DL-3-n-butylphthalide protects H9c2 cardiomyoblasts from ischemia/reperfusion injury by regulating HSP70 expression via PI3K/AKT pathway activation

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Abstract. DL-3-n-butylphthalide (NBP) is commonly used to treat ischemic strokes due to its antioxidative and anti-inflammatory effects. The present study aimed to examine the protective effects of NBP on myocardial ischemia-reperfusion injury (MIRI) by establishing a MIRI model in H9c2 cells. Cell viability assay using Cell Counting Kit-8, lactate dehydrogenase (LDH) cytotoxicity and lipid peroxidation malondialdehyde (MDA) content were assessed to detect cell activity, degree of cell injury and oxidative stress reaction. Reverse transcription-quantitative PCR was used to quantify the expression of inflammatory factors in H9c2 cells. Western blotting and immunofluorescence staining were used to detect the protein expression of PI3K/AKT and heat shock protein 70 (HSP70). The present results indicated that NBP significantly increased cell viability during ischemia-reperfusion. Moreover, NBP inhibited the release of LDH and the production of MDA. NBP treatment also significantly decreased the expression of inflammatory factors at the mRNA level. Additionally, NBP activated the PI3K/AKT pathway and upregulated the expression of HSP70 compared with cells in the MIRI model. LY294002, a PI3K inhibitor, reversed the protective effects of NBP and suppressed the expression of HSP70. The present study demonstrated that NBP protected H9c2 cells from MIRI by regulating HSP70 expression via PI3K/AKT pathway activation.

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Introduction

With the wide application of intervention and cardiac surgery in heart disease, myocardial ischemia-reperfusion injury (MIRI) has increased, becoming an urgent problem in the medical field. The main consequence of ischemia is bioenergetic exhaustion caused by an insufficient transport of oxygen and nutrients (1). MIRI is a phenomenon in which the ischemic injury of tissues and organs is not alleviated, but further aggravated after blood perfusion; it is caused by an increase in oxygen free radical content, calcium overload, inflammatory cell infiltration and other phenomena associated with ischemia-reperfusion, leading to cardiomyocyte necrosis and apoptosis (2). However, timely reperfusion is effective to resuscitate myocardial tissue and improve clinical prognosis, paradoxically, this type of reperfusion therapy causes more severe myocardial injury than simple ischemia (3). A previous study has reported that certain interventions, such as primary unloading, could limit infarct size after acute myocardial infarction (4). In addition, a number of anti-ischemia-reperfusion drugs have emerged, but their effect in clinical application has not been exceptional (5). Therefore, the mechanism of reperfusion injury needs to be further elucidated.

Heat shock proteins (HSPs) are highly conserved proteins. The expression of HSPs is induced to protect body functions during stress exposure (6). HSP70 is an endogenous protective protein that plays a protective role in MIRI (7,8). The PI3K/AKT signaling pathway has also been indicated to serve an important role in the protection of cardiomyocytes in MIRI (9-11). The PI3K family is divided into class I, II and III forms, class I being more commonly detected in MIRI (12). Previous studies have indicated that apoptosis

during reperfusion is an important cause of fatal cardiomyocyte injury. The remedial kinase pathway against apoptosis during reperfusion is called the reperfusion injury salvage kinase (RISK) pathway (13). The PI3K/AKT pathway is one of the RISK signaling pathways (14), which functions through the regulation of cell morphology, cardiomyocyte survival, apoptosis, protein synthesis and metabolic integration (15). Previous studies have identified a close association between the PI3K/AKT pathway and HSP70, and this mutual regulation has been demonstrated in brain and lung tissues (16,17). However, the association between PI3K/AKT and HSP70 in the myocardium requires further elucidation.

DL-3-n-butylphthalide (NBP) is a natural product extracted from celery seeds (18). Previous studies have revealed that NBP promoted vasodilation and improved cerebral microcirculation by promoting the production of nitric oxide (19,20). Furthermore, NBP has been indicated to stabilize the blood-brain barrier and mitochondrial membrane structure by inhibiting inflammation and oxidative stress (21-24). Interestingly, NBP has also been hypothesized to play an important role in MIRI by reducing myocardial infarction and the incidence of arrhythmia (25,26). Recent studies have indicated that NBP significantly improved cardiac function by regulating the PI3K/AKT pathway in MIRI, and that HSP70 exhibited protective effects on MIRI (7,27). However, the role of HSP70 in the cardioprotective effect of NBP remains unknown. Furthermore, further research is required to explore the relationship between HSP70 and the PI3K/AKT signaling pathway.

Therefore, the present study aimed to explore whether NBP protected against MIRI by reducing inflammation and oxidative stress through the PI3K/AKT signaling pathway and HSP70. The association between NBP, PI3K/AKT and HSP70 was also explored.

