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ORIGINAL RESEARCH

Protopine regulates oxidative stress, apoptosis and autophagy in H9C2 cardiomyocytes under hypoxic conditions

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Abstract

The heart is highly sensitive to oxygen deprivation, and hypoxia can lead to cardiomyocyte damage and subsequent cardiac dysfunction. Protopine Pro), an isoquinoline alkaloid derived from various herbs, exhibits diverse biological activities. However, the protective mechanisms of Pro against hypoxia-induced cardiomyocyte damage are not well understood. In this study, Pro was observed to stimulate cell proliferation in H9C2 cardiomyocytes under hypoxia/reoxygenation (H/R) conditions and mitigate apoptosis typically associated with H/R exposure. Despite a notable increase in oxidative stress following H/R treatment, Pro treatment effectively reduced this effect. Additionally, Pro was found to enhance autophagy in H/R-treated H9C2 cells, as evidenced by higher microtubule-associated protein 1 light chain 3 (LC3)-II/LC3-I ratios and increased LC3B fluorescence intensity. The activation of the phosphatase and tensin homolog deleted on chromosome ten (PTEN)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway by Pro was also demonstrated. Overall, Pro influences oxidative stress, apoptosis, and autophagy in H9C2 cardiomyocytes under H/R conditions by activating the PTEN/PI3K/Akt pathway, suggesting it as a promising agent to mitigate hypoxia-induced cardiomyocyte damage.

Keywords

Protopine; H9C2 cardiomyocytes; Hypoxia; Autophagy; PTEN/PI3K/Akt pathway

1. Introduction

The formation of plaques on the arterial wall reduces cardiac blood flow, leading to myocardial damage and hypoxia, which can culminate in myocardial infarction (MI) [1]. Hypoxia-induced myocardial damage can occur in various conditions, including high altitude, congenital heart disease, coronary artery disease, and cardiopulmonary bypass [2], and frequently results in cardiomyocyte necrosis and apoptosis through multiple signaling pathways, contributing to cardiac dysfunction and adverse clinical outcomes [3]. Thus, it is crucial to identify effective drugs and strategies to safeguard cardiomyocytes from damage induced by hypoxia.

Protopine (Pro), an isoquinoline alkaloid present in plants from the Papaveraceae and Corydalis families in northeastern Asia [4], exhibits a range of biological activities. Like other natural alkaloids, Pro has demonstrated anti-angiogenic, anti-inflammatory, and anti-tumor properties. For example, Pro modulates the toll-like receptor 4 (*TLR4*) pathway to inhibit cell apoptosis and inflammation, thus improving lipopolysaccharide (LPS)-induced acute kidney injury [5]. In Alzheimer's disease models, Pro suppresses histone deacetylase 6 (*HDAC6*), facilitating tau proteasomal degradation [6]. In liver carcinoma, Pro affects the reactive oxygen species (ROS)/PI3K/Akt pathway to induce apoptosis through the intrinsic pathway [7]. Additionally, Pro can reduce inflammation and oxidative stress by inhibiting the NLR family, pyrin domain containing 3 (NLRP3) and nuclear factor kappa-B (NF-κB) pathways, alleviating LPS-induced intestinal epithelial cell injury [8]. Despite these observations, the significance and mode of action of Pro in hypoxia/reoxygenation (H/R)-induced cardiomyocyte damage remain poorly understood.

Autophagy can be activated in response to cellular hypoxia, ischemia or nutrient deficiency [9]. It has been reported that cardiomyocyte autophagy is rapidly induced during the early stages of MI, potentially offering protection and rescue to cardiomyocytes in the infarct border zone [10]. Nevertheless, the impact of Pro on autophagy regulation throughout the progression of MI has not been extensively studied.

This research seeks to investigate how Pro influences the progression of MI and connections to the PTEN/PI3K/Akt signaling pathway to provide new perspectives on potential therapeutic approaches to enhance MI outcomes.

2. Materials and methods

2.1 Cell lines and treatment

Rat H9C2 cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 37 °C incubator with 5% carbon dioxide (CO_2). To establish the MI model, H9C2 cells were subjected to H/R conditions. Specifically, cells were incubated in a hypoxic environment (95% nitrogen (N_2) and 5% CO_2) for 21 hours, followed by reoxygenation in a normoxic environment (95% air and 5% CO_2) for 6 hours. Control cells were maintained under normal conditions (95% air and 5% CO_2) [11].

