

Piperine protects against myocardial ischemia/reperfusion injury by activating the PI3K/AKT signaling pathway

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Abstract. Piperine (PIP) exerts numerous pharmacological effects and its involvement in endoplasmic reticulum (ER) stress (ERS)-led apoptosis has garnered attention. The present study focused on whether PIP played protective effects on hypoxia/reoxygenation (H/R)-induced cardiomyocytes by repressing ERS-led apoptosis. The potential molecular mechanisms in association with the PI3K/AKT signaling pathway were investigated. Primary neonatal rat cardiomyocytes (NRCMs) were isolated and randomized into four groups: Control + vehicle group, control + PIP group, H/R + vehicle group and H/R + PIP group. The H/R injury model was constructed by 4 h of hypoxia induction followed by 6 h of reoxygenation. A total of 10 μ M PI3K/AKT inhibitor LY294002 was supplemented to the cells during the experiments. Cell viability and myocardial enzymes were detected to evaluate myocardial damage. A flow cytometry assay was performed to assess apoptotic response. Western blot analysis was performed to detect the expression of related proteins including PI3K, AKT, CHOP, GRP78 and cleaved caspase-12. The results showed that H/R markedly promoted myocardial damage as shown by the increased release of lactate dehydrogenase and creatine kinase levels, but a reduction in cell viability. In addition, ERS-induced apoptosis was markedly promoted by H/R in NRCMs, as shown by the increased apoptotic rates and expression of C/EBP-homologous protein, endoplasmic reticulum chaperone BiP and caspase-12. PIP administration reversed cell injury and ERS-induced apoptosis in H/R. Mechanistic studies concluded that the apoptosis-inhibitory contributions and cardio-favorable effects of PIP were caused partly by the activation of the PI3K/AKT signaling pathway,

which was verified by LY294002 administration. To conclude, PIP can reduce ERS-induced apoptosis by activating the PI3K/AKT signaling pathway during the process of H/R injury, which could be a potential therapeutic target for the treatment of myocardial ischemia/reperfusion injury.

Introduction

Acute myocardial infarction (AMI) remains one of the leading causes of mortality and disability worldwide (1,2). Currently, the most effective way to rescue dying cardiomyocytes induced by AMI is timely revascularization (3). However, re-establishing blood flow to the ischemic area yields additional myocardial damage, which is termed as myocardial ischemia/reperfusion (I/R) injury (MIRI) (3). Although effective strategies such as percutaneous coronary intervention, intravenous thrombolysis and coronary artery bypass grafting timely limits infarct expansion and decrease in-hospital mortality, the subsequent occurrence of chronic heart failure and cardiac remodeling has gradually increased (1-3). Thus, MIRI affects the clinical efficacy of revascularization strategies, and is an important factor that causes the deterioration of myocardial function and electrophysiological status (1-4). Novel approaches for attenuating MIRI need to be explored in order for the maximal benefits of myocardial revascularization under AMI to be achieved.

Conventionally, the endoplasmic reticulum (ER) is involved in the biosynthesis, enfoldment, processing, excretion and transportation of proteins (3,5,6). However, the disruption of ER homeostasis caused by MIRI, which is referred to as ER stress (ERS), leads to inaccurate synthesis and assembly of proteins (3,5,6). Increasing evidence suggested that continuous activation of ERS and the consequent elevation of apoptotic cascade events act as crucial pathogenic mechanisms in MIRI (3,5,6). Despite the confirmed benefits of ERS-induced apoptosis against MIRI, the molecular mechanisms of MIRI and its related therapeutic approaches remain to be elucidated (3,5,6). Targeting ERS-related apoptosis may be a possible therapeutic approach for MIRI.

Piperine (PIP) is a phenolic substance found in black and long pepper that exerts multiple pharmacological effects, such as liver protection and tumor inhibition (7,8). In recent

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years, the protective effect of PIP in I/R injury has received much attention (9,10). Vaibhav *et al* (10) found that piperine treatment inhibited I/R-induced neuronal dysfunction and that its protective function is achieved by reducing inflammation. Zou *et al* (9) also verified the neuroprotective effects of PIP in response to cerebral I/R injury, the underlying mechanism of which is strictly related to the regulation of complement and coagulation cascades. However, the exact effects of PIP in MIRI are not clear and the possible pathological mechanisms associated with ERS-related apoptosis remains to be elucidated.

Accumulating data have proven that activation of the PI3K/AKT signaling pathway acts as a crucial molecular mechanism by which PIP exerts pleiotropic contributions under various pathological conditions (11-13). The activity of the PI3K/AKT pathway has multiple consequences in response to MIRI; in particular, it has anti-apoptotic effects against I/R injury related to ERS (3,5,6). Further studies to investigate whether the anti-apoptotic effects produced by PIP in a PI3K/AKT-related manner play a role in cardioprotection during MIRI are required. The present study showed that PIP administration repressed myocardial apoptosis and ERS in injury caused by 4 h of hypoxia followed by 6 h of reoxygenation (H/R), and that this may be related to activation of the PI3K/AKT pathway. Thus, the present study provided novel insights into the benefits and potential mechanisms of PIP against MIRI, and may offer a novel therapeutic target for MIRI treatment.

