Puerarin protects against myocardial ischemia/reperfusion injury by inhibiting endoplasmic reticulum stress and apoptosis via activating the AMPK/NRF2 pathway

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Research Article

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Abstract

Objective

Puerarin was reported to protect against myocardial ischemia/reperfusion (IR) injury. The current study aims to investigate the protective effects of puerarin against myocardial IR injury and the underlying mechanisms.

Methods

The hearts were subjected to the ligation of the left anterior descending coronary artery and reperfusion. Before reperfusion, pretreatment with puerarin (100 mg/kg), with or without the AMPK inhibitor Compound c (0.25 mg/kg). Myocardial infarct size, the serum creatine kinase-MB (CK-MB) activity, cardiac function, apoptotic cell death, and the endoplasmic reticulum stress, as well as activation of the AMPK/NRF2 pathway were observed.

Results

Treatment with puerarin significantly decreased the myocardial infarct size, the serum CK-MB activity and notably alleviated IR-induced cardiac dysfunction. Moreover, puerarin reduced myocardial IR-induced apoptotic cell death. In addition, puerarin inhibited myocardial IR-induced endoplasmic reticulum stress and activated the AMPK/NRF2 pathway. More importantly, the AMPK inhibitor Compound c prevented these puerarin-induced cardioprotective effects and activation of the AMPK/NRF2 pathway, as well as apoptosis and the endoplasmic reticulum stress activation.

Conclusion

Our results show that puerarin alleviates myocardial IR injury by inhibiting apoptosis and endoplasmic reticulum stress via the AMPK/NRF2 pathway.

1. Introduction

Coronary artery disease, especially acute myocardial infarction presenting as ST-segment elevation, is the leading cause of death and disability worldwide [1]. Timely blood flow recovery by means of primary percutaneous coronary intervention (PCI) is the most effective way to maintain cardiac function and reduce myocardial ischemic damage [2]. However, the restoration of blood reperfusion will aggravate the damage caused by ischemia, which is known as myocardial ischemia/reperfusion (IR) injury [3]. Unfortunately, there is currrently no effective therapy for reducing myocardia IR injury and preventing the onset of heart failure. Therefore, new treatments are needed for the improvement of myocardia IR injury treatment.

Endoplasmic reticulum is a vital organelle for protein folding and post-translational modification. Endoplasmic reticulum stress (ERS) disrupts ER homeostasis and leads to accumulation of unfolded or misfolded proteins [4]. Moderate ERS plays an important role in recognition and degradation of misfold protein [5]. However, excessive or severe ERS will induce cell apoptosis by activating proapoptotic protein Bax while inhibiting antiapoptotic protein Bcl2 [6]. Many studies have shown that ERS is an important pathological factor involved in myocardial IR injury and cardiac remodeling progression [7–9].

Adenosine monophosphate-activated protein kinase (AMPK), a serine-threonine protein kinase that acts as a critical energy sensor and regulates cellular metabolism in diabetes, cardiovascular diseases and cancer [10–12]. AMPK activation modulates glucose metabolism, apoptosis, ERS, mitochondrial dysfunction and oxidative stress during myocardia IR [13–15]. Nuclear factor E2-associated factor 2 (NRF2) is a pivotal regulator for maintaining the redox balance that translocates into the nucleus and controls the initiation of the transcriptional expression of downstream antioxidant enzymes [16, 17]. It has been reported that the AMPK/NRF2 signaling pathway is associated with the cardioprotective effects against myocardia IR injury [13, 18].

Puerarin (Pue), as the major bioactive ingredient extracted from the root of traditional Chinese medicine *Pueraria lobate*, plays an important role in the treatment of cardiovascular and other diseases, such as stroke, angina pectoris, myocardia IR, diabetes and hypertension [19–21]. Pue has been shown to exert protective effects against myocardia IR by reducing infarct size and improving cardiac function [22]. Some studies have reported that the protective effects of Pue in myocardial hypoxia/reoxygenation were related to Akt-dependent autophagy inhibition and PKCε activation [23, 24]. Our previous study showed Pue exerting protective effects against myocardia IR injury by inhibiting inflammation and the NLRP3 inflammasome via the SIRT1/NF-κB pathway [25]. However, the role of the AMPK/NRF2 pathway in Pue-induced cardioprotection against myocardia IR has not been elucidated. Therefore, the aims of this present study were to investigate whether Pue could regulate ERS and apoptosis on myocardia IR in mice and to identify the molecular mechanisms responsible for these putative effects.

2. Materials And Methods

2.1 Animals

Male C57/bl6 mice, weighing 25–30 g, were purchased from the Laboratory Animal Center of the AIR Force Medical University and were housed in an SPF environment with a 12 h light/dark cycle and free access to food and water. This study was approved by the Research Commission on Ethics of the Air Force Medical University, and all experimental procedures were designed and performed according to the Guidelines for the Care and Use of Laboratory Animals by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 80 – 23, revised in 1996).

2.2 Materials

4’,6-diamidino-2-phenylindole dihydrochloride (DAPI), evans blue, puerarin, and triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A creatine kinase-MB (CK-MB) activity assay kit was purchased from Nanjing Jiancheng Bioengineering (Nanjing, China). Compound c was purchased from Selleck (Houston, Texas, USA). Primary antibodies against Bcl2 (#3498), Bax (#14796), p-PERK (#3179), PERK (#5683), IRE1α (#3294), ATF6 (#65880), CHOP (#5554), AMPK and ACC antibody sampler kit (#9957), and β-actin (#3700) were purchased from Cell Signaling Technology (Boston, MA, USA). Primary antibodies against p-IRE1α (ab48187), NRF2 (ab62352), and HO-1 (ab13248) were purchased from Abcam (Cambridge, MA, USA). Goat anti-mouse (SA00001-1) and goat anti-rabbit (SA00001-2) secondary antibodies were purchased from the Proteintech Company (Wuhan, China).

2.3 Myocardial IR injury model

The myocardial IR procedure was performed as previously described [26]. Briefly, mice were anesthetized with 2% isoflurane mixed with pure oxygen (1 L/min) via an isoflurane vaporizer, and then the left anterior descending (LAD) coronary artery was ligated for 30 min with a 6 − 0 silk suture slipknot, and the slipknot was released to reperfuse for 3 h (western blotting) or 24 h (cardiac function, myocardial infarct size, TUNEL staining, CK-MB activity) to induce myocardial IR injury. The same surgical procedures except ligation of LAD was performed in the mice of sham group. The mice were divided into the following groups (n = 15): sham group (Sham); sham group with Pue treatment (Pue); myocardial IR group (IR); IR group treatment with Pue (Pue + IR); or IR group treatment with Pue and Compound c (Pue + CC + IR). Pue (100 mg/kg) and Compound c (0.25 mg/kg) were intraperitoneally injected 15 min or 20 min prior to reperfusion respectively. The concentrations of Pue and Compound c used were based on previous studies [13, 25].