After H/R, Pro (10, 20 or 40 μmol/L, S388301, Shanghai Selleck Chemicals Co., Ltd., Shanghai, China) was administered to the H9C2 cells for 24 hours [7].

The experimental groups included: Control, H/R, H/R + 10 μmol/L Pro, H/R + 20 μmol/L Pro, and H/R + 40 μmol/L Pro.

2.2 Cell counting kit-8 (CCK-8) assay

H9C2 cells (1000 cells/well) were plated in a 96-well plate and treated with 10 μL of CCK-8 solution (CK04, Dojindo Laboratories, Kumamoto, Japan) for 4 hours. Cell viability was measured by absorbance at 450 nm using a microplate reader (680, Bio-Rad, Hercules, CA, USA). The cell survival rate was calculated as follows:

$$Cell\ survival\ rate\ (\%) = [(As - Ab)/(Ac - Ab)] \times 100\%$$

Where As is the absorbance of the experimental well (cells, medium, CCK-8 solution and drug), Ac is the absorbance of the control well (cells, medium and CCK-8 solution without drug), and Ab is the absorbance of the blank well (medium and CCK-8 solution without cells or drug).

2.3 5-Ethynyl-2′-deoxyuridine (EDU) assay

H9C2 cells (1×10^5) were incubated in a 96-well plate for 24 hours, then treated with 50 μM EDU reagent (C10310, RiboBio, Guangzhou, Guangdong, China) for 2 hours. After staining with Apolo and 4,6-diamino-2-phenyl indole (DAPI), EDU-positive cells were examined under a fluorescence microscope (M165, Leica, Wetzlar, HE, Germany).

2.4 Flow cytometry

H9C2 cells (1×10^5 cells/well) were cultured in a 6-well plate for 24 hours. After incubation, cells were resuspended and stained with fluorescein Isothiocyanate (FITC) Annexin V (5 μL, 556547, BD Biosciences, Franklin Lakes, NJ, USA) and propidium iodide (PI; 5 μL) to assess apoptosis. Flow cytometric analysis was performed using a flow cytometer (BD FACSCanto II, BD Biosciences, San Jose, CA, USA).

2.5 Detection of ROS, MDA, SOD and GSH

The levels of reactive oxygen species (ROS; ab186027, Abcam, Shanghai, China), malondialdehyde (MDA; ab118970), superoxide dismutase (SOD; ab65354), and glutathione (GSH; ab65322) were quantified according to the manufacturer's protocols.

2.6 Immunofluorescence (IF) assay

H9C2 cells (1×10^5 per well) were seeded in a 24-well plate and incubated for 24 hours. They were then fixed with 4% paraformaldehyde, blocked with 5% bovine serum albumin (BSA), and permeabilized with 0.2% Triton X-100. After incubation with primary antibody against microtubule-associated protein 1 light chain 3B (LC3B, 1 μg/mL, ab192890, Abcam, Shanghai, China) and secondary antibody (1:1000, ab6717), immunofluorescence images were obtained using an Olympus microscope (BX53, Olympus, Tokyo, Japan).

2.7 Western blot

Proteins from H9C2 cells were extracted with radio immunoprecipitation assay (RIPA) buffer (P0013B, Beyotime, Shanghai, China) and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). They were transferred to polyvinylidene fluoride (PVDF) membranes (FFP33, Beyotime), blocked with non-fat milk, and probed with primary antibodies overnight. After incubation with secondary antibodies (1:1000, ab7090), protein bands were detected using a chemiluminescence kit (89880, Thermo Fisher Scientific, Waltham, MA, USA) and visualized with an enhanced chemiluminescence system (Thermo Fisher Scientific). Band intensity was measured with ImageJ. The primary antibodies used are illustrated in **Supplementary Table 1**.

2.8 Statistical analysis

Data are shown as mean ± standard deviation (SD). Experiments were done in triplicate. Differences between groups were analyzed using one-way one-way analysis of variance (ANOVA) in GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA), with significance set at $p < 0.05$.