Materials and methods

Chemicals and reagents. PIP was purchased from Sigma-Aldrich; Merck KGaA. The purity of PIP, which was >98%, was tested using high-performance liquid chromatography as previously described (12). DMEM, PBS, trypsin, FBS and collagenase type II were purchased from Gibco; Thermo Fisher Scientific, Inc. The CCK-8 assay and PI3K/AKT inhibitor LY294002 were purchased from Dojindo Molecular Technologies, Inc. and Selleck Chemicals, respectively. Lactate dehydrogenase (LDH) and creatine-kinase (CK) were detected using commercially available ELISA kits (Nanjing Jiancheng Bioengineering Institute; cat. A020-2-2 for LDH; cat. A032-1-1 for CK). The following primary antibodies were purchased from Abcam: Phosphorylated (p)-PI3K (1:600; cat. no. ab32509), PI3K (1:400; cat. no. ab191606), p-AKT (1:1,000; cat. no. ab182651), endoplasmic reticulum chaperone BiP (GRP78; 1:500; cat. no. ab21685), cleaved caspase-12 (1:600; cat. no. ab62484) and GAPDH (1:1,000; cat. no. ab181602). Antibodies against AKT (1:600; cat. no. 9272) and C/EBP-homologous protein (CHOP; 1:600; cat. no. 2895) were purchased from Cell Signaling Technology, Inc. Horseradish peroxidase-conjugated rabbit anti-rat immunoglobulin G secondary antibody was purchased from BIORSS. The BCA Protein Assay kit was purchased from Pierce; Thermo Fisher Scientific, Inc.

Neonatal rat cardiomyocyte (NRCM) culture. NRCMs were isolated from the ventricles of 1-3-day-old Sprague-Dawley rats as previously described (14). Animals were bred in a standard environment with controlled temperature (20-25°C), humidity (40-60%) and light condition (12 h light/dark

cycle). The experiments and all animal care outlined in the present study were performed in adherence with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011) (1,2), and were approved by the Institutional Animal Care and Use Committee from the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology. For cardiomyocyte isolation, all rats were sacrificed by decapitation. Subsequently, the hearts were quickly removed and large blood vessels were carefully excised. The obtained ventricles were rinsed in ice-cold PBS three times to remove residuary blood and placed in a dish with ice-cold PBS. Heart tissues were digested with 0.08% collagenase type II and 0.125% trypsin at room temperature for 5 min. NRCMs were then centrifuged at 3,000 x g for 10 min at 37°C and resuspended in DMEM containing 10% FBS and 1% penicillin/streptomycin (HyClone; GE Healthcare Life Sciences) in a humidified incubator at 37°C with 5% CO₂ and 95% O₂.

Construction of a H/R injury model and experimental groups. H/R injury was established as previously described with minor modifications (15). Cultured NRCMs were washed twice with PBS and preserved in serum-free DMEM. Subsequently, the cells were incubated at 37°C in an anaerobic chamber for 4 h. The cells were then moved into a normal incubator with 95% O₂ and 5% CO₂ at 37°C for an additional 6 h for reoxygenation.

To determine the possible function of PIP in MIRI, different dosage gradients of PIP (1, 5, 10 and 20 µM) were administered to first measure the most effective dose against MIRI. A total of 16 neonatal rats were used in each group (n=4 per group). Another experiment was then designed to explore the precise roles and molecular mechanisms of PIP in response to H/R. Primary cardiomyocytes were randomly divided into four groups: The control + vehicle group, which consisted of NRCMs cultured in normoxic conditions without PIP treatment; the control + PIP group, which consisted of NRCMs cultured in normoxic conditions with PIP treatment; the H/R + vehicle group, which consisted of NRCMs cultured in H/R conditions without PIP treatment and the H/R + PIP group, which consisted of NRCMs cultured in H/R conditions with PIP treatment. During this experiment, 20 neonatal rats were used for each group (n=5 per group). Additionally, to detect the underlying mechanism of the association with the PI3K/AKT pathway, cells were pre-treated with 10 µM LY294002 for 30 min prior to PIP administration, and were then subjected to H/R intervention. During this experiment, 24 neonatal rats were used for each group (n=6 per group).

Cell viability assay. The CCK-8 assay was used to measure cell viability in each group as previously described (16). The optical density values were detected at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA). Cell viability was evaluated as a percentage relative to the control group.

Measurement of markers of myocardial injury. After the indicated procedures, the medium in each group was collected using a sterile pipette. Commercially available biochemical kits were used in line with the manufacturer's instructions to detect LDH and CK.