Materials and methods

Materials and reagents. NBP (cat. no. H20100041) was purchased from CSPC Enbipu Pharmaceutical Co., Ltd. LY294002 (cat. no. A8250; PI3K inhibitor) was purchased from APeXBIO Technology LLC. FBS (cat. no. SFBS) was purchased from Bovogen Biologicals Pty Ltd. DMEM (low-glucose, cat. no. 30021; high-glucose, cat. no. 30022) was purchased from HyClone; Cytiva. ChamQ Universal SYBR qPCR Master Mix (cat. no. Q711-02) and HiScript II Q RT SuperMix for qPCR + gDNA wiper (cat. no. R223-01) were purchased from Vazyme Biotech Co., Ltd. All antibodies and reagents were of analytical grade and are commercially available.

Cell culture. H9c2 cells (cat. no. CL-0089) were obtained from Procell Life Science & Technology Co., Ltd. H9c2 cells ($1x10^6$ cells/ml) were seeded in 25-cm^2 cell-culture flasks containing high-glucose DMEM (10% FBS; 1% penicillin/streptomycin). The cells were cultured in a cell incubator with 95% air and 5% CO_2 at 37˚C. After H9c2 cells were cultured for 24 h, they were randomly assigned to four groups: i) Control group (CON); ii) MIRI group; iii) NBP pretreatment group (MIRI + NBP); and iv) PI3K inhibitor group (MIRI + NBP + LY294002). CON cells were cultured at 37˚C with 95% air. MIRI cells were incubated at 37˚C with 5% CO_2, 93% N_2 and 2% O_2 for 6 h, then reoxygenated for 4 h. MIRI + NBP cells were pretreated with 100 μM NBP for 2 h before being subjected to hypoxia for 6 h, followed by reoxygenation for 4 h. MIRI + NBP + LY294002 cells were pretreated with 10 μM LY294002 (28) for 1 h before treatment with NBP for 2 h, followed by hypoxia for 6 h and reoxygenation for 4 h.

Construction of the H9c2 MIRI model. To simulate the *in vivo* model of ischemia-reperfusion injury, the cell culture medium was replaced with low-glucose DMEM without FBS, and the cells were incubated at 37˚C with 5% CO_2, 93% N_2 and 2% O_2. A total of 10 μM of LY294002 was added 1 h before NBP treatment. NBP pretreatment lasted for 2 h before hypoxia. After MIRI, the medium was replaced with high-glucose DMEM (10% FBS; 1% penicillin/streptomycin) and the cells were cultured in an incubator (37˚C; 5% CO_2; 95% air) for 4 h.

Determination of optimal NBP concentration and cell viability. H9c2 cells ($1x10^4$ cells/well) were seeded in 96-well plates for 24-48 h, and then pretreated with different doses of NBP (1, 50, 100, 200, 300 and 500 μM). After reperfusion, the original medium was discarded and 100 μl medium containing 10 mg/ml Cell Counting Kit-8 (CCK-8; cat. no. CA1210-100; Beijing Solarbio Science & Technology Co., Ltd.) were added to each well. The absorbance at 450 nm was measured after culture for 1 h without light. The final cell viability of each group was calculated with the following formula: Cell viability (%)=(experimental group-blank)/(MIRI only group-blank) x100.

Determination of oxidation index. After ischemia-reperfusion treatment, total proteins were extracted using RIPA lysis buffer (cat. no. R0020; Beijing Solarbio Science & Technology Co., Ltd.). BCA Protein Assay Kit (cat. no. CW0014; CoWin Biosciences) was used to measure the protein concentration. According to the instructions of the Lipid Peroxidation malondialdehyde (MDA) Assay Kit (cat. no. S0131; Beyotime Institute of Biotechnology), freshly prepared MDA detection solution was added to blank, standard and sample tubes, heated at 100˚C for 15 min, centrifuged at room temperature and 1,000 x g for 10 min, and 200 μl of each supernatant were placed in a 96-well plate. The absorbance was determined at 530 nm using microplate reader (Bio-Rad Laboratories, Inc.) and the absolute value of MDA of each group was calculated. The MDA content per weight of protein (nmol/mg) was calculated according to the protein concentration of each group.