3. Results

3.1 Pro accelerates cell proliferation in H/R-mediated H9C2 cells

The structural formula of Pro is shown in Fig. 1a. The results showed that treatment with Pro at concentrations of 10, 20 or 40 μmol/L did not significantly alter the survival rates of H9C2 cells under normal conditions. However, H/R treatment reduced cell survival, an effect that was reversed by Pro treatment at all concentrations tested (Fig. 1b). The 5-Ethynyl-2′-deoxyuridine (EDU) assay demonstrated a significant decrease in cell proliferation from 28% to 9% following H/R treatment. Pro treatment at 20 μmol/L and 40 μmol/L partially restored proliferation to 19% and 22%, respectively (Fig. 1c). These results indicate that Pro can enhance cell proliferation in H/R-mediated H9C2 cells.

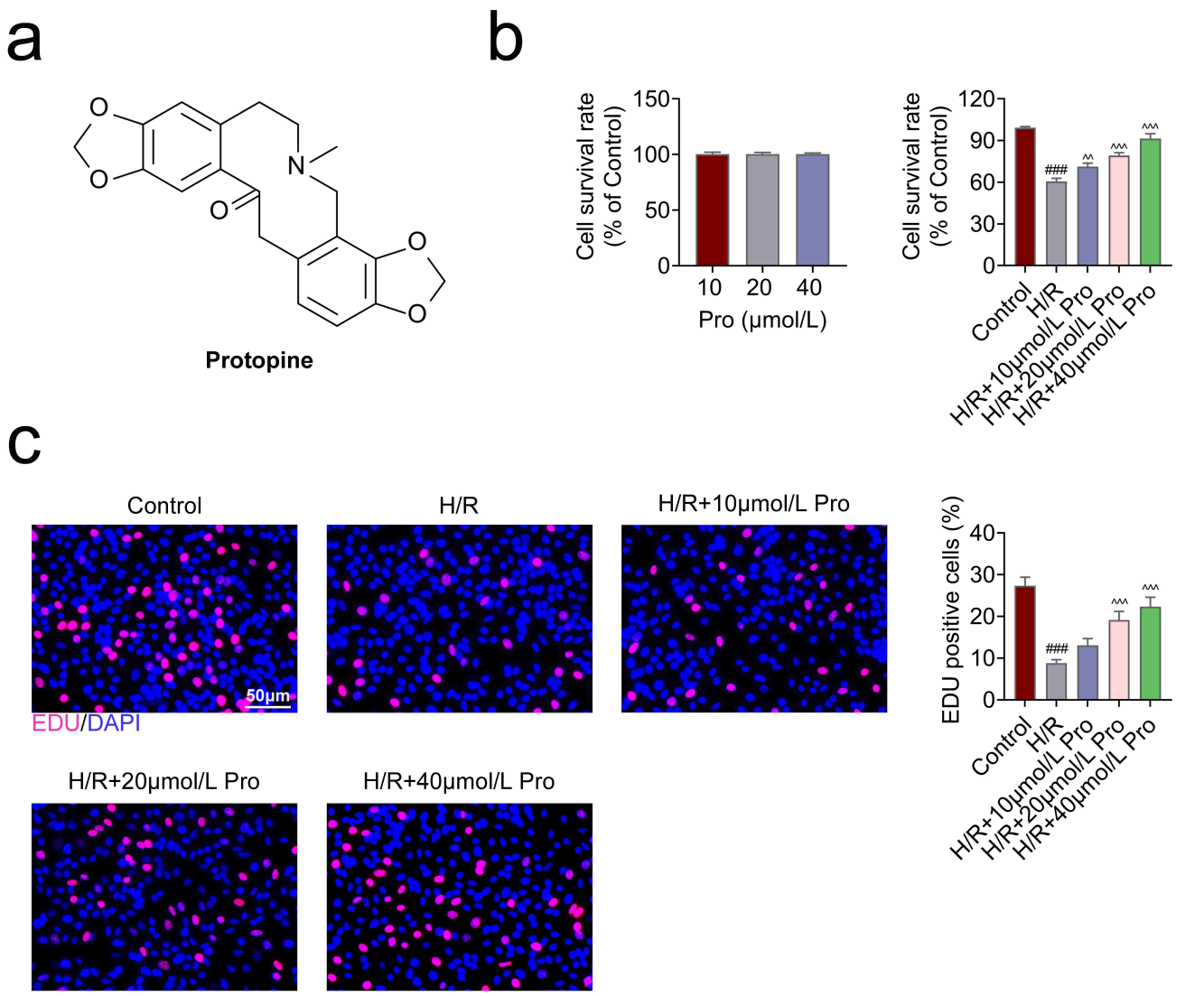

FIGURE 1. Pro accelerated cell proliferation in H/R-mediated H9C2 cells. (a) The chemical structure of Pro is shown. (b) Cell survival rates were evaluated using the CCK-8 assay in the Control, H/R, H/R + 10 μmol/L Pro, H/R + 20 μmol/L Pro, and H/R + 40 μmol/L Pro groups. (c) Cell proliferation was assessed using the EDU assay across the same groups. Data are presented as mean ± SD; $^{\#\#\#}p < 0.001$ versus Control, $\wedge\wedge p < 0.01$, $\wedge\wedge\wedge p < 0.001$ versus H/R. Pro: Protopine; H/R: hypoxia/reoxygenation; EDU: 5-Ethynyl-2′-deoxyuridine; DAPI: 4,6-diamino-2-phenyl indole.

