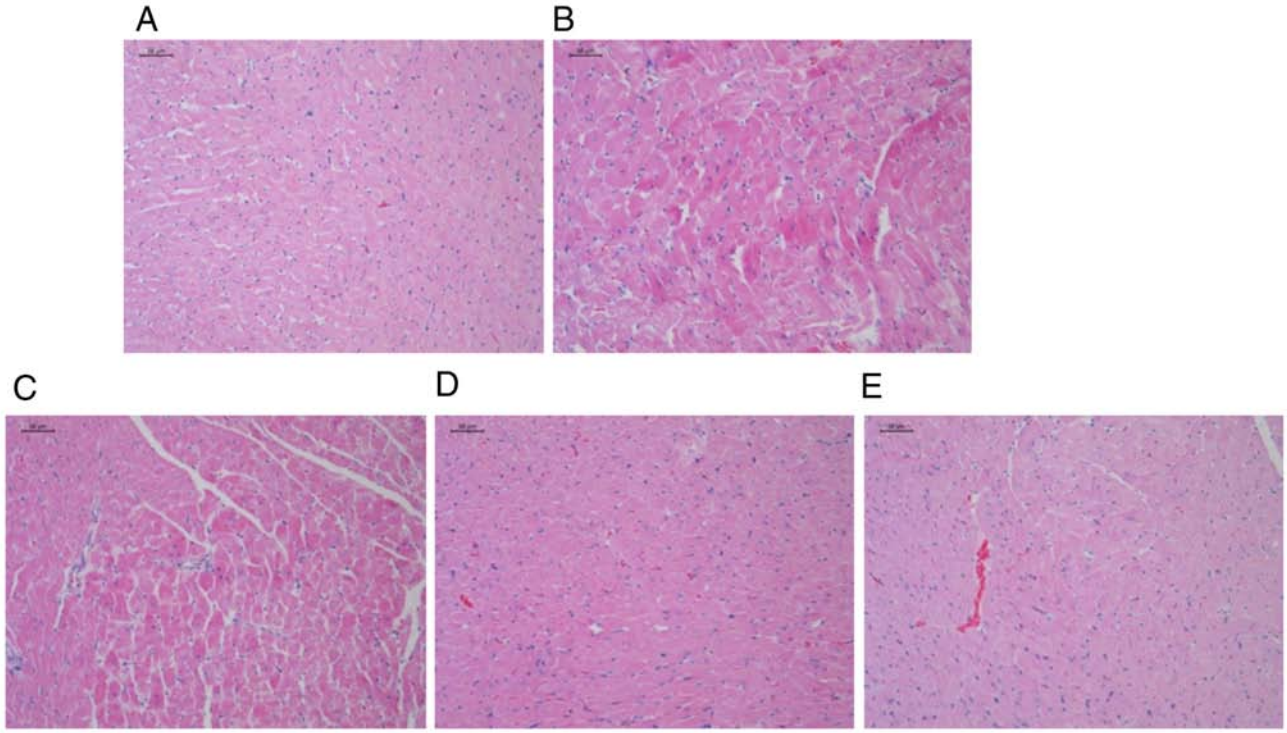

Figure 2. Representative images of hematoxylin and eosin staining in the heart tissues. (A) control group; (B) intrauterine hypoxia group; (C) Astragaloside IV 20 mg/kg group; (D) Astragaloside IV 40 mg/kg group; (E) Astragaloside IV 80 mg/kg group (scale bars, 50 μm).

HW/BW (Fig. 1A), LVM/BM (Fig. 1B), HM/TL (Fig. 1C) and LVM/TL (Fig. 1D) ratios compared with those in the model group.

AS-IV prevents the induction of myocardial damage in rats born from mothers with IHU. Microscopic investigation of the H&E-stained heart tissues from the Ctrl group demonstrated typical features of the normal endocardium and myocardium, with normal quantities and distribution of the vascular endomysium among cardiac cells. By contrast, heart tissues from rats in the IHU group showed focal areas of sub-endocardium degeneration, which were mainly found in the left ventricle. In addition, several focal areas of mononuclear cellular infiltrations could be observed, where there was an accumulation of fibrous tissues in the endomysium and increased thickness of the myocardium in the left ventricle; however, treatment with AS-IV 40 and 80 mg/kg prevented these morphometric changes (Fig. 2). Meanwhile, the marker of cardiac hypertrophy, β-myosin heavy chain (MyHC) expression level was also increased in heart tissues from rats born from mothers with IHU. AS-IV treatment decreased the upregulated MyHC (Fig. 4A).