Determination of lactate dehydrogenase (LDH) in the culture medium. According to the instructions of the LDH Cytotoxicity Assay Kit (cat. no. C0017; Beyotime Institute of Biotechnology), after the ischemia-reperfusion treatment, the culture medium of each group was centrifuged for 5 min at room temperature and 400 x g, and 120 μl supernatant of each sample were placed in a 96-well plate. LDH detection solution (60 μl) was added to each well for a 30-min incubation at room temperature. The optical density was measured at 490 nm using a microplate reader, and the amount of LDH (mU/ml) released by the cells in each group was determined.

mRNA expression of IL-1β and TNF-α in H9c2 cells. The mRNA expression of inflammatory factors in H9c2 cells was determined via reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol® reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.), and the RNA concentration was quantified in each group. Complementary DNA (cDNA) was obtained from RNA using HiScript II Q RT SuperMix for qPCR + gDNA wiper according to the manufacturer's instructions. A PCR Master Mix of 10 μl (4.8 μl cDNA template; 5 μl qPCR Master Mix; 0.2 μl gene primers) and a LightCycler® 480 system II [Roche Diagnostics (Shanghai) Co., Ltd.] were used to perform qPCR. The following thermocycling conditions were used for qPCR: Pre-denaturation at 95˚C for 5 min; 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. The expression levels were normalized to GAPDH. The primers used were as follows: TNF-α forward, 5'-TGA TCGGTCCCAACAAGGA-3' and reverse, 5'-TGCTTGGTG GTTTGCTACGA-3'; IL-1β forward, 5'-GGGATGATGACG ACCTGC-3' and reverse, 5'-CCACTTGTTGGCTTATGTT-3'; GAPDH forward, 5'-GTTACCAGGGCTGCCTTCTC-3' and reverse, 5'-ACCAGCTTCCCATTCTCAGC-3'. The mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (29).

Western blot analysis. After establishing the MIRI model, the original medium was discarded and H9c2 cells were washed three times using TBS with Tween-20 (TBST; cat. no. T1085; Beijing Solarbio Science & Technology Co., Ltd.). A total of 120 μl RIPA lysis buffer was added to each 25-cm^2 culture flask. The lysates were centrifuged at 4˚C and 12,000 x g for 5 min. BCA Protein Assay Kit was used to measure protein concentration. The protein samples were mixed with 5X loading buffer (cat. no. P1015; Beijing Solarbio Science & Technology Co., Ltd.) at a 4:1 ratio, and subsequently boiled at 100˚C for 5 min. A total of 30 μg protein/sample were separated by 10% SDS-PAGE. The proteins were transferred onto PVDF membranes (MilliporeSigma), which were then blocked with 5% non-fat milk at room temperature for 2 h. The membranes were incubated at 4˚C overnight with primary antibodies as follows: Anti-PI3K (1:10,000; cat. no. 09-482; MilliporeSigma); anti-AKT (1:2,000; cat. no. 05-591; MilliporeSigma); anti-phosphorylated (p)-AKT (1:7,000; cat. no. ab81283; Abcam); anti-HSP70 (1:1,000; cat. no. ab181606; Abcam) or anti-GAPDH (1:10,000; cat. no. ab181602; Abcam). After washing with TBST three times, the goat anti-rabbit immunoglobulin G (IgG; H+L) horseradish peroxidase (HRP; 1:5,000; cat. no. GAR007; MultiSciences Biotech Co., Ltd.) or goat anti-mouse IgG (H+L) HRP (1:5,000 dilution, GAM007; MultiSciences Biotech Co., Ltd.) was incubated with the membrane for 2 h at room temperature, and the membranes were subsequently washed three times with TBST. Protein bands were visualized using ECL detection reagents (cat. no. CW0049M; CWBIO). The relative band intensity was measured by Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Immunofluorescence staining. Cells were cultured for 24 to 48 h before the MIRI model was established. Subsequently, the cells were washed three times with PBS and fixed with 4% paraformaldehyde (cat. no. P1110; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 30 min, then blocked with 10% goat serum (cat. no. SL038; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 30 min. The cells were incubated with the primary antibody overnight at 4˚C using PI3K (1:5,000; cat. no. 09-482; MilliporeSigma), phosphorylated (p)-Akt (1:200; cat. no. ab81283; Abcam), and HSP70 (1:50; cat. no. ab181606; Abcam). The cells were then washed and incubated with goat anti-mouse IgG-FITC (1:500; cat. no. SA0015; Beijing Solarbio Science & Technology Co., Ltd.) or goat anti-rabbit IgG-FITC (1:500; cat. no. SA0025; Beijing Solarbio Science & Technology Co., Ltd.) for 30 min without light at 37˚C. Nuclei were stained with a DAPI solution (cat. no. C0065; Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C for 5 min. The cells were mounted with an anti-fluorescence attenuator and the staining was observed under a fluorescence microscope.

Statistical analysis. SPSS v20.0 statistical software (IBM Corp.) was used to analyze the data. Results are presented as the mean ± SEM of three experimental repeats. Significant differences were determined using one-way ANOVA followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of different concentrations of NBP on the viability of H9c2 cells. The viability of H9c2 cells treated with different NBP doses was examined via the CCK-8 colorimetric assay. Compared with the MIRI group, 100 μM NBP increased cell viability ($P<0.05$; Fig. 1A). However, NBP concentrations ≥200 μM slightly decreased cell viability (Fig. 1A). The present results indicated that 100 μM NBP was the optimal concentration for protecting cells from MIRI.