3.2 Pro inhibits cell apoptosis in H/R-triggered H9C2 cells

H/R treatment significantly increased cell apoptosis from 8% to 32%, while Pro treatment at 20 μmol/L and 40 μmol/L reduced apoptosis to 21% and 17%, respectively (Fig. 2a). Western blot analysis revealed that H/R treatment decreased B-cell lymphoma-2 (BCL-2) protein levels but increased Bax and Cleaved caspase-3 levels. Pro treatment mitigated these effects, restoring BCL-2 levels and reducing Bax and Cleaved caspase-3 levels (Fig. 2b). Thus, Pro effectively inhibits apoptosis in H/R-triggered H9C2 cells.

3.3 Pro suppresses oxidative stress in H/R-stimulated H9C2 cells

H/R treatment elevated levels of ROS and MDA while decreasing SOD and GSH levels. Pro treatment reversed these changes, indicating that Pro may effectively suppress oxidative stress in H/R-stimulated H9C2 cells (Fig. 3).

3.4 Pro enhances autophagy in H/R-induced H9C2 cells

H/R treatment led to a decreased LC3-II/LC3-I protein ratio and increased levels of P62, indicating impaired autophagy. Pro treatment counteracted these effects, increasing the LC3-II/LC3-I ratio and reducing P62 levels (Fig. 4a). Additionally, the fluorescence intensity of LC3B decreased from 100% to 5% following H/R treatment but was significantly increased to 15%, 22% and 46% with Pro treatment (Fig. 4b,c). These findings suggest that Pro can promote autophagy in H/R-induced H9C2 cells.

