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Nicotinamide protects chronic hypoxic myocardial cells through regulating mTOR pathway and inducing autophagy

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Abstract. – OBJECTIVE: To determine the protective effect of nicotinamide on chronic hypoxic myocardial cells and its underlying mechanism.

MATERIALS AND METHODS: The H9C2 cell lines were taken as objects of study, and were divided into blank group, hypoxia group and nicotinamide treatment group. The cell viability, apoptosis level, autophagy level and mammalian target of rapamycin (mTOR) pathway activity in each group were detected via Cell Counting Kit-8 (CCK8) assay, Hoechst staining, immunofluorescence staining, Polymerase Chain Reaction (PCR) and Western blotting, respectively.

RESULTS: Nicotinamide could protect the viability of normoxic and chronic hypoxic myocardial cells. Besides, it could also inhibit the expression of caspase3 messenger ribonucleic acid (mRNA) in chronic hypoxic myocardial cells, and reduce the expression of apoptosis-related proteins. Furthermore, it could induce the mRNA expression of autophagy-associated gene 5 (ATG5) and increase the expression of autophagy-related proteins. Further study on the mechanism of nicotinamide showed that nicotinamide could inhibit the activity of the mTOR pathway, thus regulating the autophagy.

CONCLUSIONS: Nicotinamide induces the autophagy of chronic hypoxic myocardial cells by regulating the mTOR pathway, thereby protecting cells from apoptosis.

Key Words:
Nicotinamide, Cell hypoxia, Autophagy, Apoptosis, mTOR.

Introduction

The heart is a highly-aerobic organ, and hypoxia affects the cardiac function. Chronic myocardial hypoxia is an important pathophysiological change in various heart diseases, including the cyanotic congenital heart disease, coronary atherosclerotic heart disease, chronic pulmonary heart disease and high-altitude heart disease[1]. The systemic hypoxia or local cardiac hypoxia caused by these diseases will lead to the decline in myocardial contraction, accelerating the progression of heart failure.

Nicotinamide, also known as NAM, is an amide compound of the B vitamin niacin, which is a component of coenzyme I and coenzyme II. Besides, it is also involved in cell respiration and energy synthesis[2]. NAM is mainly applied in the clinical treatment of myocardial conduction block, viral myocarditis, etc., with significant efficacy. Studies[3] have demonstrated that the cardiac ischemia and hypoxia injury in mice can be alleviated by NAM-rich feed, but its mechanism of myocardial protection remains unclear. NAM can modulate the cellular energy metabolism by regulating the adenosine monophosphate-activated protein kinase (AMPK) pathway, thereby protecting hypoxic cells and reducing apoptosis[4]. AMPK, as an "energy sensor" of cells, plays an important role in maintaining cellular energy metabolism balance and homeostasis[5]. The activation of AMPK can increase glucose uptake, enhance fatty acid oxidation and inhibit protein biosynthesis, thereby increasing the cellular energy reserve[6].

The mammalian target of rapamycin (mTOR) pathway is a downstream target of AMPK, and the regulation on this pathway exerts an important effect on cell viability[7]. The mTOR protein is the focus of various intracellular signaling pathways, whose activity has an important influence on cell growth, differentiation and proliferation. In addition, the mTOR protein is also an upstream regulatory protein of the autophagy pathway, and the activation of the mTOR pathway can inhibit autophagy[8].

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Autophagy is a metabolic process in which cells degrade cellular content and keep cell viability in virtue of lysosomes. Generally, vesicles with double-membrane structure wrap the cellular content to form autophagosomes, and then fuse with lysosomes to form autophagic lysosomes, ultimately degrading cellular content through various enzymes[9]. Autophagy is an important way to control the cell mass, through which cells meet the metabolic needs, and remove and renew cellular content and organelles[10]. Studies have found that the autophagy level of myocardial cells markedly increases after hypoxia, which is conducive to removing the damaged organelles in damaged cells and alleviating apoptosis[11].