NBP inhibits the oxidative stress response during MIRI. When ischemia-reperfusion injury occurs in cardiomyocytes, oxidative stress also plays an important role (30). An MDA kit was used to explore the effect of NBP on the oxidative stress index after MIRI and the role of PI3K in this process. The oxidation level in the MIRI group was significantly higher compared with the CON group ($P<0.05$). However, NBP pretreatment significantly decreased oxidative stress ($P<0.05$). In contrast, LY294002 eliminated the antioxidant effect of NBP ($P<0.05$; Fig. 1B). Namely, NBP significantly decreased the oxidative stress response of H9c2 cells during MIRI, but this antioxidant protective effect was eliminated by blocking PI3K.

NBP protects H9c2 cells from MIRI. In vitro, the destruction of the cell membrane structure caused by apoptosis or necrosis leads to LDH release into the culture medium (31). Therefore, LDH activity can indirectly indicate the degree of cell damage. To explore the effect of NBP on MIRI and the involvement of PI3K in MIRI, H9c2 cell viability was determined via CCK-8 colorimetric assay, while an LDH kit was used to determine the LDH content in the culture medium. Compared with the CON group, the cell viability of the MIRI group was significantly lower ($P<0.05$). However, the cell viability of the MIRI + NBP group was higher than that of the MIRI group ($P<0.05$). By contrast, addition of the PI3K inhibitor LY294002 decreased cell viability ($P<0.05$; Fig. 1C). Furthermore, the LDH content