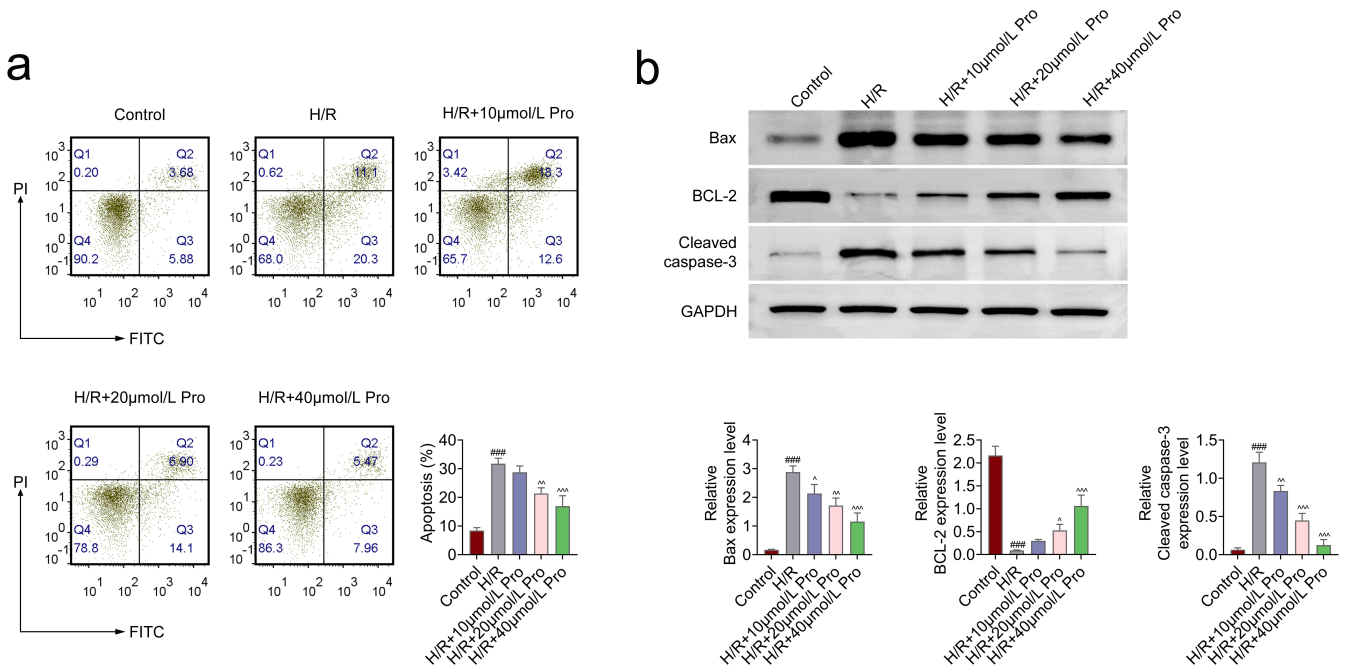

FIGURE 2. Pro reduced cell apoptosis in H/R-triggered H9C2 cells. (a) Cell apoptosis was analyzed by flow cytometry in the Control, H/R, H/R + 10 μmol/L Pro, H/R + 20 μmol/L Pro, and H/R + 40 μmol/L Pro groups. (b) Western blotting was used to measure the protein levels of Bax, BCL-2 and Cleaved caspase-3. Data are presented as mean ± SD; $^{\#\#\#}p < 0.001$ versus Control, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ versus H/R. Pro: Protopine; H/R: hypoxia/reoxygenation; PI: propidium iodide; FITC: fluorescein isothiocyanate; BCL-2: B-cell lymphoma-2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

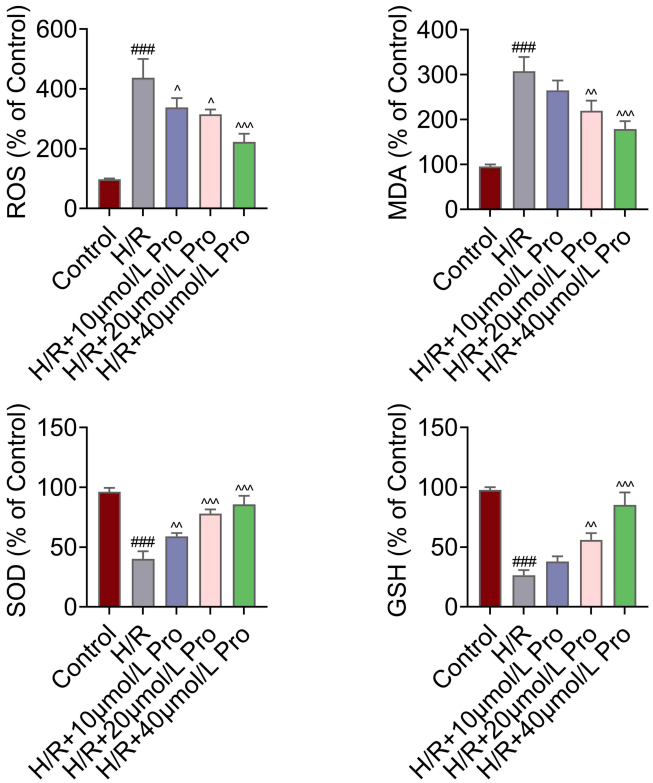

FIGURE 3. Pro attenuated oxidative stress in H/R-stimulated H9C2 cells. Groups were separated into Control, H/R, H/R + 10 μmol/L Pro, H/R + 20 μmol/L Pro, and H/R + 40 μmol/L Pro. Levels of ROS, MDA, SOD and GSH were measured using commercial kits. Data are presented as mean ± SD; $^{\#\#\#}p < 0.001$ versus Control, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ versus H/R. Pro: Protopine; H/R: hypoxia/reoxygenation; ROS: reactive oxygen species; MDA: malondialdehyde; SOD: superoxide dismutase; GSH: glutathione.