2.4 Echocardiography

Cardiac function was determined at 24 h after operation by Doppler echocardiography with a 15-Hz linear transducer (Vevo 2100, VisualSonics, Toronto, Ontario, Canada). Briefly, the mice were anesthetized with 2% isoflurane and placed on a heating pad. M-mode echocardiography was performed and recorded to assess cardiac function. The left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were determined by Vevo LAB 3.1.0 software.

2.5 Activity measurement of CK-MB

At the end of 24 h reperfusion, the serum was collected by centrifugation of blood at 3000 g for 10 min. The activity of CK-MB was measured spectrophotometrically according to the manufacturer’s instructions and calculated based on the manufacturer’s instructions.

2.6 Triphenyltetrazolium chloride/Evans blue double staining

After 24 h of reperfusion, the mice were anesthetized with 2% isoflurane, and then the LAD was reoccluded at the same position and 1% Evans blue dye was injected into the hearts via the aorta. The hearts were collected and frozen on dry ice for 30 min. The tissues were measured by 2% TTC solution at

37 ℃ for 15 min. Then the slices were fixed in 4% paraformaldehyde for 24 h. The area at risk (AAR) and infarct size were determined by using Image Pro Plus software (Media Cybernetics, MD, USA).

2.7 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit (Roche Applied Science, Penzberg, Germany) was used for detection of the apoptosis levels according to the manufacturer's instructions. The percentage of TUNEL-positive cell nuclei was determined by the ratio of green fluorescence to blue fluorescence, multiplied by 100.

2.8 Western blotting

The proteins were isolated from left ventricles tissue samples for western blotting detection. Protein concentrations were determined using Pierce BCA protein assay kit (Thermo Fisher, Waltham, MA, USA), and then the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene diflfluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with 5 % nonfat milk in TBST at room temperature for 2 h; subsequently, the membranes were then incubated with primary antibodies against Bcl2 (1:1000), Bax (1:1000), p-PERK (1:1000), PERK (1:1000), p-IRE1α (1:1000), IRE1α (1:1000), ATF6 (1:1000), CHOP (1:1000), p-AMPK (1:1000), AMPK (1:1000), p-ACC (1:1000), ACC (1:1000), NRF2 (1:1000), HO-1 (1:1000), and β-actin (1:5000) at 4°C overnight. After that, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and then visualized by a ChemiDocXRS Imaging System (Bio-Rad Laboratory, Hercules, CA, USA). β-actin was used as the internal control.

2.9 Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). All data were analyzed using GraphPad Prism 8.0 (GraphPad software, Inc., San Diego, CA, USA). The statistical significance of differences between two groups was determined by one-way ANOVA followed by Tukey's correction. A $P < 0.05$ was considered statistically significant.

3. Results

3.1 Pue reduced IR-induced cardiac dysfunction and myocardial injury

To examine the protective effects of Pue against myocardial IR injury, the hearts were subjected to myocardial IR operation. As shown in Fig. 1, under our experimental conditions, there were no differences in the markers of cardiac function and myocardial injury between the Sham group and Pue group (Figures. 1A-G). Moreover, there were no differences in the area at risk of the left ventricle between any of the groups (Figures. 1E). However, there was a significant decrease of LVEF and LVFS in IR, whereas the myocardial infarct size and the serum CK-MB activity showed a significant increase (Figures. 1A-G). As

expected, IR treatment with Pue markedly improved LVEF and LVFS, and reduced the myocardial infarct size and the serum CK-MB activity (Figures. 1A-G). These data demonstrated that Pue protects against cardiac dysfunction and myocardial IR injury.

3.2 Pue inhibited IR-induced cardiomyocyte apoptosis

As apoptosis is an important pathological character of myocardial IR injury, we assessed the protective effects of Pue against myocardial apoptosis. As shown in Fig. 2, TUNEL staining and western blotting analysis indicated that IR treatment significantly increased the percent of TUNEL-positive cells and the protein level of Bax, and decreased the protein level of Bcl2 compared to those of the Sham group (Figures. 2A-D). Treatment with Pue reduced the percent of TUNEL-positive cells and the protein level of Bax, and increased the protein level of Bcl2 in IR mice (Figures. 2A-D). These results further confirmed the protective effects of Pue on myocardial IR injury were associated with inhibiting apoptosis.

3.3 Pue alleviated IR-induced ERS

Given that ERS plays an important role in myocardial IR injury progression, we assessed the effect of Pue on IR-induced ERS. As shown in Fig. 3, western blotting analysis showed that the levels of phosphorylation of PERK and IRE1 and the protein levels of ATF6 and CHOP were notably increased in the IR group (Figures. 3A-E). Interesting, Pue treatment significantly decreased the levels of phosphorylation of PERK and IRE1 and the protein levels of ATF6 and CHOP (Figures. 3A-E). These results suggested that Pue attenuated IR-induced ERS.

3.4 Pue influenced the AMPK/NRF2 pathway in myocardial IR injury

To further determine the mechanisms underlying the protective effects of Pue on IR injury, the activities of AMPK and NRF2, and their substrates were detected. As shown in Fig. 4, western blotting analysis showed that the levels of phosphorylation of AMPK and ACC markedly decreased in the IR group (Figures. 4A-C). In addition, the protein levels of NRF2 and HO-1 were also decreased in the IR group (Figures. 4A and 4D-E). However, Pue treatment significantly increased the levels of phosphorylation of AMPK and ACC, and the protein levels of NRF2 and HO-1 (Figures. 4A-E). Therefore, these results suggested that Pue influenced the AMPK/NRF2 pathway in myocardial IR injury.

3.5 Compound c abolished Pue-mediated alleviation of IR-induced cardiac dysfunction and myocardial injury

To explore whether AMPK signaling was implicated in the Pue-mediated alleviation of IR-induced cardiac dysfunction and myocardial injury, the mice were cotreated with Pue and the AMPK inhibitor Compound c followed by IR operation. As shown in Fig. 5, IR treatment significantly decreased LVEF and LVFS, and increased the myocardial infarct size and the serum CK-MB activity compared to those of the sham group

(Figures. 5A-G). Consistent with previous results, IR treatment with Pue markedly improved LVEF and LVFS, and reduced the myocardial infarct size and the serum CK-MB activity (Figures. 5A-G). However, Compound c restrained the effects of Pue on LVEF and LVFS, the myocardial infarct size and the serum CK-MB activity (Figures. 5A-G). These results demonstrated that Pue alleviated IR-induced cardiac dysfunction and myocardial injury by regulating AMPK signaling.

3.6 Compound c prevented Pue-alleviated IR-induced apoptosis

Next, Compound c was used to assess whether the anti-apoptosis effects of Pue were bound up with AMPK signaling. As shown in Fig. 6, Pue treatment markedly inhibited the percent of TUNEL-positive cells and the protein level of Bax, and increased the protein level of Bcl2 in IR mice (Figures. 6A-D). Nevertheless, Compound c prevented the effects of Pue on the percent of TUNEL-positive cells and the protein levels of Bax and Bcl2 (Figures. 6A-D). Altogether, these results indicated that the anti-apoptosis effects of Pue in myocardial IR were bound up with AMPK signaling.