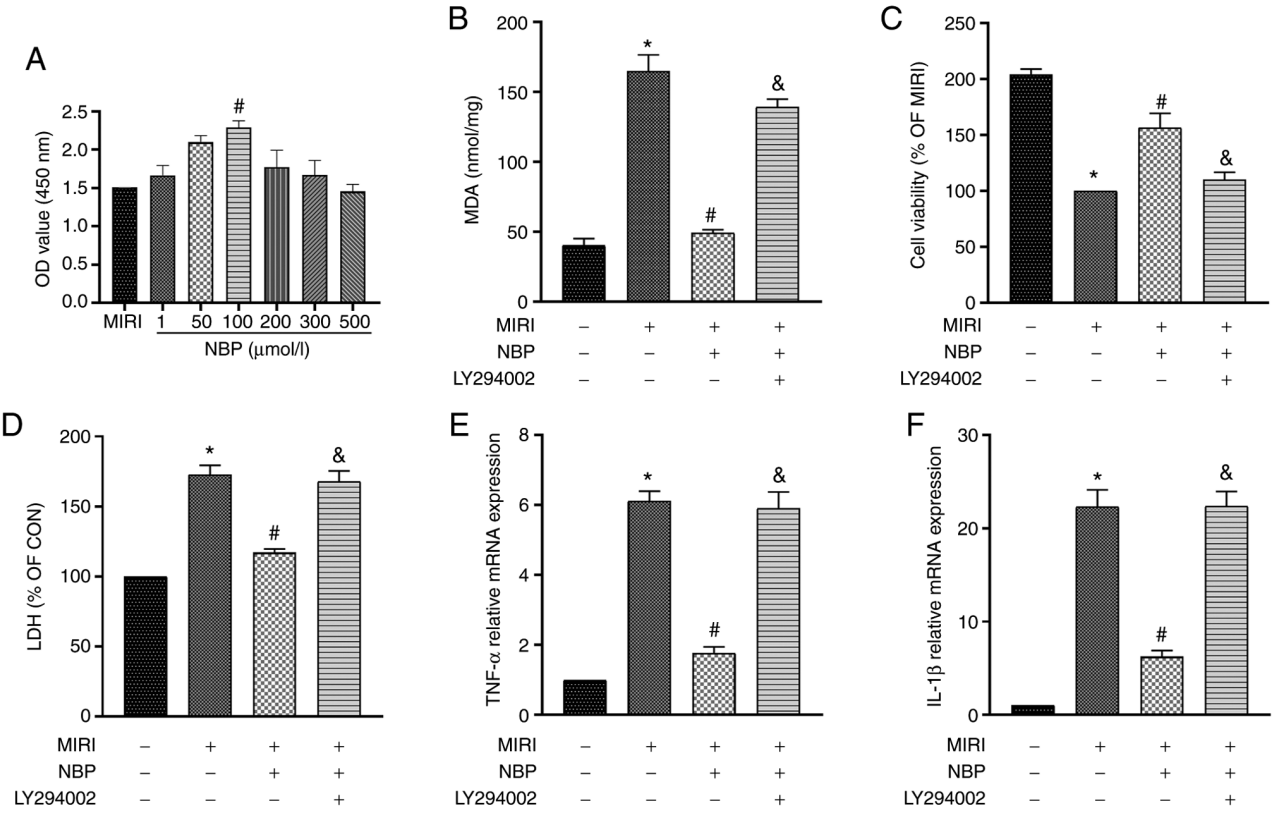

Figure 1. Effects of NBP on H9c2 cell viability, degree of oxidative stress reaction, level of cell injury and mRNA expression of TNF-α and IL-1β in different groups after MIRI. (A) Cell viability after treatment with different NBP concentrations. (B) Lipid peroxidation MDA content was determined via an MDA assay kit. (C) Cell viability percentages. (D) The release of LDH was determined using an LDH release assay kit. mRNA expression of (E) TNF-α and (F) IL-1β was assessed by reverse transcription-quantitative PCR in each group. Results are expressed as the mean ± SEM. *P<0.05 vs. CON; $^{\#}$P<0.05 vs. MIRI; $^{\&}$P<0.05 vs. MIRI + NBP. NBP, DL-3-n-butylphthalide; MIRI, myocardial ischemia-reperfusion injury; OD, optical density; MDA, malondialdehyde; LDH, lactate dehydrogenase; CON, control.

of the MIRI group was significantly higher than that of the CON group (P<0.05). Pretreatment with NBP decreased the LDH release (P<0.05), but addition of LY294002 reversed this effect (P<0.05; Fig. 1D). Thus, NBP significantly increased the viability of H9c2 cells after ischemia-reperfusion injury and reduced H9c2 cell injury. However, the PI3K inhibitor LY294002 reversed the protective effect of NBP on H9c2 cells.

NBP inhibits inflammation by decreasing the TNF-α and IL-1β mRNA expression. Activation of inflammatory cytokines during MIRI aggravates the injury of cardiomyocytes (32). Therefore, to determine the association between the protective effect of NBP during MIRI and inflammatory factors, the mRNA expression of TNF-α and IL-1β was examined in H9c2 cells via RT-qPCR. Compared with the CON group, the expression of TNF-α and IL-1β in the MIRI group significantly increased (P<0.05). However, the expression of these genes decreased in the MIRI + NBP compared with the MIRI group (P<0.05). By contrast, addition of LY294002 increased the expression of TNF-α and IL-1β compared with the MIRI + NBP group (P<0.05; Fig. 1E and F). Therefore, it was revealed that NBP effectively reduced the expression of inflammatory factors during MIRI, while the application of the PI3K inhibitor LY294002 reversed this anti-inflammatory effect.

NBP activates the HSP70 and PI3K/AKT signaling pathway. During MIRI, the expression of the stress-protective proteins HSP70 and PI3K/AKT was upregulated. Western blotting and immunofluorescence staining were used to determine the protein expression of HSP70 and PI3K/AKT after NBP treatment. The expression levels of p-AKT and PI3K in the MIRI group were significantly increased compared with the CON group (P<0.05). In the MIRI + NBP group, the expression of p-AKT and PI3K was increased compared with the MIRI group (P<0.05). By contrast, in the MIRI + NBP + LY294002 group, the expression of p-AKT and PI3K decreased compared with the MIRI + NBP group (P<0.05; Fig. 2A-D). This tendency was also observed in the immunofluorescence assay (Fig. 2E and F). Additionally, the expression level of HSP70 in the MIRI group increased compared with the CON group (P<0.05). NBP pretreatment further increased the expression of HSP70 (P<0.05), whereas LY294002 decreased it compared with the MIRI + NBP group (P<0.05; Fig. 3A and B). This tendency was also observed in the immunofluorescence assay (Fig. 3C). The present results indicated that NBP pretreatment could increase the expression level of PI3K, p-AKT and HSP70 following MIRI, and that the inhibition of the PI3K pathway also altered HSP70 expression.

Discussion

MIRI mechanism involves several processes, including energy metabolism disorder, oxidative stress, calcium overload, inflammation, apoptosis and autophagy (33). In the clinic, the