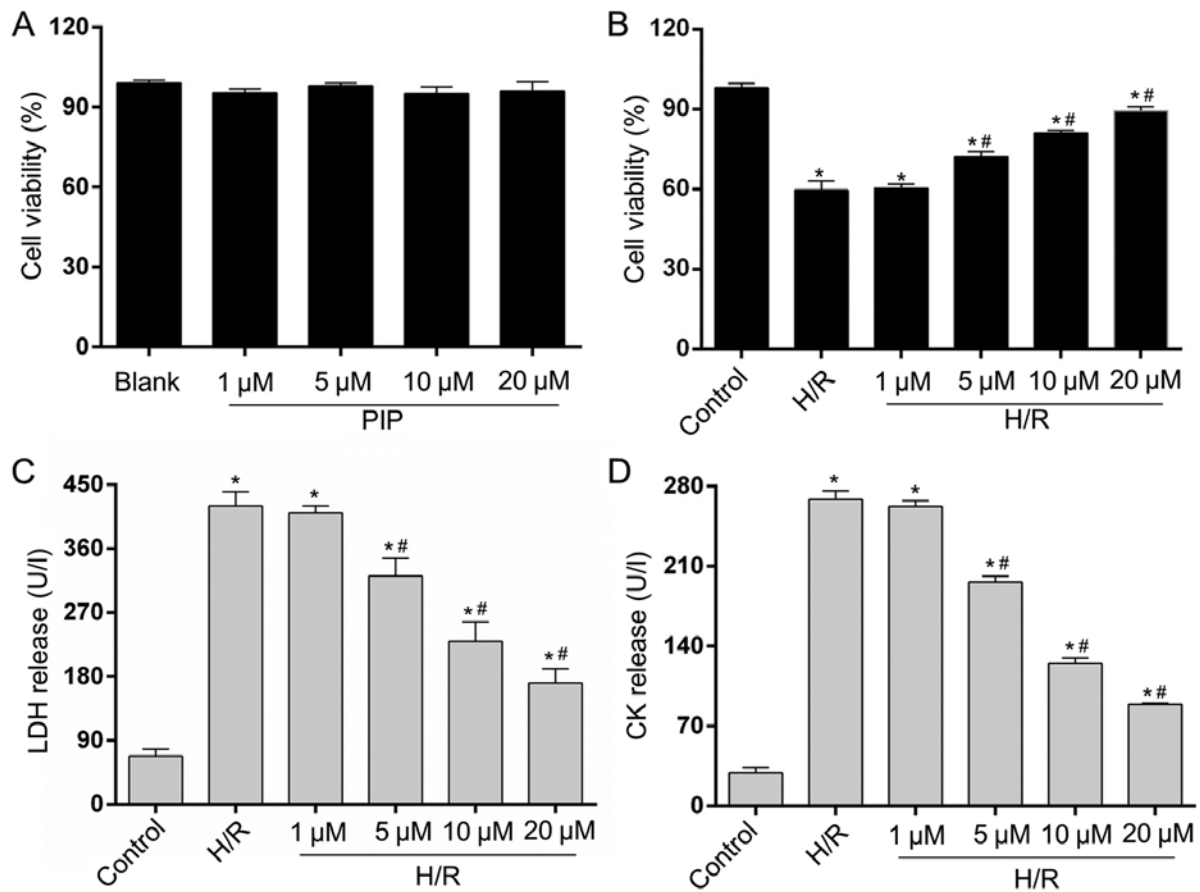


Figure 1. PIP attenuates H/R-induced NRCM injury. (A) The cytotoxicity of PIP (1, 5, 10, and 20 μ M) on NRCM viability assessed by a CCK-8 assay. The effect of PIP on (B) cell viability, (C) LDH release and (D) CK release following H/R insult. n=4. *P<0.05 vs control; and #P<0.05 vs. the H/R group. PIP, piperine; NRCM, neonatal rat cardiomyocyte; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; CK, creatine-kinase.

Apoptosis detection. Apoptosis of NRCMs was measured by a flow cytometry assay as previously described (16,17). Cells were maintained in 100 μ l of binding buffer, which included 5 μ l Annexin V-APC and 5 μ l 7-aminoactinomycin D. Stained cells were observed using a flow cytometer (Beckman Coulter), followed by the fluorescence-activated cell sorting on a flow cytometric assay (CytoFLEX; Beckman Coulter, Inc., Brea, CA, USA). NRCMs positive for 7-AAD or Annexin V-APC were recognized as apoptotic cells.