3.7 Compound c abrogated Pue-alleviated IR-induced ERS

In order to further confirm whether the anti-ERS effects of Pue in IR were related to AMPK signaling, Compound c was employed. As shown in Fig. 7, Pue treatment significantly decreased the levels of phosphorylation of PERK and IRE1 and the protein levels of ATF6 and CHOP (Figures. 7A-E). It was demonstrated that the suppression of the levels of phosphorylation of PERK and IRE1 and the protein levels of ATF6 and CHOP by Pue were rescued by inhibition of AMPK with Compound c (Figures. 7A-E). These results proved that the anti-ERS effects of Pue in myocardial IR were related to AMPK signaling.

3.8 Compound c reversed the activation effect of Pue on AMPK/NRF2 pathway in IR injury

To determine the regulation of the AMPK/NRF2 pathway by Pue, we examined whether inhibition of AMPK with Compound c could reverse the effect of Pue on the AMPK/NRF2 pathway. As shown in Fig. 8, Pue treatment significantly increased the levels of phosphorylation of AMPK and ACC, and the protein levels of NRF2 and HO-1 (Figures. 8A-E). However, the enhancement of the levels of phosphorylation of AMPK and ACC, and the protein levels of NRF2 and HO-1 induced by Pue was almost rescued by inhibition of AMPK with Compound c (Figures. 8A-E). Taken together, these results established that the protective effect of Pue on myocardial IR injury was dependent on the AMPK/NRF2 pathway.

4 Discussion

Myocardial reperfusion injury following acute myocardial infarction is the leading cause of high morbidity and mortality due to the lack of effective treatment [2]. Though myocardial IR injury is a complex pathophysiological process, apoptosis and ERS are believed that the main mechanisms [6, 27].

In the current study, we further confirmed that Pue plays a protective role in myocardial IR. In addition, the AMPK inhibitor Compound c significantly blocked these Pue-related alterations in ERS, apoptosis and the AMPK/NRF2 pathway in mice exposed to myocardial IR, suggesting that the AMPK/NRF2 pathway is involved in the protective effects of Pue against myocardial IR injury.

Timely reperfusion by restoration of blood flow to the ischemic myocardium became the standard treatment for acute myocardial infarction patients [1]. Although reperfusion is essential for rescuing ischemic myocardium, it is also associated with additional cardiomyocyte death, a phenomenon known as myocardial reperfusion injury [3]. Pue has been reported to protect against myocardial IR injury [22, 24, 25]. The protective effects of Pue in myocardial IR were related to autophagy inhibition, ROS reduction, the mitochondrial membrane potential recovery and oxidative stress inhibition [22–24]. Our previous study showed Pue exerting protective effects against myocardial IR injury by inhibiting inflammation and NLRP3 inflammasome [25]. In line with those of previous studies from our and other groups, the present study further demonstrated that Pue treatment significantly reduced myocardial IR-induced increases in myocardial infarct size and the serum CK-MB activity and improved myocardial IR-induced cardiac dysfunction, indicating that Pue attenuated myocardial IR injury. However, the AMPK inhibitor Compound c blocked the protective effects of Pue against myocardial IR injury, suggesting that AMPK signaling is involved in Pue-mediated cardioprotection.

Numerous studies have demonstrated that apoptosis plays an important role in cardiomyocyte death and is proposed to contribute to final infarct size [28, 29]. A pivotal event in the initiation of apoptotic cell death is ERS [6]. Reactive oxygen species (ROS), Ca^{2+} overload, inflammation, and metabolic derangements are potent inducers of the ERS, all of which are present in myocardial IR [9, 30, 31]. As expected, our results showed that IR treatment significantly increased the number of TUNEL-positive cells, and the protein level of Bax and decreased the protein level of Bcl2. Meanwhile, we also observed that the levels of phosphorylation of PERK and IRE1 and the protein levels of ATF6 and CHOP were notably increased in the IR group, indicating that excessive ERS involved in myocardial IR. Moreover, Pue pretreatment attenuated IR-induced ERS. More importantly, Compound c abrogated Pue-alleviated IR-induced ERS, suggesting that the anti-ERS effects of Pue are related to AMPK signaling.

AMPK orchestrates various celluar biological processes such as energy metabolism, cell proliferation, inflammation, and apoptosis [10, 12]. It is accepted that AMPK plays a key part in IR injury. Given that NRF2 is an essential component of the adaptive response to cardiomyocytes stress during ischemia and reperfusion [16]. Next, we explored the effect of Pue on the activation of the AMPK/NRF2 pathway in myocardial IR. In this study, we demonstrated that Pue treatment could lead to the activation of AMPK and its substrate protein ACC. Meanwhile, Pue increased NRF2 and HO-1 proteins expression. However, the AMPK inhibitor Compound c abolished Pue-induced the activation of the AMPK/NRF2 pathway. These results demonstrated that the cardioprotective effects of Pue against myocardial IR could through the activation of the AMPK/NRF2 pathway.

In conclusion, our study further demonstrates that Pue protects against myocardial IR injury. Moreover, to the best of our knowledge, this is the first report to describe cardiomyocyte ERS and apoptosis and the AMPK/NRF2 pathway involved in Pue-mediated alleviation of myocardial IR injury. These findings provide a new insight to the Pue-induced protective effects against myocardial IR injury.

Declarations

Author contributions

Animal experiments: Jingxiao Yang, Pengyun Liu and Ximing Zhu; Biochemical experiments: Jingxiao Yang, Pengyun Liu and Jingyuan Chen; Echocardiography: Zikuan Wang; Data analysis: Yi Chu and Jingyuan Chen; Supervision: Fangfang Wang; Writing: Jingxiao Yang and Fangfang Wang.

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Availability of data and material

All data, models, or code generated or used during the study are available from the corresponding author by request.

Ethics Approval

The animal experiments were approved by the Research Commission on Ethics of the Air Force Medical University, and all experimental procedures were designed and performed according to the Guidelines for the Care and Use of Laboratory Animals by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 80-23, revised in 1996).