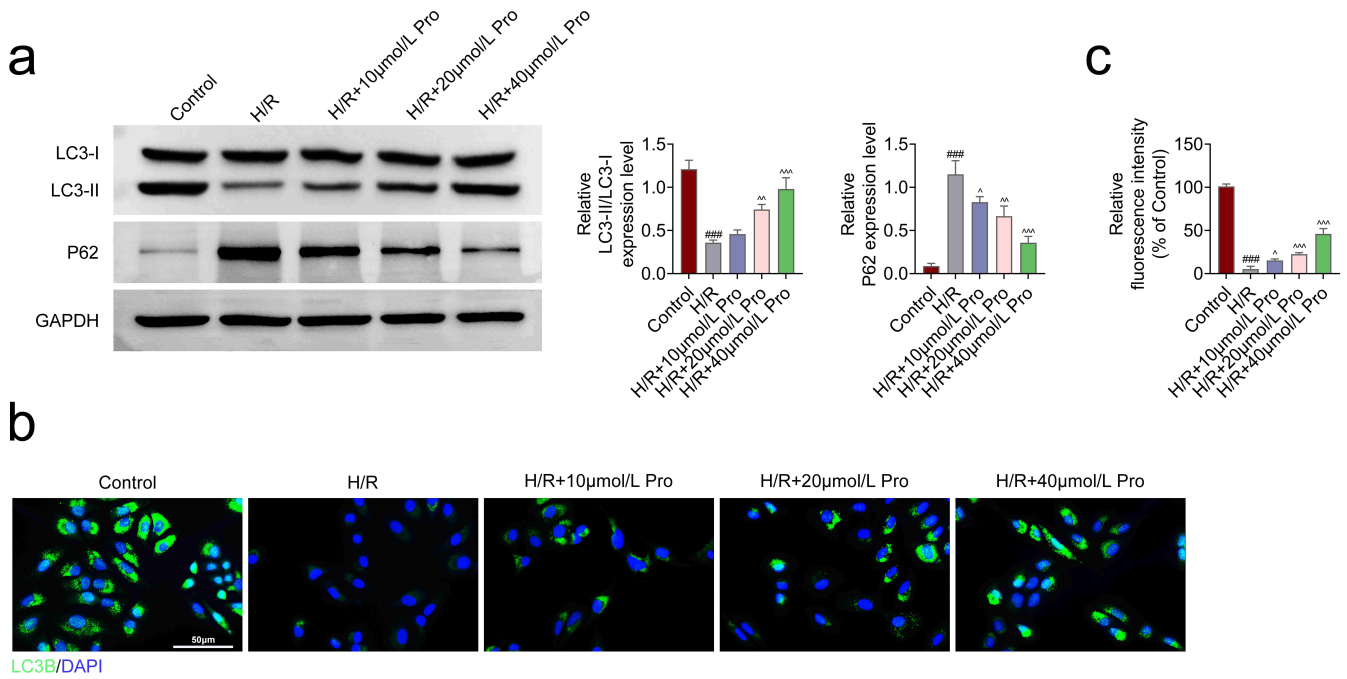

FIGURE 4. Pro enhanced autophagy in H/R-induced H9C2 cells. (a) Western blot analysis was conducted to determine the protein levels of LC3-I, LC3-II and P62. (b,c) Immunofluorescence staining was used to assess LC3B fluorescence intensity. Groups included Control, H/R, H/R + 10 μmol/L Pro, H/R + 20 μmol/L Pro, and H/R + 40 μmol/L Pro. Data are presented as mean ± SD; $^{\#\#\#}p < 0.001$ versus Control, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ versus H/R. Pro: Protopine; H/R: hypoxia/reoxygenation; LC3: microtubule-associated protein 1 light chain 3; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DAPI: 4,6-diamino-2-phenyl indole.