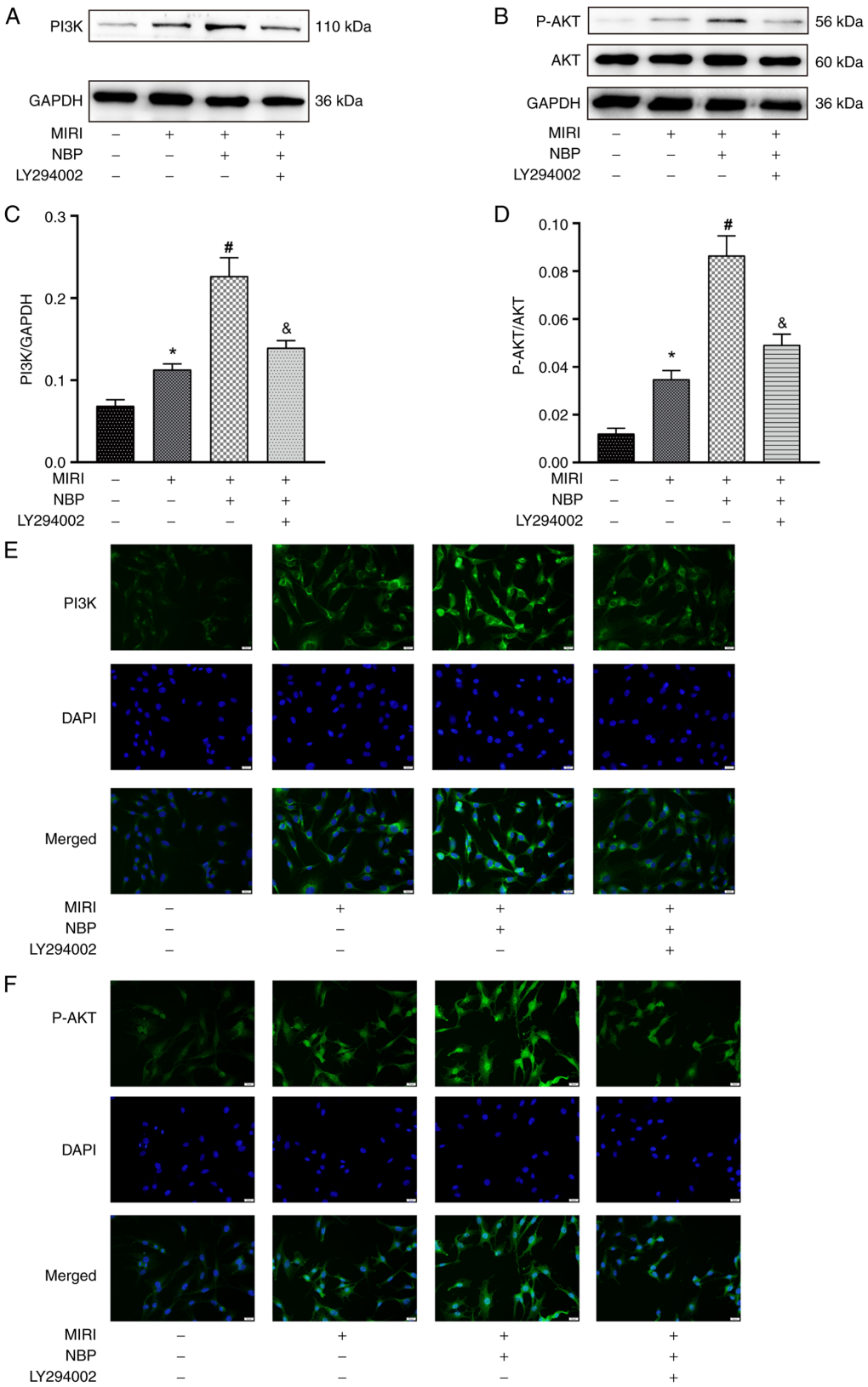

Figure 2. Protein expression of PI3K, AKT and p-AKT in H9c2 cells in different groups after MIRI using western blotting and immunofluorescence. (A) PI3K and (B) AKT and p-AKT expression in H9c2 cells of each group assessed via western blotting. Quantification of the relative expression of (C) PI3K and (D) p-AKT. Results are expressed as the mean ± SEM. *P<0.05 vs. control; #P<0.05 vs. MIRI; &P<0.05 vs. MIRI + NBP. Immunofluorescence staining demonstrating (E) PI3K and (F) p-AKT expression in H9c2 cells of each group. Green represents PI3K or p-AKT and blue represents the nuclei. Scale bar, 20 μm. NBP, DL-3-n-butylphthalide; MIRI, myocardial ischemia-reperfusion injury; p, phosphorylated.

best treatment for patients with myocardial infarction is immediate reperfusion, causing a paradoxical situation because of its adverse effects (34). Therefore, an in-depth understanding of the mechanism of MIRI has become a priority.