Western blotting. Western blotting was used to detect protein expression as previously described (3,16). Myocardial samples were homogenized and lysed in RIPA buffer (Beyotime Institute of Biotechnology) at a mass/volume ratio of 100 mg/ml. Protein was then extracted and protein concentration was detected using a BCA kit (Beyotime Institute of Biotechnology). Then, 10% SDS-PAGE was performed to separate the proteins, and 50 μ g of extracted proteins in each group were electrophoretically transferred onto PVDF membranes (EMD Millipore). Next, the membranes were blocked by 5% skimmed milk in TBST for 2 h at 37°C. Subsequently the membranes were incubated with antibodies against CHOP, GRP78, caspase-12, PI3K, p-PI3K, AKT and p-AKT overnight at 4°C. GAPDH was used as the internal reference protein. The horseradish peroxidase-conjugated secondary antibodies were added for another 2 h at 37°C. The protein bands were visualized using an ECL kit (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was performed to detect mRNA levels as previously described (3,16). Total RNA was extracted from NRCMs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturers protocol, after the indicated treatments. qPCR was performed using a SYBR-Green Master Mix kit (cat. no. 4472918; Thermo Fisher Scientific, Inc.) on a 7500 ABI Prism system (Applied Biosystems). The following thermocycling conditions were used for the qPCR: Initial denaturing at 50°C for 2 min, 40 cycles of denaturation at 95°C for 30 sec and final extension at 60°C for 30 sec. The mRNA expression levels of CHOP, GRP78 and caspase-12 were normalized to GAPDH levels. The fold-change of the mRNA levels relative to the control cells was calculated using the $2^{-\Delta\Delta C_q}$ method (3-5). The following primer pairs were used for the qPCR: CHOP forward, 5'-TAGCTTGCTGACTGAGGAGC-3' and reverse, 5'-CTTCAGCAAGCTGTGCCAAT-3'; GRP78 forward, 5'-GATAATCAGCCACCGTAACAAT-3' and reverse, 5'-GCAAACCTCTCGCGTCATT-3' and caspase-12 forward, 5'-CATTGCCAATTCCGACAAAC-3' and reverse, 5'-CCTTCCTTCTCCATCACTGGA-3'; GAPDH forward, 5'-ACAGCAACAGGGTGGTGGAC-3' and reverse, 5'-TTTGAGGGTGCAGCGAACTT-3'.

Statistical analysis. SPSS 17.0 (SPSS, Inc.) was used for statistical analysis. Data were expressed as the mean \pm SD. The Student's t-test was used for comparisons within groups.