In this work, the model of chronic hypoxic myocardial cells was established using chronic hypoxic H9C2 cells, and the apoptosis, autophagy and the mTOR pathway in each group were observed after the treatment with NAM.

Materials and Methods

Cells and Experimental Materials

H9C2 cells were purchased from the Shanghai Institute of Cell Research, Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and experimental antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Other reagents were purified in analytical grade.

Cell Culture

The cell suspension was transferred into a 10 mL centrifuge tube and added with 5 mL of DMEM containing 10% FBS, followed by centrifugation at 1000 rpm for 10 min. Then, the supernatant was discarded, and an appropriate amount of DMEM containing 10% FBS was added and mixed evenly. The cells were inoculated into a 25 mL culture flask (1×10^5/mL) and incubated in an incubator with 5% CO_2 at 37°C. The medium was replaced after 24 h.

Cell Hypoxia

The cells in the hypoxia group were cultured in a hypoxic incubator containing 94% N_2, 5% CO_2 and 1% O_2 at 37°C for 48 h to establish the myocardial ischemia-hypoxia model *in vitro*[13]. The cells in the blank group were still cultured in the normal incubator.

Cell Counting Kit-8 (CCK-8)

The cells in the logarithmic growth phase were digested, collected, adjusted into cell suspension at a concentration of 1×10^5/mL, and then inoculated into a 96-well plate (100 μL/well). Three repeated wells were set in the experiment, and the blank control wells were also set at the same time. After inoculation overnight, the good cell adherence was confirmed *via* microscopic observation. After the grouping and treatment, 20 μL of methyl thiazolyl tetrazolium (MTT; Sigma-Aldrich, St. Louis, MO, USA) was added into each well, followed by culture at 37°C for another 4 h. Then, the supernatant was carefully discarded, and 150 μL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added into each well and mixed evenly. The optical density (OD) value of each well was determined at a wavelength of 570 nm using a microplate reader. The experiment was repeated 3 times.

Lactate Dehydrogenase (LDH)

The cells in the logarithmic growth phase were digested, collected, adjusted into the cell suspension at a concentration of 1×10^5/mL, and then inoculated into a 96-well plate (100 μL/well). Three repeated wells and the blank control wells were set in the experiment. After the cell adherence, the culture plate was placed into the incubator with 5% CO_2 for incubation at 37°C for 24 h. After grouping, 20 μL supernatant was taken from each well. Besides, the corresponding reagent was added (according to instructions of the kit (Solarbio, Beijing, China)), mixed evenly and placed at room temperature for 3 min, followed by zero setting with 440 nm double distilled water using the 1 cm-light-path cuvette. The OD value of each tube was determined using the microplate reader. 1 gmoL pyruvic acid produced in the reaction system after the action of 1,000 mL of the culture solution and substrate at 37°C for 15 min was taken as 1 unit. The LDH content in the medium was calculated according to the formula.

Monodansylcadaverine (MDC) Staining

The cells in the logarithmic growth phase were taken and inoculated into the 6-well plate (10×10^6/mL). Three repeated wells were set in each group. After grouping, the cells were fixed with methanol, stained with MDC, washed, suspended and collected, followed by analysis using flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) and measurement of the ratio

of MDC-positive cells *via* CellQuest software. A small number of cells were resuspended, applied on a glass slide and covered with a cover glass, followed by observation under an inverted fluorescence microscope immediately at an excitation wavelength of 450-490 nm and photography.