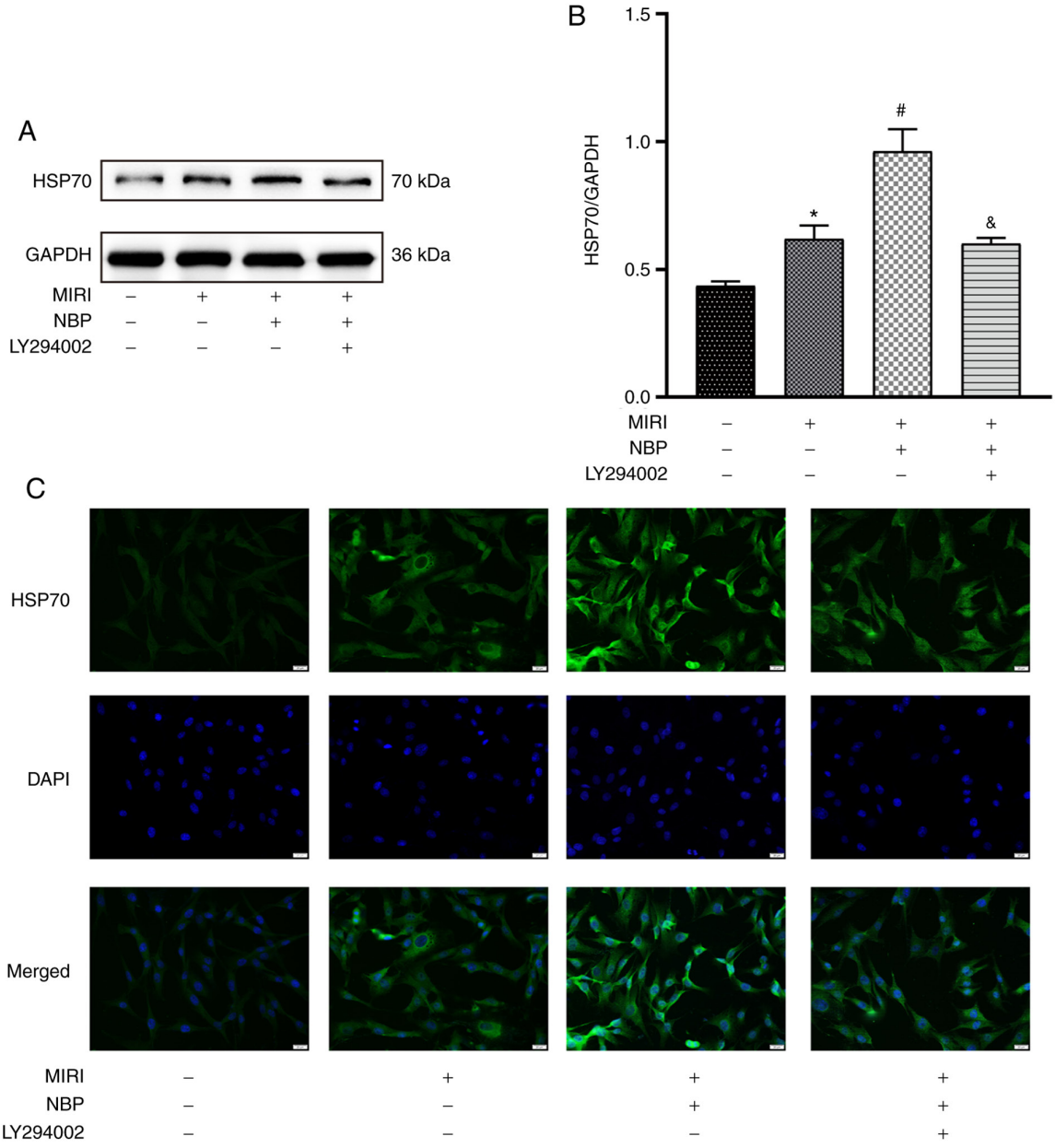

Figure 3. Protein expression of HSP70 in H9c2 cells in different groups after MIRI using western blotting and immunofluorescence. (A) HSP70 expression in H9c2 cells of each group and (B) quantification of the relative expression. Results are expressed as the mean ± SEM. *P<0.05 vs. control; $^{\#}$P<0.05 vs. MIRI; $^{\&}$P<0.05 vs. MIRI + NBP. (C) Immunofluorescence staining demonstrating HSP70 expression in H9c2 cells of each group. Green represents HSP70 and blue represents the nuclei. Scale bar=20 μm. NBP, DL-3-n-butylphthalide; MIRI, myocardial ischemia-reperfusion injury; HSP70, heat shock protein 70.

As a drug used for the treatment of stroke, NBP has been widely studied in the nervous system. For instance, a previous study has indicated that NBP could reduce nerve cell death during cerebral ischemia by inhibiting inflammation, oxidative stress, autophagy and apoptosis (35). NBP has a wide range of functions, as it can protect the nervous system and delay the onset and progression of diabetic cataract (25,36). Furthermore, a previous study has revealed that NBP exhibited a protective effect against MIRI, and its mechanism may be associated with the regulation of the mitochondrial apoptosis and the AKT/Nuclear factor erythroid 2-related factor 2 signaling pathways (37). However, the specific mechanism underlying the NBP effect on MIRI needs to be further explored.

As a protective protein under stress, HSP70 has been indicated to play a protective role in cardiomyocytes during MIRI (38). However, it is unclear whether HSP70 is involved in the NBP protective effect on cardiomyocytes. The present results indicated that NBP regulated the expression of HSP70 via the PI3K/AKT signaling pathway, thereby protecting H9c2 cells from MIRI. The present study provided a basis for the application of NBP in the clinical treatment of cardiovascular diseases, and elucidated the protective mechanism of NBP to a certain extent.