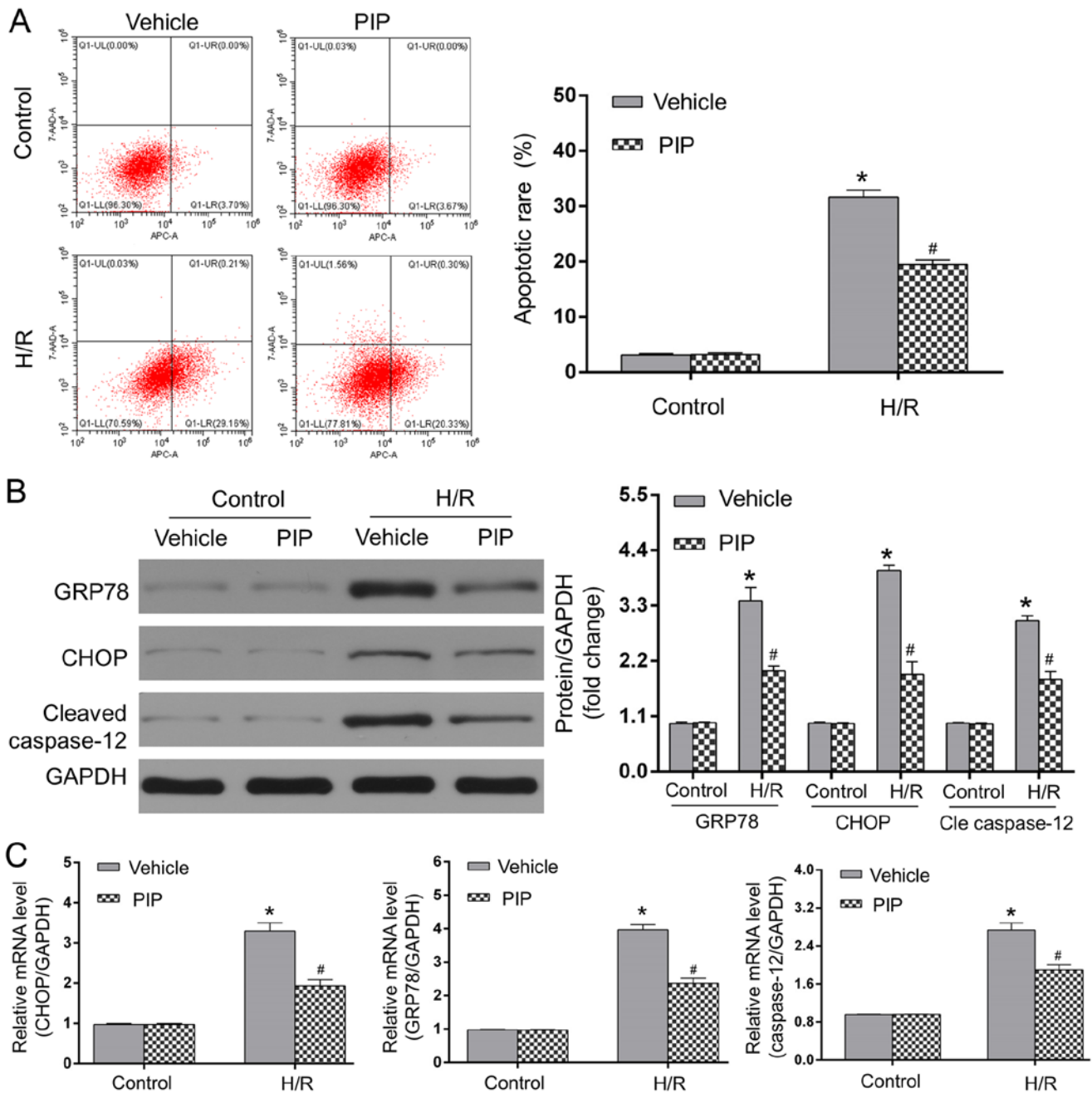


Figure 2. PIP treatment alleviates ERS-induced apoptosis during H/R injury. (A) Apoptotic cells were monitored by flow cytometry. (B) Protein levels of GRP78, CHOP and caspase-12 were detected by western blotting. The left panel shows representative blots and the right panel shows quantitative results of western blotting. (C) mRNA levels of GRP78, CHOP and caspase-12 were detected by reverse transcription-quantitative PCR. $n=4$. * $P<0.05$ vs. the vehicle + control group; # $P<0.05$ vs. the vehicle + H/R group. PIP, piperine; ERS, endoplasmic reticulum stress; H/R, hypoxia/reoxygenation; GRP78, endoplasmic reticulum chaperone BiP; CHOP, C/EBP-homologous protein; Cle caspase-12, cleaved caspase-12.

The statistical comparisons among multiple groups were performed using analysis of variance (ANOVA) followed by a post hoc Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PIP attenuates H/R-induced NRCM injury. A CCK-8 assay was performed to assess cell viability (17). The four different PIP concentrations (1, 5, 10 and 20 μM) had no effect on cell viability and exhibited very low cytotoxicity in control NRCMs (Fig. 1A). To evaluate the possible effects of PIP on H/R injury,

NRCMs underwent 4 h of hypoxia and 6 h of reoxygenation. As shown in Fig. 1B, cell viability was reduced following H/R insult compared with controls. In addition, the various doses of PIP (5, 10 and 20 μM) increased cell viability compared with H/R-induced cells. Cell viability significantly increased following treatment with 5 and 10 μM PIP, and the highest improvement in cell viability compared with the H/R group was obtained following treatment with 20 μM PIP. LDH and CK release were detected to assess NRCM death. The release of LDH (Fig. 1C) and CK (Fig. 1D) caused by H/R injury was limited starting at a concentration of 5 μM . Minimal levels of LDH and CK were measured in cells treated with 20 μM PIP

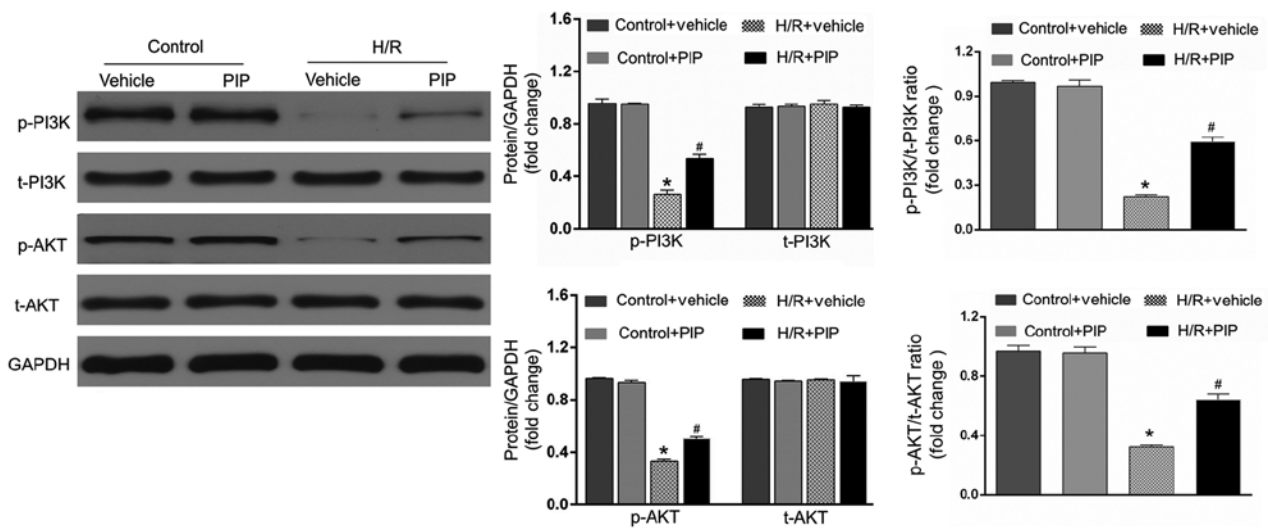


Figure 3. PIP pretreatment activates the PI3K/AKT signaling pathway. The left panel shows representative western blots of p-PI3K, PI3K, p-Akt and Akt. The right panel shows corresponding bar graphs of densitometric analysis in neonatal rat cardiomyocytes detected by western blotting. $n=4$. * $P<0.05$ vs. the vehicle + control group and # $P<0.05$ vs. the vehicle + H/R group. PIP, piperine; p-PI3K, phospho-PI3K; t-PI3K, total PI3K; p-AKT, phospho-AKT; t-AKT, total AKT; H/R; hypoxia/reoxygenation.

compared with other concentrations. No statistical difference in LDH and CK release or cell viability was found between the 1 μ M PIP-treated and H/R groups. A total of 20 μ M PIP was used for subsequent experiments. The results suggested that PIP could increase cell survival and repress cell damage in NRCMs under H/R injury.