Polymerase Chain Reaction (PCR)

After the treatment, the cells in each group were collected, and the total ribonucleic acid (RNA) was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). The sample concentration was measured, based on which the reverse transcription system was added for reverse transcription reaction. The first 40 cycles were used for complementary deoxyribonucleic acid (cDNA) synthesis. Besides, the conditions of the reverse transcription reaction were set for PCR amplification. After each cycle, the fluorescence signal was collected in a real-time way, and the amplification and dissociation curves were recorded. Primer sequences used in this study were as follows: caspase3, F: 5'-CCTCCTTACTCAGGACCC-3', R: 5'-TGTGTCCGCTAGGGAAGA-3'; ATG5, F: 5'-GCCTGTAAACATCGCGACTG-3', R: 5'-ATTGCCCGTCGTGGAGG-3'; mTOR, F: 5'-AGCGGCATTCCTGGACAA-3', R: 5'-CGTGAAACGAGACAGTTCT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blotting

The cells in each group were taken and washed twice with D-Hank's solution, and then D-Hank's solution was sucked dry using the absorbent paper. 150 μL of pre-cooled lysis buffer was added into each group, followed by lysis on ice for 30 min. The protein in each group was collected into the Eppendorf (EP; Eppendorf, Hamburg, Germany) tube using the cell scraper and centrifuged at 12,000 rpm and 4°C. The supernatant was obtained and transferred into a new EP tube. After determination of the protein concentration using bicinchoninic acid (BCA), 5 × loading buffer was added and mixed evenly, and the protein was heated at 100°C for 6 min. 30 μL of proteins were added into the loading wells of the prepared separation gel and spacer gel, followed by electrophoresis in electrophoresis buffer under appropriate voltage. After that, the gel was pressed tightly against the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and transferred onto the membrane under a constant voltage of 100 V in transfer buffer at 0°C for 60 min. Then, the PVDF membrane was sealed with 5% skim milk powder at room temperature for 1 h, cut according to the molecular weight and incubated with the primary antibody in a refrigerator at 4°C overnight. On the next day, the PVDF membrane was taken and washed with Tris-Buffered Saline with Tween-20 (TBST; Sigma-Aldrich, St. Louis, MO, USA), and the secondary antibody IgG (1:5000) was added for incubation at room temperature for 1 h. After that, the membrane was washed again with TBST, followed by image development using the Tannon 5200 fluorescence immune image development system and measurement of gray level.

Statistical Analysis

Data were expressed as mean ± standard deviation, and Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for analysis. One-way analysis of variance was adopted for the comparison among groups by Post-Hoc Test (Least Significant Difference), and independent-samples *t*-test was utilized for the pairwise comparison. $p<0.05$ suggested that the difference was statistically significant. Data were presented as the percentage in the control group.

Results

NAM Reduced Apoptosis of Chronic Hypoxic Myocardial Cells

Myocardial cells were treated with NAM in different doses (5, 10 and 20 mM) for 16 h, and the cell viability, toxicity and apoptosis ratio under normoxia and chronic hypoxia were detected. The results of Cell Counting Kit-8 assay revealed that the cell viability increased in a dose-dependent manner after NAM was added. There was a statistically significant difference between 10 mM group and 5 mM group ($p<0.05$), but there was no statistically significant difference between 20 mM group and 10 mM group (Figure 1A and 1C). The cell toxicity was detected *via* LDH. The results of LDH showed that the release of LDH in cells was decreased in a dose-dependent manner after NAM was added. 10 mM group exhibited no statistically significant difference from the blank group and 5 mM group ($p<0.05$), but there was no statistically significant difference between 20 mM group and 10 mM group (Figure 1B and 1D).

The myocardial cells were divided into the blank group, hypoxia group and NAM treatment