Conflicts of interest The authors declare no conflicts of interest.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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Figures

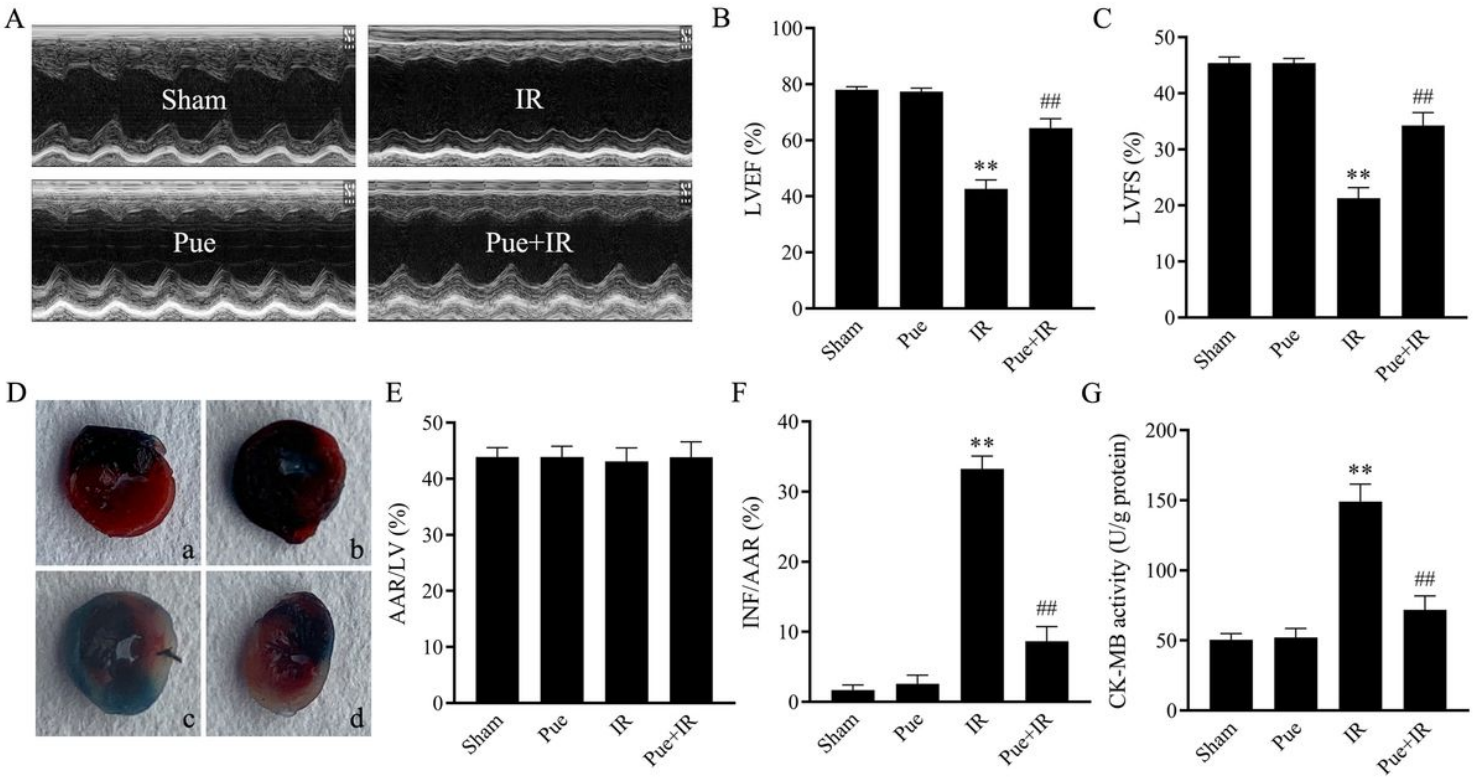

Figure 1

Puerarin alleviated myocardial ischemia/reperfusion injury. A. Representative echocardiography images; B. LVEF; C. LVFS. D. Representative Evens blue and TTC staining of heart slices; E-F. Analysis of infarct size (INF) and area at risk (AAR); G. Analysis of the serum CK-MB activity. The data are presented as the mean ± SD, n=6. **P < 0.01 vs. the Sham group, ##P < 0.01 vs. the IR group.