AS-IV prevents the changes in the LV hemodynamics of rats born from mothers with IHU. Cardiac catheterization was performed at the end of the treatment. During cardiac catheterization, increases in the SBP, DBP, LVSP, LVEDP, dP/dt maximum and heart rate (HR) were observed in the IHU group compared with those in the control group (Fig. 3A-E). Treatment with 80 mg/kg AS-IV suppressed the increased SBP, DBP, LVSP, LVEDP, dP/dt maximum and HR induced by IHU (Fig. 3A-F). However, there were no differences in all the cardiac catheterization parameters between the AS-IV 20 mg/kg treatment group and the IHU group. These results suggested that AS-IV could prevent changes in the LV hemodynamics of rats born from mothers with IHU in a dose-dependent manner.

AS-IV prevents the activation of PKCβII/Egr-1 signaling in rats born from mothers with IHU. PKCβII and Egr-1 were reported to be associated with cardiomyocyte injury (20,21). Furthermore, AS-IV was shown to exert suppressive effects on PKCβII and Egr-1 activity and mRNA expression (12). In the present study, ERK1/2 phosphorylation and Egr-1 protein expression were markedly increased in rats born from mothers with IHU (Fig. 4A). Similarly, Egr-1 mRNA expression was also significantly increased compared with that in the control group (Fig. 4B). In addition, using immunohistochemistry assays, p-ERK1/2 levels were increased in the IHU group (Fig. 4C). In the AS-IV treatment groups, these increased levels of ERK1/2 phosphorylation and Egr-1 expression were significantly decreased compared with those in the IHU group (Fig. 4A-C).

Discussion

In the present study, treatment with 40 and 80 mg/kg AS-IV significantly decreased the HW/BW, LVM/BM, HM/TL and LVM/TL ratios in rats born from mothers with IHU. H&E staining demonstrated that AS-IV 40 and 80 mg/kg prevented the pathological changes induced by IHU. According to the LV hemodynamics experiments, AS-IV 80 mg/kg treatment ameliorated the increased SBP, DBP, LVSP, LVEDP, dP/dt maximum and HR induced by IHU. Mechanistically, ERK1/2 phosphorylation and Egr-1 protein expression levels were increased in rats born from mothers with IHU, which were reversed by AS-IV treatment.