PIP treatment alleviates ERS-induced apoptosis during H/R injury. Myocardial H/R injury is related to the activation of ERS-induced apoptosis and PIP was indicated to be involved in ERS progression (3,18,19). As an ER chaperone, GRP78 is crucial for myocardial apoptosis during H/R, particularly since ERS can drive the apoptotic cascade, including the activation of caspase-12 in a CHOP-dependent manner (3). To determine whether 20 μ M PIP affects H/R injury in an ERS-related manner, the protein and mRNA expressions of GRP78, CHOP and caspase-12 in apoptotic NRCMs were assessed. As shown in Fig. 2A, minimal apoptotic rates were observed in the control + vehicle and control + PIP groups, whereas compared to the controls, H/R injury significantly increased cardiomyocyte apoptosis. PIP treatment significantly decreased the number of apoptotic cells under H/R conditions compared with vehicle-treated cells following H/R ($P<0.05$). The expression of ERS-related proteins, CHOP, GRP78 and caspase-12 in each group was detected using western blotting and RT-qPCR. As shown in Fig. 2B and C, H/R injury elevated protein and mRNA expressions of GRP78, CHOP and caspase-12 compared with the control groups ($P<0.05$). However, PIP administration significantly decreased the levels of these proteins and mRNAs in response to H/R injury compared with those in the H/R + vehicle group ($P<0.05$). Therefore, the above data indicated that PIP mitigated H/R-led ERS and subsequent apoptosis in NRCMs.

PIP activates the PI3K/AKT signaling pathway. To further investigate the underlying mechanisms behind PIP in alleviating I/R injury, the expression of genes involved in the PI3K/AKT signaling pathway was determined. As shown in Fig. 3, H/R

significantly downregulated the expression of p-PI3K and p-AKT compared with the control group ($P<0.05$). However, pretreatment with PIP at the onset of H/R increased the expression of p-PI3K and p-AKT compared with the H/R + vehicle group. No significant difference in the expression of PI3K and AKT was observed between the different groups. Therefore, it can be speculated that H/R injury is ameliorated by PIP, possibly through activating the PI3K/AKT signaling pathway.

PI3K/AKT repression reversed the inhibitory effects of PIP on H/R injury. To further validate whether the PI3K/AKT pathway served as a causative mediator of PIP-induced repression of H/R, NRCMs were pre-administrated with the specific PI3K/AKT inhibitor LY294002 and subjected to H/R injury. The results showed that LY294002 rescued H/R-induced cell death and ERS-related apoptosis, as demonstrated by the restored LDH and CK activity (Fig. 4B), apoptotic rate (Fig. 4C), restored GRP78, CHOP and caspase-12 mRNA levels (Fig. 4D) and suppression of cell viability (Fig. 4A). PIP lost the ability to reverse these alternations in the presence of LY294002 under H/R insult (Fig. 4A-D). Furthermore, in the presence of LY294002, there was no significant difference in these alterations with or without PIP administration. Thus, these findings indicated that PIP pretreatment alleviates ERS-induced apoptosis in H/R-exposed NRCMs mainly by activating the PI3K/AKT pathway.

Discussion

Previous studies found that PIP could alleviate pressure overload-led cardiac remodeling and neuronal I/R injury (9,12), but its role in MIRI and the possible mechanisms in association with the PI3K/AKT pathway remain to be elucidated. The present study observed that PIP treatment reduced myocardial H/R injury, which was characterized by the upregulation of cell activity and the decreased release of LDH and CK. In addition, PIP administration significantly alleviated myocardial