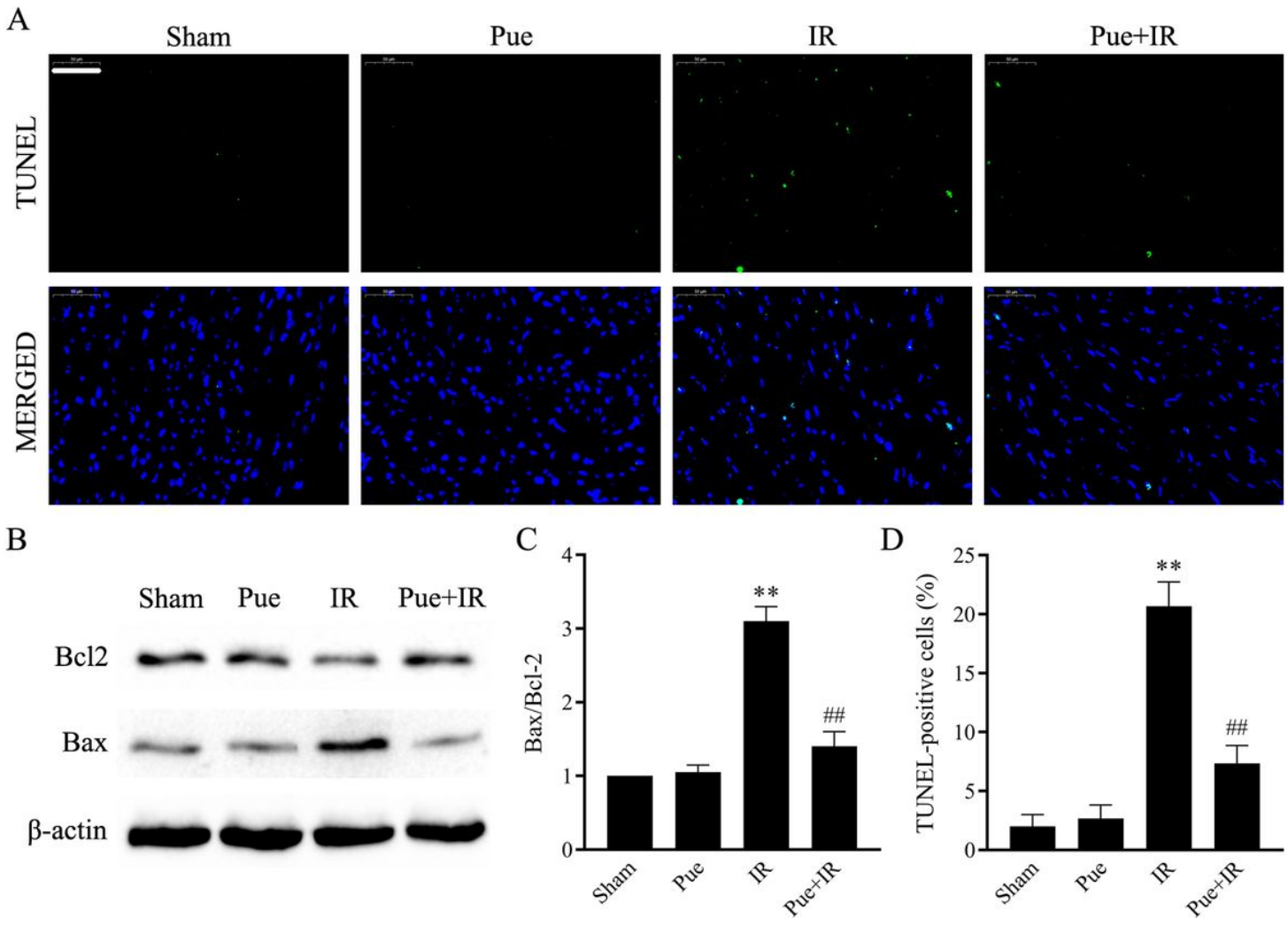

Figure 2

Puerarin inhibited myocardial IR-induced apoptotic cell death. A. Representative TUNEL staining of heart slices (magnification: 400×); B. Representative immunoblots of Bcl2, Bax, and β-actin; C. Analysis of Bax/Bcl2 levels; D. Analysis of TUNEL-positive cells. The data are presented as the mean ± SD, n=6. **P < 0.01 vs. the Sham group, ##P < 0.01 vs. the IR group.

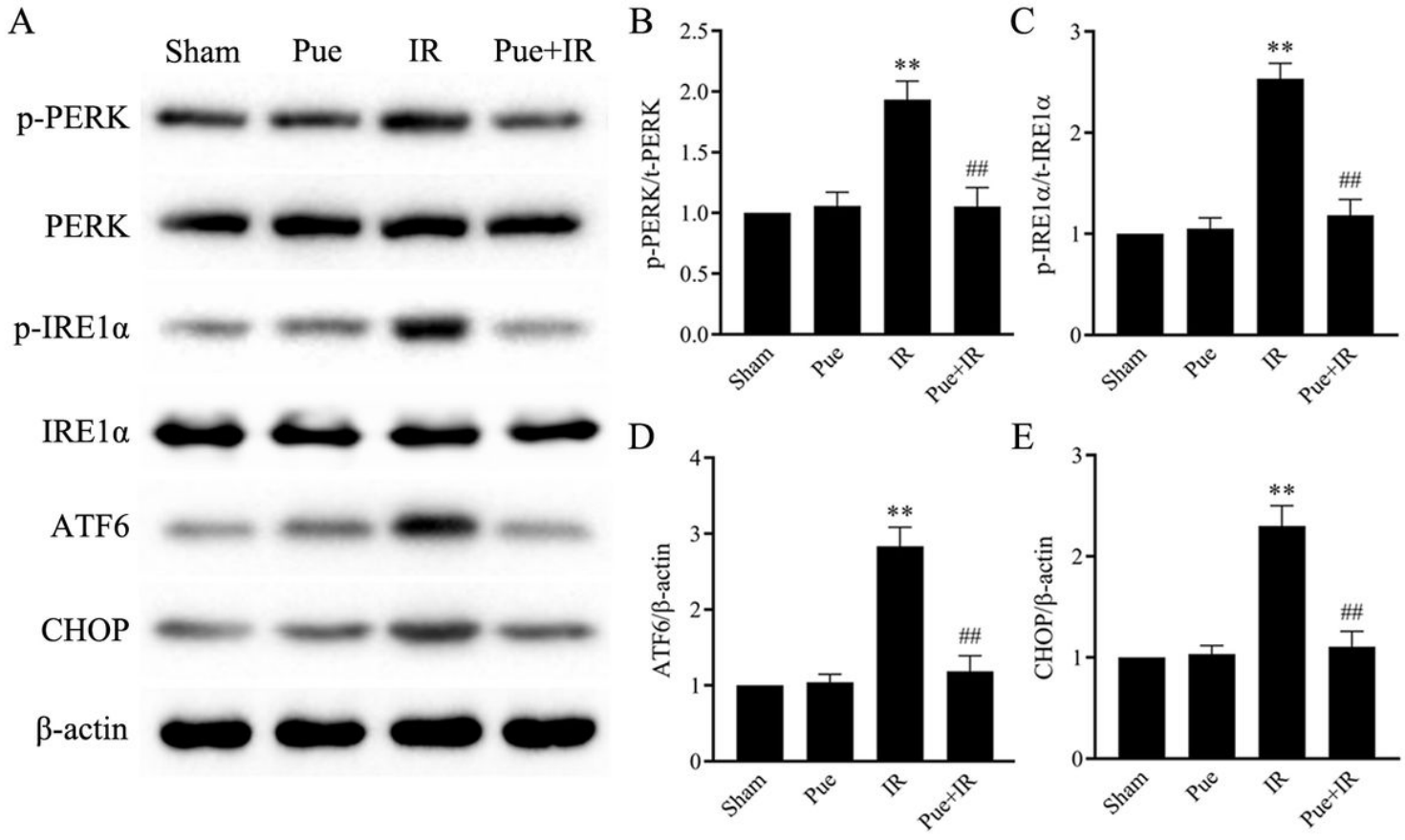

Figure 3

Puerarin reduced myocardial IR-induced endoplasmic reticulum stress. A. Representative immunoblots of p-PERK. PERK, p-IRE1α, IRE1α, ATF6, CHOP, and β-actin; B. Analysis of p-PERK expression level; C. Analysis of p-IRE1α expression level; D. Analysis of ATF6 expression level; E. Analysis of CHOP expression level. The data are presented as the mean ± SD, n=6. **P < 0.01 vs. the Sham group, ##P < 0.01 vs. the IR group.