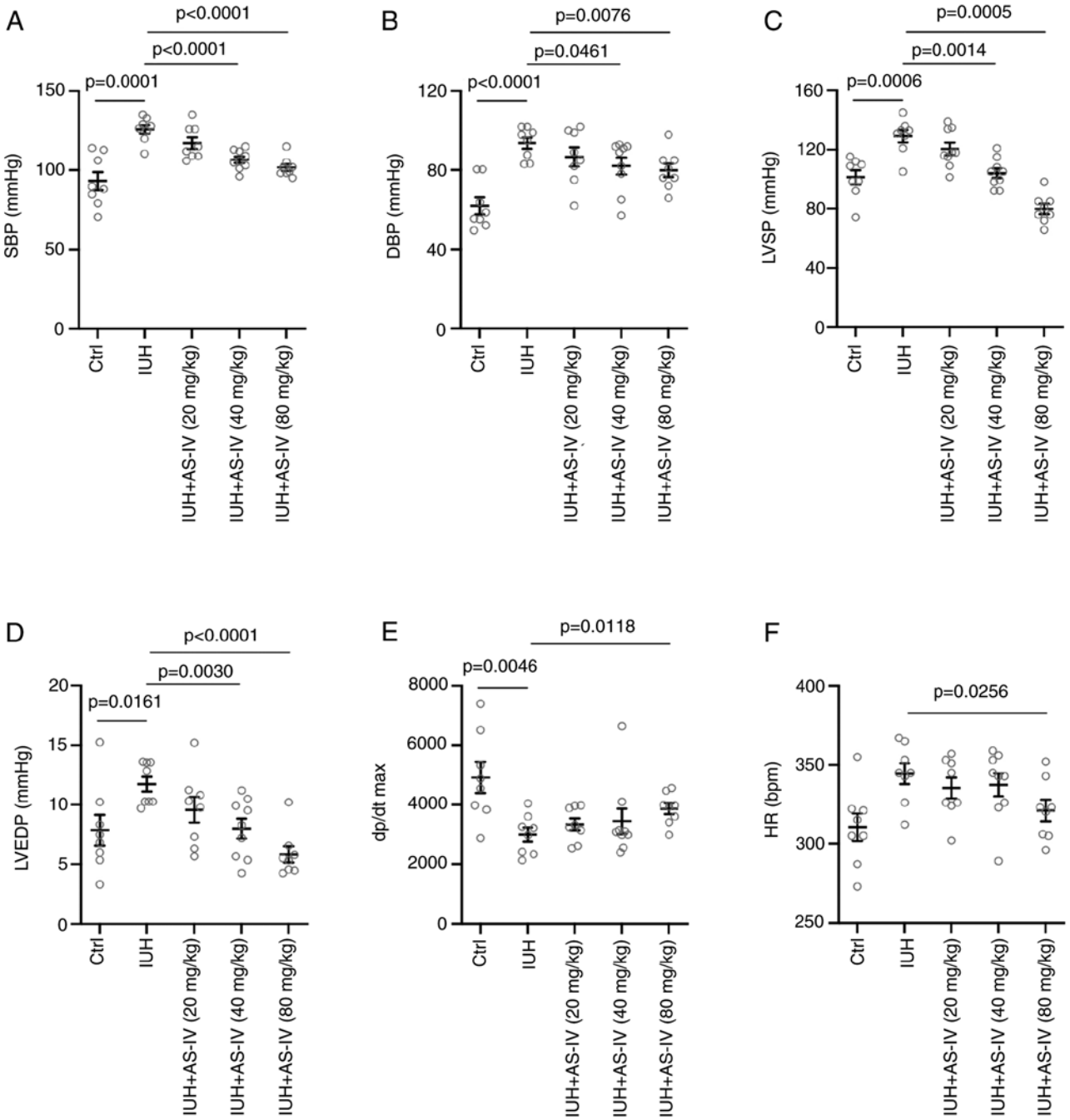

Figure 3. Astragaloside IV reverses cardiac dysfunction associated with intrauterine hypoxia. Echocardiography measurements of (A) Systolic blood pressure, (B) Diastolic blood pressure, (C) Left ventricular systolic pressure, (D) Left ventricular end-diastolic pressure, (E) Maximal slope of the systolic pressure increment and (F) Heart rate. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. IUH, intrauterine hypoxia; Ctrl, control group; AS-IV, astragaloside IV; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; dp/dt max, the maximal slope of the systolic pressure increment; HR, heart rate.

Intrauterine hypoxia was shown to occur when the fetus is deprived of an adequate supply of oxygen, which may be due to a variety of reasons, such as prolapse or occlusion of the umbilical cord, placental infarction, maternal diabetes (prepregnancy or gestational diabetes) and maternal smoking (22). Although the role of oxygen in the development of the fetus remains controversial, it was proposed that the lack of oxygen *in utero* may be responsible for increasing the risk of cardiovascular disorders in the offspring (23). Data from the present study indicated that after placing the pregnant rats in a plexiglass chamber with a maternal oxygen supply of 10%, their offspring exhibited pathological features of cardiac hypertrophy when growing up. This suggested that IHU could increase the risk of cardiac hypertrophy development in children and young adults.

AS-IV is the most abundant saponin and a marker compound found in *Astragali Radix*, a Chinese herb prescribed in compound formulas for medicinal purposes or dietary purposes as a functional food in China and other eastern Asian countries (19). Its use was associated with its reported potent immune-promoting and anti-aging effects (24). A recent pharmacological study investigated the properties of AS-IV, including its cardioprotective, angiogenic, hepatoprotective, neuroprotective, anti-inflammatory