3.5 Pro activates the PTEN/PI3K/Akt pathway

Fig. 5a,b shows that H/R treatment reduced the protein levels of PTEN, p-PI3K/PI3K, p-Akt/Akt and p- mammalian target of rapamycin (mTOR)/mTOR. However, treatment using Pro was found to effectively reverse these effects, indicating that Pro activates the PTEN/PI3K/Akt signaling pathway.

4. Discussion

Natural products, including Pro derived from plants, show promise for treating various diseases [12], but the mechanisms of Pro's cardioprotective effects in hypoxia-induced damage are not yet fully understood [5, 6, 13, 14].

Herein, our findings indicate that Pro enhances cell proliferation and reduces apoptosis in H/R-treated H9C2 cells. These observations are consistent with the broader context of oxidative stress modulation in myocardial injury research. For example, histidine triad nucleotide binding protein 2 (*HINT2*) knockdown exacerbates oxidative stress in MI models [15], while compounds such as Icariside II and Dioscin mitigate oxidative stress through distinct pathways. Icariside II activates the nuclear factor erythroid 2-related factor 2 (Nrf2)/silent information regulator 3 (SIRT3) pathway, which suppresses oxidative stress and alleviates MI [16]. Similarly, Dioscin alleviates oxidative stress and inflammation by regulating the bone morphogenetic protein 4 (BMP4)/nicotinamide adenine dinucleotide phosphate oxidase 1 (NOX1) pathway [17]. lncRNA cancer susceptibility candidate 2 (*CASC2*) has also been shown to reduce oxidative stress in MI by targeting specific microRNAs [18]. Our results corroborate these findings by demonstrating that Pro reduces oxidative stress in H/R-treated cells.

Autophagy and apoptosis are essential processes for cellular homeostasis and often share regulatory mechanisms [19, 20]. The interaction between these processes can influence disease outcomes. In the context of myocardial injury, autophagy plays a significant role. For instance, Idebenone modulates excessive autophagy through the ROS-adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK)-mTOR pathway, thereby protecting cardiac function [21]. Additionally, activating transcription factor 3 (ATF3) and amphiregulin have been implicated in regulating autophagy and myocardial fibrosis [22, 23], while Ivabradine has been shown to enhance autophagy and mitigate MI [24]. Our study extends these findings by showing that Pro promotes autophagy in H/R-induced H9C2 cells, as evidenced by increased LC3-II/LC3-I ratios and enhanced LC3B fluorescence intensity.

The PTEN/PI3K/Akt signaling pathway is crucial in regulating myocardial injury and has been the target of various therapeutic interventions. For instance, Astragaloside IV and miR-26a-5p have been shown to modulate this pathway, providing cardioprotection [25] and improving myocardial injury [26]. Additionally, Atorvastatin affects this pathway to reduce myocardial apoptosis [27]. The effects of Pro on the PTEN/PI3K/Akt pathway in MI are novel, with our study