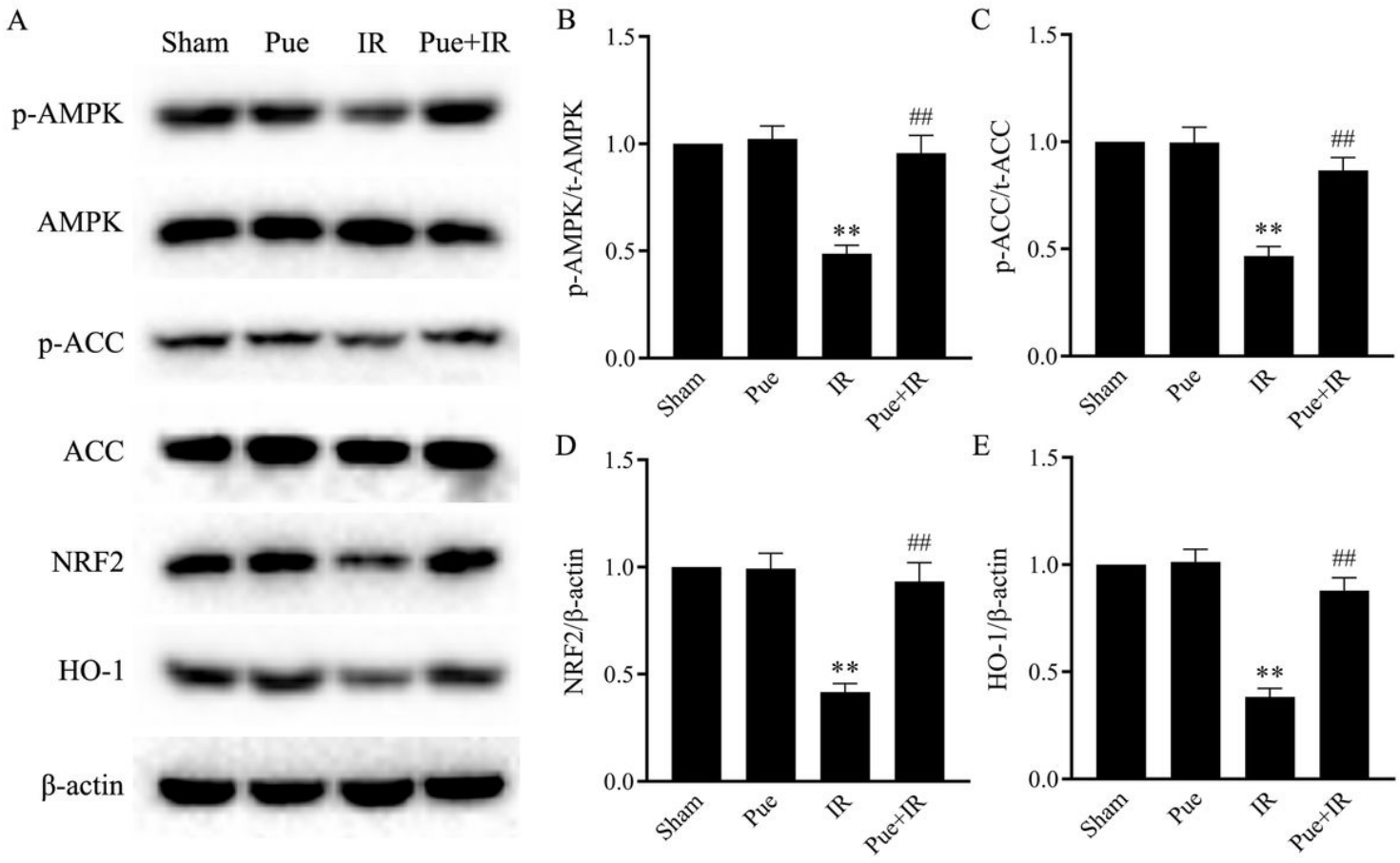

Figure 4

Puerarin influenced the AMPK/NRF2 pathway in myocardial IR. A. Representative immunoblots of p-AMPK. AMPK, p-ACC, ACC, NRF2, HO-1, and β-actin; B. Analysis of p-AMPK expression level; C. Analysis of p-ACC expression level; D. Analysis of NRF2 expression level; E. Analysis of HO-1 expression level. The data are presented as the mean ± SD, n=6. **P < 0.01 vs. the Sham group, ##P < 0.01 vs. the IR group.

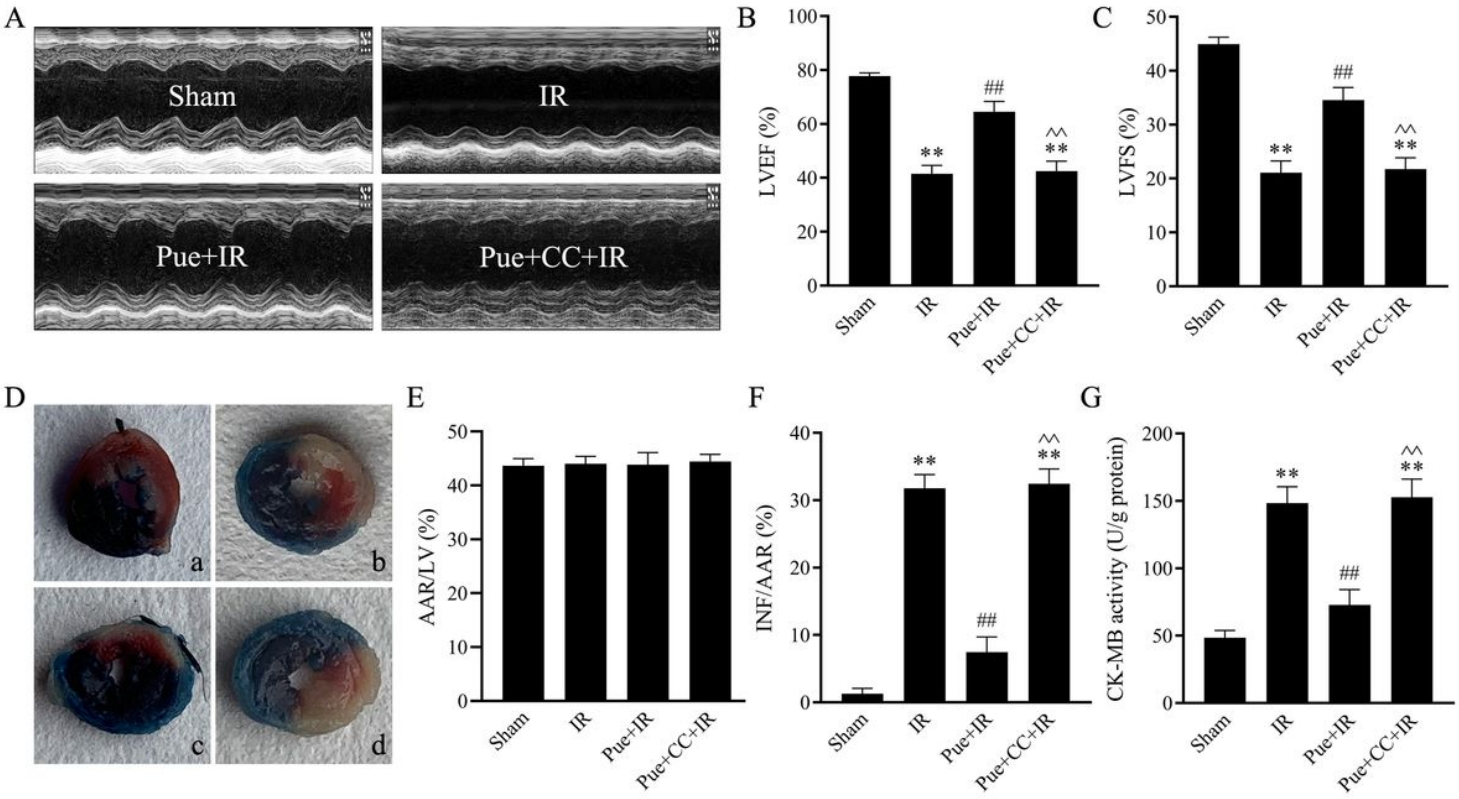

Figure 5

Compound c blunted the protective effects of puerarin on myocardial IR injury. A. Representative echocardiography images; B. LVEF; C. LVFS. D. Representative Evens blue and TTC staining of heart slices; E-F. Analysis of infarct size (INF) and area at risk (AAR); G. Analysis of the serum CK-MB activity. The data are presented as the mean ± SD, n=6. **P < 0.01 vs. the Sham group, ##P < 0.01 vs. the IR group; ^^P < 0.01 vs. the Pue+IR group.