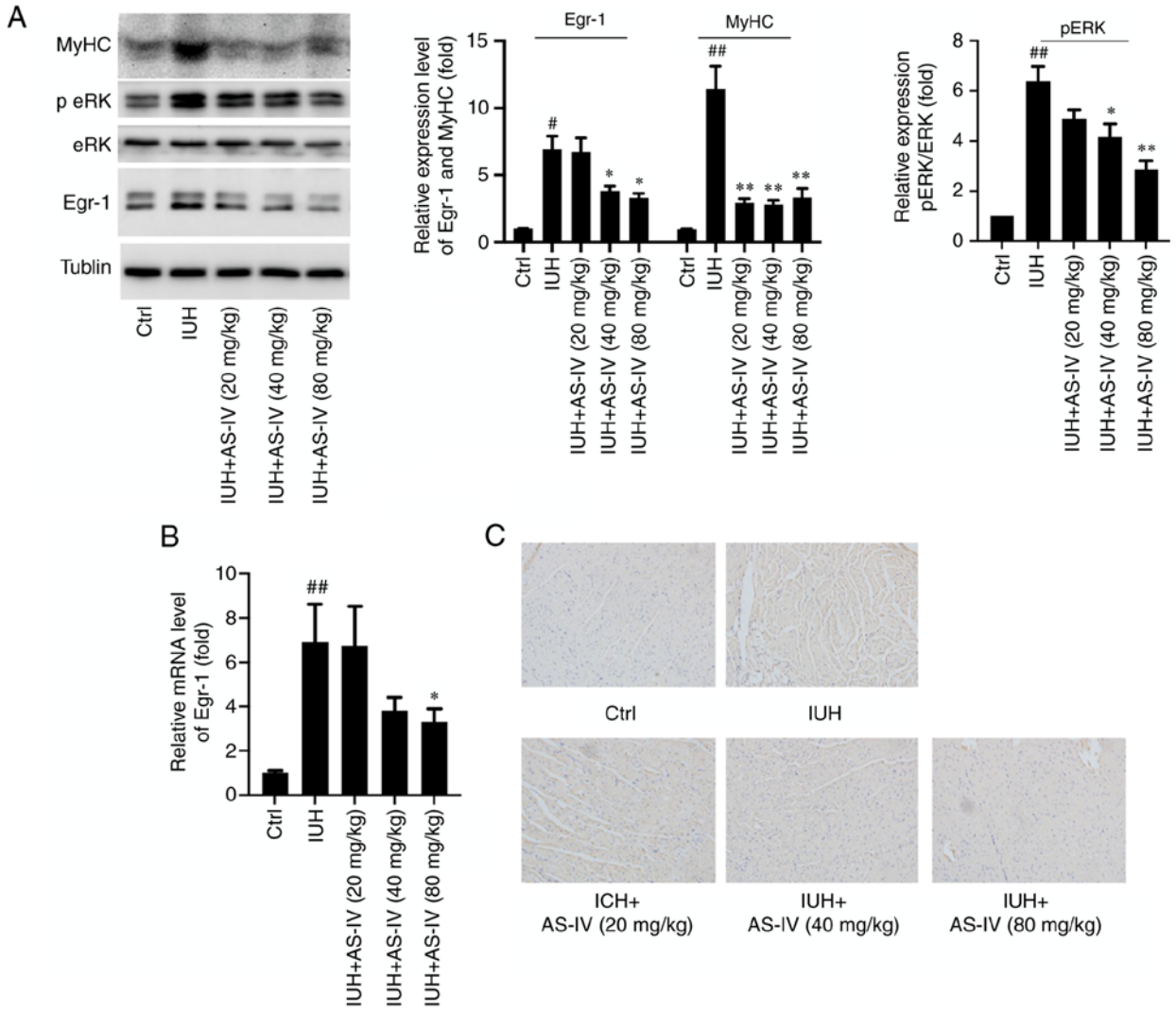

Figure 4. Astragaloside IV prevents the activation of PKCβII/Egr-1 in rats born from mothers with intrauterine hypoxia. (A) ERK1/2 phosphorylation, MyHC and Egr-1 levels were detected using western blotting. (B) Egr-1 mRNA expression was measured using reverse transcription-quantitative PCR. Data are presented as fold changes relative to the Egr-1 levels in control tissues. (C) Representative immunohistochemistry assay images of PKCβII staining in the rat heart tissues. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. Ctrl; *P<0.05, **P<0.01 vs. IUH group. IUH, intrauterine hypoxia; Ctrl, control group; AS-IV, astragaloside IV; PKCβII, protein kinase C β type isoform 2; Egr-1, early growth response 1; p-, phosphorylated.

and immunoregulatory effects (25). The present study revealed that AS-IV could prevent the increases in the heart function indices, LV hemodynamics parameters, pathological changes and MyHC levels induced by IHU. Therefore, AS-IV may be a potential compound for preventing IHU-mediated cardiac hypertrophy. However, the present study had certain limitations, since it did not include the echocardiography analysis of rat cardiac function and the analysis of other markers of cardiac hypertrophy, such as atrial natriuretic and brain natriuretic peptides; therefore, further research is needed.

PKCβII isoform overexpression was shown to lead to LV hypertrophy in mice (26,27). The present immunochemistry assay showed that AS-IV treatment reversed the increases in PKCβII expression in the heart tissues mediated by IHU. It is also noteworthy that ERK1/2 and Egr-1 were indicated as downstream targets of PKCβII (28). The present western blotting and RT-qPCR results suggested that the p-ERK1/2 protein levels and Egr-1 protein and mRNA levels were increased upon IHU induction. By contrast, the AS-IV treatment groups reversed the increased ERK1/2 phosphorylation and Egr-1 expression. These results suggested that the regulatory mechanism downstream of AS-IV may be mediated by PKCβII/Egr-1 signaling.

In conclusion, the present study demonstrated that AS-IV could attenuate cardiac hypertrophy in rats born from mothers with IHU through the PKCβII/Egr-1 pathway, with the underlying mechanism requiring further study.

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Availability of data and materials

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

Authors' contributions

YZ and HL performed data analysis and interpretation and wrote the manuscript. YZ, MW, YD and BH conducted the experiments. All authors have read and approved the final manuscript. YZ and HL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The animal study protocol was approved by the Medical Ethics Committee of Zhejiang Provincial People's Hospital (approval no. 2020022).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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