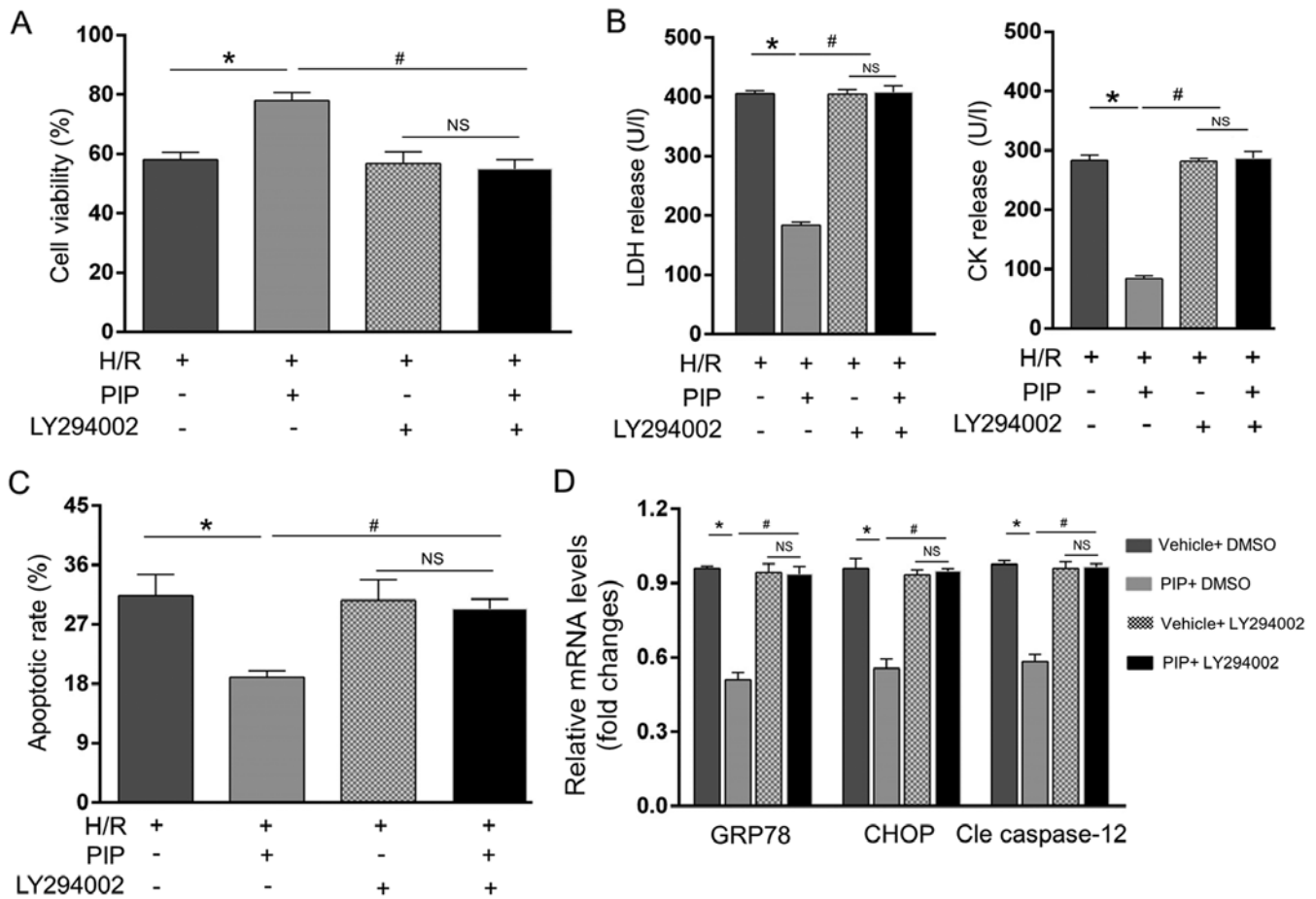


Figure 4. PI3K/AKT inhibition reverses the inhibitory effects of PIP on H/R. CCK-8 and ELISA assays were used to detect (A) cell viability and (B) LDH and CK release, respectively. Apoptosis was measured by (C) flow cytometry and (D) GRP78, CHOP and caspase-12 mRNA levels were detected by reverse transcription-quantitative PCR. $n=4$. * $P<0.05$ vs. the H/R group and # $P<0.05$ vs. the PIP + H/R group. NS, not significant; PIP, piperine; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; CK, creatine-kinase; GRP78, endoplasmic reticulum chaperone BiP; CHOP, C/EBP-homologous protein; Cle caspase-12, cleaved caspase-12.

apoptosis and inhibited the protein expression of ERS-related molecules, such as GRP78, CHOP and caspase-12. Mechanistic studies observed that PIP markedly promoted the PI3K/AKT signaling pathway, while LY294002 reversed its protective function against H/R-induced damage and ERS-related apoptosis. The above results therefore indicated that PIP ameliorated H/R-induced and ERS-related apoptosis mainly via the PI3K/AKT-dependent pathway.

Evidence has suggested that PIP has pleiotropic impacts on a variety of pathological conditions, such as glutamate release from rat hippocampal nerve terminals, lupus nephritis, 6-hydroxydopamine-induced Parkinson's disease and prostate cancer development, possibly due to its multiple bioactivities (20-23). Recent findings revealed that PIP also seems to be beneficial in the treatment of cardiovascular disease. In a rat model of isoproterenol (ISO)-induced myocardial infarction, Dhivya *et al* (24) demonstrated that PIP pretreatment acted as a potent therapeutic agent with antioxidant and anti-dyslipidemic actions against ISO-caused myocardial ischemia. In addition, Ma *et al* (12) indicated that pretreatment with PIP can attenuate pathological cardiac fibrosis following pressure overload and ISO injection. Latest research indicated that PIP exerts a pivotal effect on the course of the pathological process of I/R (9). For example, in a rat model of cerebral I/R injury, Zou *et al* (9) showed that PIP pretreatment

protected neurocytes from I/R damage by the regulation of complement and coagulation cascades. Vaibhav *et al* (10) found that PIP administration rescued ischemic penumbral zone neurons via its anti-inflammatory properties, thereby suppressing ischemic cell death. However, to the best of our knowledge, the involvements and potential mechanisms of PIP in MIRI remain to be elucidated. A recent study has demonstrated that PIP is an important mediator in the initiation and development of ERS and subsequent apoptotic activation (18). Yaffe *et al* (25) demonstrated that PIP treatment inhibited the apoptosis of human colon cancer cells triggered by ERS. PIP was verified to regulate ERS progression and thus is a possible therapeutic agent that limited ERS-related renal apoptosis and cell death (19). In terms of the possible involvement of PIP in I/R injury and ERS-related apoptosis, it was hypothesized that PIP could exert protective roles on cardiomyocytes and play inhibitory roles in ERS-related apoptosis in response to H/R insult. Consequently, the present research provided evidence that PIP has an advantageous role in H/R-injured NRCMs and can also alleviate myocardial I/R disorder.