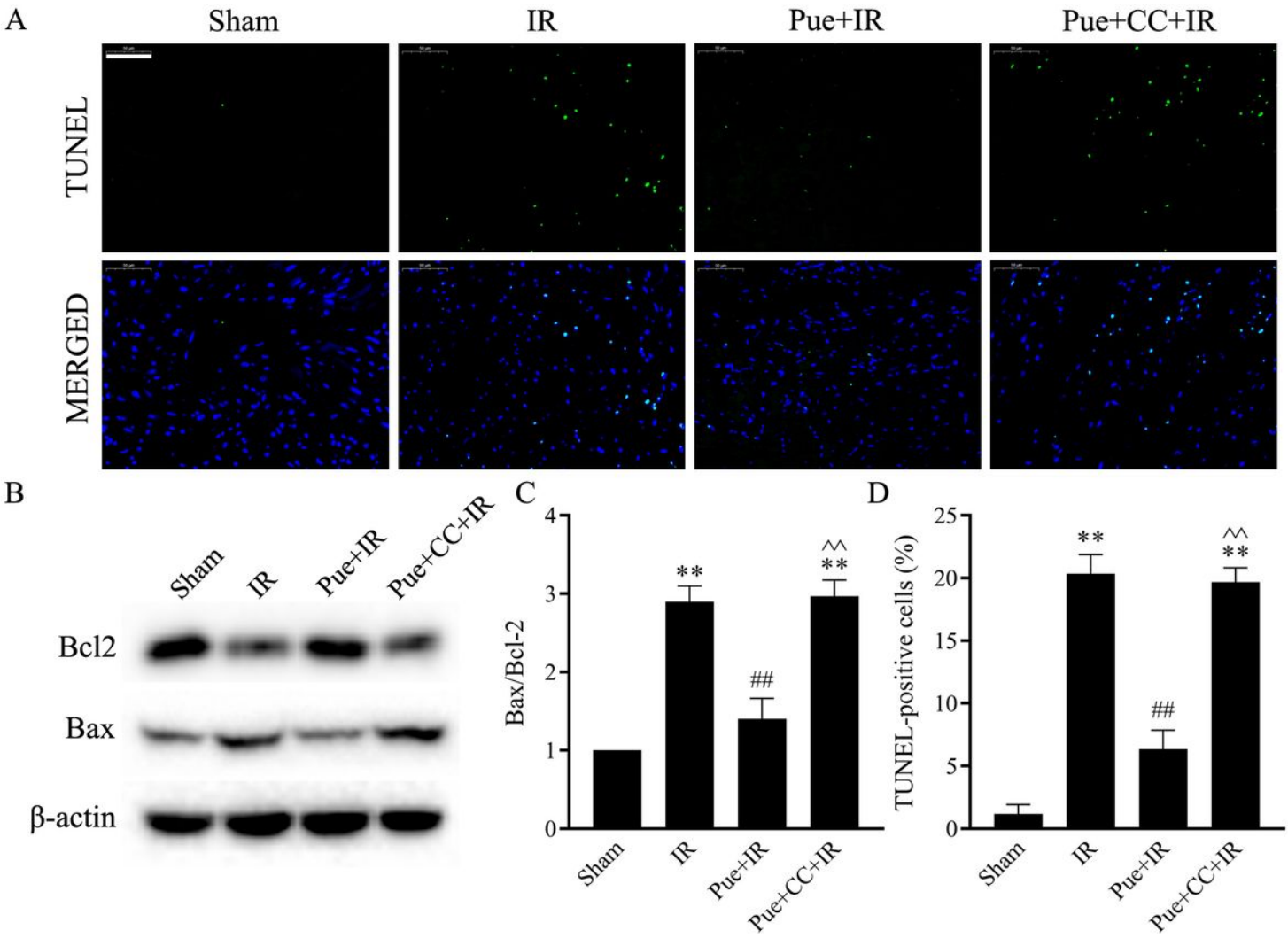

Figure 6

Compound c blocked the effects of puerarin on myocardial IR-induced apoptotic cell death. A. Representative TUNEL staining of heart slices (magnification: 400×); B. Representative immunoblots of Bcl2, Bax, and β-actin; C. Analysis of Bax/Bcl2 levels; D. Analysis of TUNEL-positive cells. The data are presented as the mean ± SD, n=6. **P < 0.01 vs. the Sham group, ##P < 0.01 vs. the IR group; ^^P < 0.01 vs. the Pue+IR group.