The current study determined the optimal concentration of NBP pretreatment against MIRI by establishing a concentration gradient of NBP, indicating that 100 μM NBP exhibited the strongest protective effect compared with the other concentrations. NBP pretreatment improved the decrease in cell viability induced by MIRI and significantly reduced LDH release, thus protecting H9c2 cells from MIRI. LY294002 treatment

reversed the protective effect of NBP, decreased cell viability and increased LDH release. The present results suggested that the protective effect of NBP on H9c2 cells depended on the activation of the PI3K signaling pathway.

During myocardial ischemia, a large number of oxygen free radicals are produced, eventually leading to the destruction of the mitochondrial structure, mitochondrial swelling, cell membrane structure damage and the disturbance of cell energy metabolism (39). MDA is the final product of reactive oxygen species oxidation of arachidonic acid, representing a biomarker of lipid peroxidation produced by oxidative stress (40). The present results indicated that the MDA content in H9c2 cells significantly increased after MIRI; however, the oxidation index indicated by the MDA content significantly decreased after NBP pretreatment. By contrast, the oxidation index increased again after LY294002 intervention. NBP inhibited oxidative stress in a process of myocardial protection, and this antioxidant mechanism was blocked by inhibiting the PI3K pathway.

A number of inflammatory cytokines are activated during MIRI, which can cause systemic inflammation (41,42). TNF-α causes local inflammation and apoptosis, eventually contributing to cardiac insufficiency and even cardiac infarction (43). IL-1 plays a central role in human autoinflammatory diseases and its target genes are numerous, such as IL-1β, IL-6 and IL-8 (44). Among those, IL-1β expression was quantified in the present study. IL-1β is a typical pro-inflammatory factor that mediates the infiltration of neutrophils and macrophages in local tissues during ischemia-reperfusion, which causes myocardial fibrosis and structural remodeling (45). To elucidate the effect of NBP on H9c2 cells, RT-qPCR was performed to determine the expression level of inflammatory cytokines. The present results indicated that the mRNA expression of IL-1β and TNF-α in the MIRI group significantly increased compared with the CON group. Pretreatment with NBP significantly inhibited the inflammatory response; however, this anti-inflammatory effect was reversed by blocking the PI3K signaling pathway. Namely, blocking the PI3K pathway eliminated the inflammatory inhibitory effect of NBP.

The protective role of the PI3K/AKT signaling pathway against MIRI is well-known. For instance, a previous study has demonstrated that activation of the insulin-induced PI3K/AKT pathway could inhibit cardiomyocyte apoptosis and protect or improve local and global cardiac function (46). Upon PI3K-induced activation, AKT activates GSK-3β through phosphorylation, this junction of several pathways plays a notable role in myocardial protection (47). Relevant experiments have confirmed that the increase of heat shock factor 1 (HSF1) results from GSK-3β phosphorylation (48,49). HSP70 is a stress-protective protein that protects cardiomyocytes during MIRI (50). HSP70 induction is mediated by the interaction with HSF1; therefore, PI3K/AKT and HSP70 may be associated through GSK-3β and HSF1 (51,52). It was previously demonstrated that there is a mutual regulation between the PI3K/AKT pathway and HSP70 in brain and lung tissues (16,17). To further elucidate the association between the PI3K/AKT pathway and HSP70 in H9c2 cells, the expression of HSP70 was examined upon application of the PI3K inhibitor LY294002. Western blotting and immunofluorescence staining indicated that MIRI increased the expression levels of HSP70, PI3K and p-AKT, and that NBP pretreatment further increased this expression. By contrast, LY294002 suppressed the expression levels of PI3K, p-AKT and HSP70. Therefore, the protective effect of NBP against MIRI was indicated to be associated with the PI3K/AKT signaling pathway and HSP70.

Certain main limitations can be taken into account in the present work. Firstly, the present regulation pathway needs to be further investigated in an animal model. Secondly, the protective effect of NBP on MIRI has not been reflected in clinical treatment, and further studies are required to explore the drug administration route and dosages. Thirdly, H9c2 cells are rat myoblasts and not cardiomyocytes; therefore, they present differences in their characteristics and protein expression compared with adult cardiomyocytes. Future studies will investigate the present hypotheses in primary cardiomyocytes.

In summary, NBP upregulated HSP70 through the PI3K/AKT pathway and reduced the inflammatory response, oxidative stress and injury of H9c2 cells, thereby attenuating MIRI. These findings may provide a novel therapeutic target for the clinical treatment of 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

WH, XDS and XTS designed the research and revised the manuscript. YY, YZ and YL conducted experiments. MW and BD analyzed the data and participated in technical editing of the manuscript. YY and XTS wrote the draft manuscript. YY and WH confirm the authenticity of all the raw data. 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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