Timely and effective reperfusion of ischemic heart tissue was confirmed to be the most efficacious remedy for AMI (1-3). However, the accompanying I/R injury largely offsets this beneficial effect (1-3). Effective methods for mitigating the adverse influences of reperfusion still need to be further determined.

Myocardial apoptosis is considered to be one of the most critical mechanisms of MIRI pathogenesis, and the ERS-elicited apoptotic cascade has drawn considerable attention (3,5). Previous findings showed that MIRI could result in severe ERS as GRP78 levels are drastically enhanced following MIRI (3). GRP78 activation then causes a depolymerization reaction between ER-transmembrane transducers and GRP78, resulting in the activation of caspase-12 and apoptosis (3). CHOP is a specific transcription factor involved in ERS-induced apoptosis (3), and prolonged ERS promotes the expression of CHOP (3). The present study demonstrated that MIRI could cause significant myocardial damage and ERS. However, PIP administration reduced GRP78, CHOP and caspase-12 expression, ameliorating the parameters relative to the severity of the H/R injury. Therefore, PIP has the ability to protect cardiomyocytes mainly by mitigating ERS-related apoptosis.

Based on the above findings, the potential molecular mechanisms by which PIP exerts its protective effects against H/R injury and ERS-related apoptosis were further explored. PI3K and its downstream target serine/threonine kinase AKT belong to a conserved family of signal transduction enzymes (3,5,6). Activation of the PI3K/AKT pathway is considered an endogenous regulatory mechanism that facilitates cell survival in response to harmful external stimuli (3,5,6). Numerous studies provided evidence that the PI3K/AKT signaling pathway plays a critical role in the pathological process of MIRI (3,5,6), and compromised ERS-related apoptosis was verified to be an important mechanism for the protective role of the PI3K/AKT activation (3,5,6). Zhang *et al* (3) showed that the PI3K/AKT axis participated in anti-I/R effects via ERS repression, resulting in reduced GRP78, CHOP and caspase-12 expression and apoptotic inhibition. Deng *et al* (26) demonstrated that activation of the PI3K/AKT signaling pathway promoted myocardial survival by attenuating ERS-induced apoptosis. Moreover, there is a close relationship between PIP and the activation of the PI3K/AKT pathway (13). Chen *et al* (13) revealed that PIP promoted Leydig cell development but inhibited spermatogenesis in rats by activating AKT. Consistent with a previous study (13), the present study validated that pretreatment with PIP not only inhibited apoptosis induced by H/R injury, but also upregulated the expression of p-PI3K and p-AKT. To further verify the causal relationship between the activation of the PI3K/AKT signaling pathway and PIP administration, LY294002 was added to cells at the onset of H/R injury. The cardioprotective efficacy and inhibitory effects of PIP on ERS-related apoptosis were significantly reversed in the presence of LY294002. Therefore, the above data indicated that PIP administration can decrease ERS-associated apoptosis and H/R-induced cell damage in a PI3K/AKT-dependent manner. Of note, the LY294002 supplement at the onset of control group was missing in this experiment. This is a limitation of the manuscript that it would be beneficial to include this group to confirm the H/R-inhibitory roles of PIP. Furthermore, the specific roles of PIP in alterations in the PI3K/AKT pathway remain controversial. Ma *et al* (12) found that PIP attenuated cardiac fibrosis via the inhibition of AKT after pressure overload, inconsistent with previous reports elucidating that PIP repressed migration and proliferation potentially via a reduction in the AKT pathway in

non-cardiomyocytes (11). These discrepancies might be partially explained by the fact that the actions of PIP on the activation of the PI3K/AKT pathway vary in a cell-type and stimulus-dependent manner.

In conclusion, results of the present study have demonstrated that pretreatment with PIP alleviated MIRI by attenuating ERS-associated apoptosis in a PI3K/AKT-dependent manner. Numerous other signaling pathways including the TLR4/NF- κ B pathway (16) and the LncRNA/miRNA pathway (2) are involved in the pathophysiological process of MIRI. Further studies are necessary to explore whether any other signaling pathways involved in the protective effect of PIP in MIRI. Taken together, the present results indicated that PIP might be a prospective therapeutic option for MIRI.

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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

YPL and YHC conceived and designed the experiments. ZC and YPL performed majority of the experiments, collected the data and finished the statistical analysis. All authors contributed to manuscript preparation and revision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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