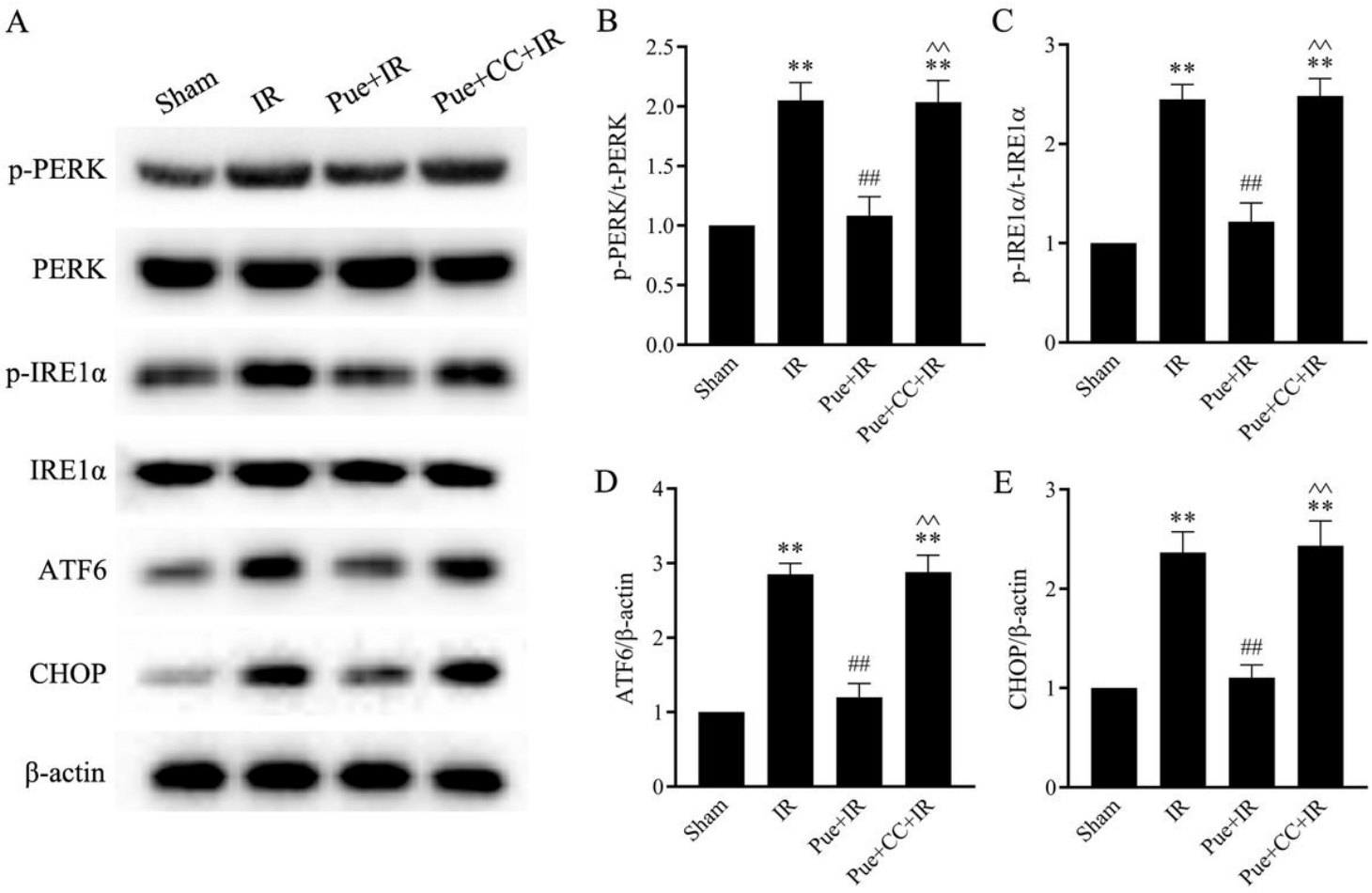

Figure 7

Compound c inhibited the effects of puerarin on myocardial IR-induced endoplasmic reticulum stress. A. Representative immunoblots of p-PERK. PERK, p-IRE1α, IRE1α, ATF6, CHOP, and β-actin; B. Analysis of p-PERK expression level; C. Analysis of p-IRE1α expression level; D. Analysis of ATF6 expression level; E. Analysis of CHOP expression level. The data are presented as the mean ± SD, n=6. **P < 0.01 vs. the Sham group, ##P < 0.01 vs. the IR group; ^^P < 0.01 vs. the Pue+IR group.

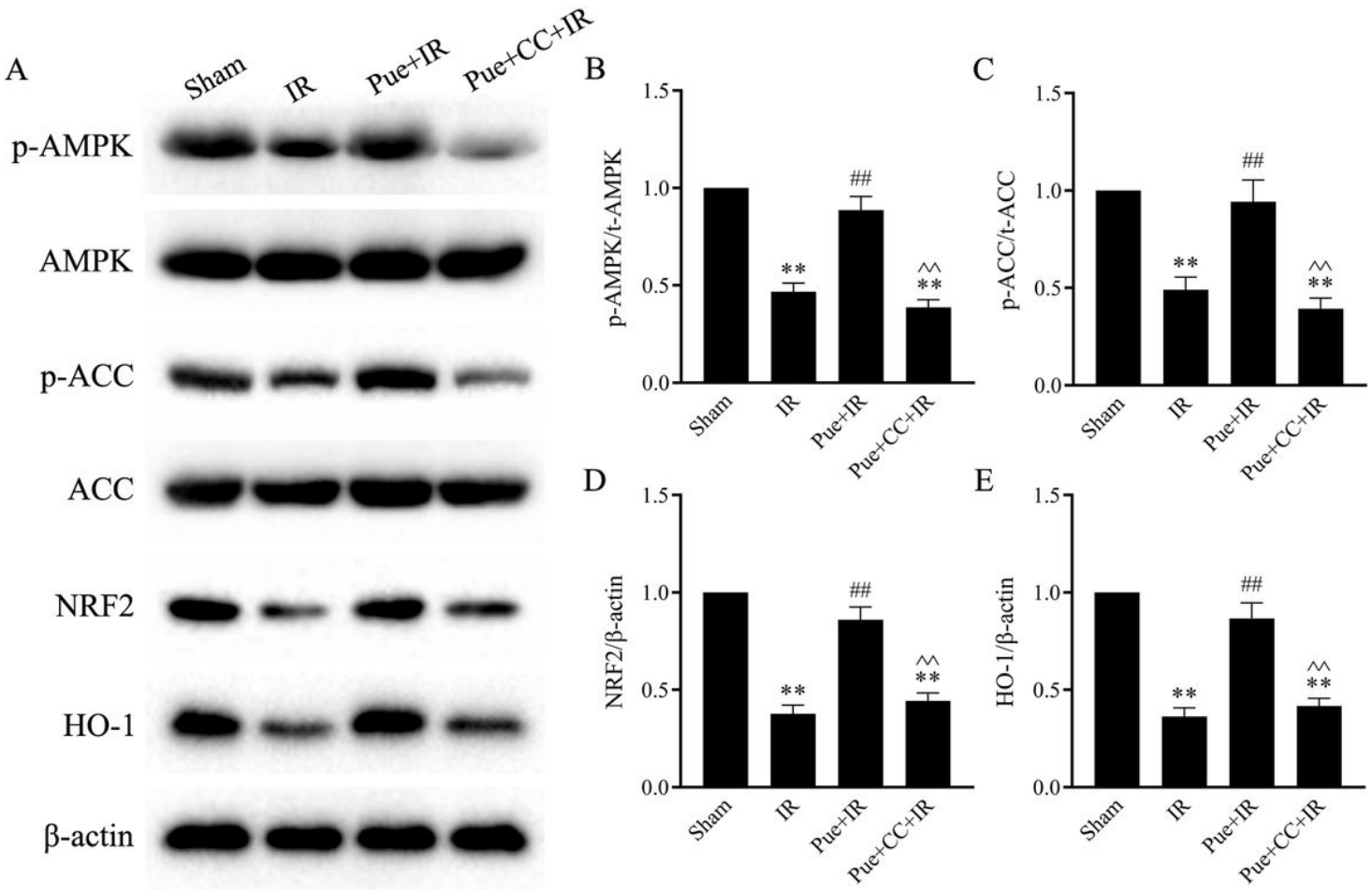

Figure 8

Compound c prevented the effects of puerarin on the AMPK/NRF2 pathway in myocardial IR. A. Representative immunoblots of p-AMPK. AMPK, p-ACC, ACC, NRF2, HO-1, and β-actin; B. Analysis of p-AMPK expression level; C. Analysis of p-ACC expression level; D. Analysis of NRF2 expression level; E. Analysis of HO-1 expression level. The data are presented as the mean ± SD, n=6. **P < 0.01 vs. the Sham group, ##P < 0.01 vs. the IR group; ^^P < 0.01 vs. the Pue+IR group.