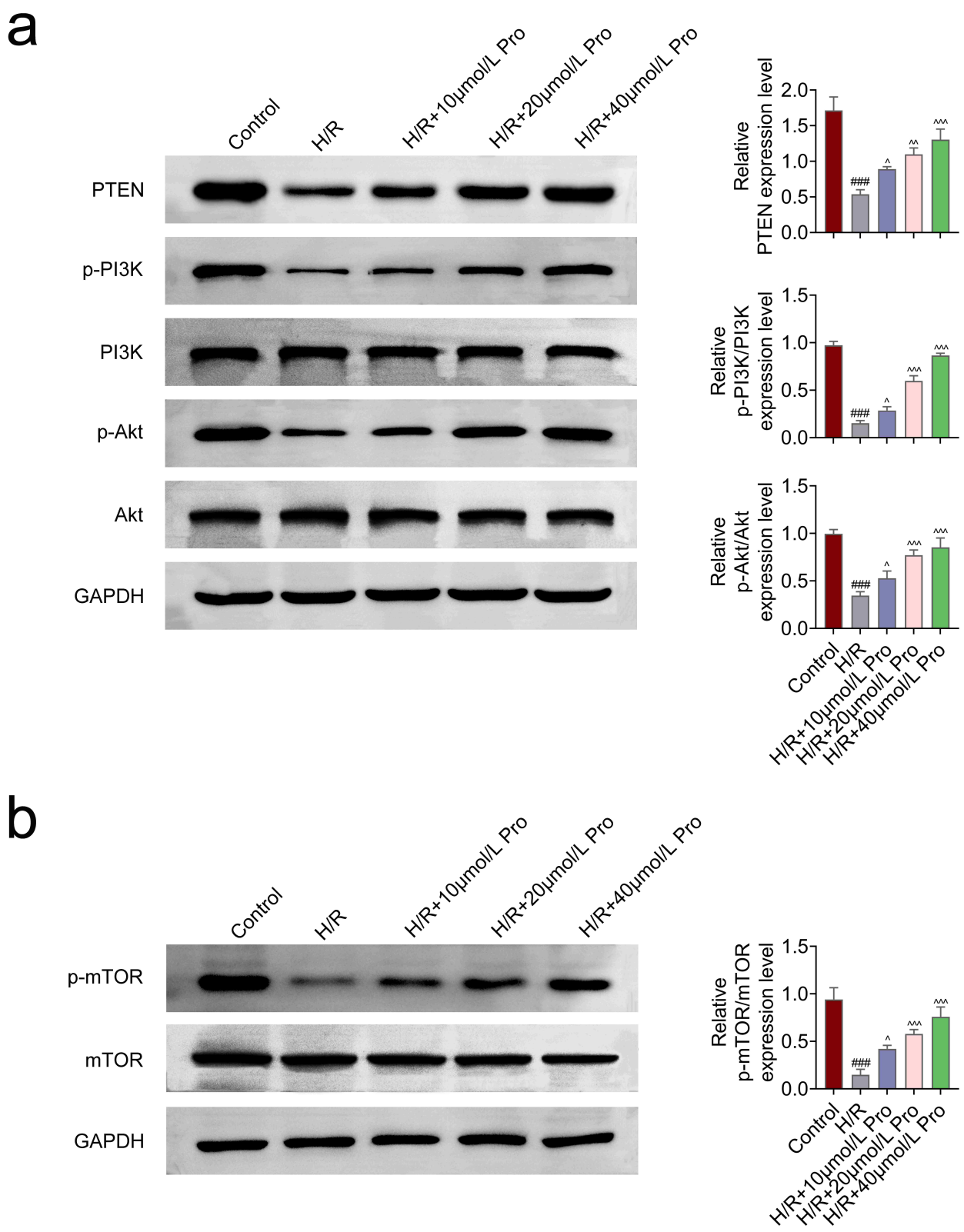

FIGURE 5. Pro activated the PTEN/PI3K/Akt pathway. (a) Western blotting was used to analyze the protein expression levels of PTEN, p-PI3K, PI3K, p-Akt and Akt. (b) Western blotting was also used to evaluate the protein levels of p-mTOR and mTOR. Groups included Control, H/R, H/R + 10 μmol/L Pro, H/R + 20 μmol/L Pro, and H/R + 40 μmol/L Pro. Data are presented as mean ± SD; $^{\#\#\#}p < 0.001$ versus Control, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ versus H/R. Pro: Protopine; H/R: hypoxia/reoxygenation; PTEN: phosphatase and tensin homolog deleted on chromosome ten; PI3K: phosphatidylinositol 3-kinase; Akt: protein kinase B; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; mTOR: mammalian target of rapamycin.

providing new evidence that Pro activates this pathway in H/R-treated H9C2 cells.

5. Conclusions

In summary, Pro reduces oxidative stress, apoptosis, and promotes autophagy in hypoxic cardiomyocytes while also activating the PTEN/PI3K/Akt pathway, suggesting its potential for myocardial protection. Further research, including clinical trials and animal studies, is needed to confirm these findings and explore additional effects.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article. The datasets used and/or analyzed are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

XWM—conducted Material preparation and experiments. JD, YW—carried out Data collection and analysis. PYZ—written the first draft of the manuscript. All authors made subsequent revisions. All authors reviewed and approved the final manuscript. All authors contributed to the study's conception and design.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found, in the online version, at https://oss.signavitae.com/mre-signavitae/article/1843472232596029440/attachment/Supplementary%20material.docx.

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