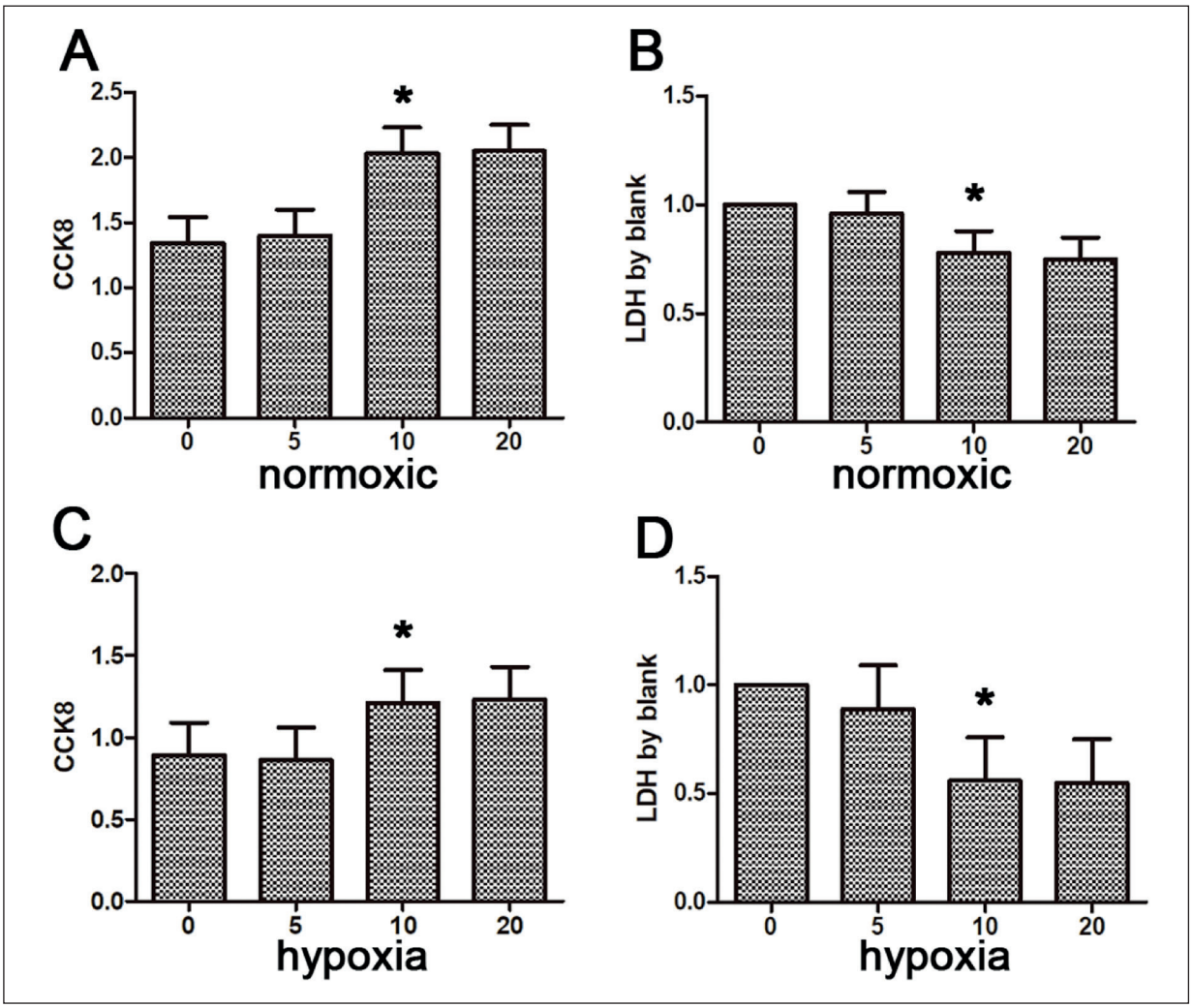

Figure 1. Effects of NAM at different concentrations on the viability of myocardial cells. ***A,*** Detection of effects of NAM at different concentrations (0, 5, 10 and 20 mM) on the viability of normoxic H9C2 cells *via* CCK-8. ***B,*** Detection of toxic effects of NAM at different concentrations (0, 5, 10 and 20 mM) on normoxic H9C2 cells *via* LDH. ***C,*** Detection of effects of NAM at different concentrations (0, 5, 10 and 20 mM) on the viability of chronic hypoxic H9C2 cells *via* CCK-8. ***D,*** Detection of toxic effects of NAM at different concentrations (0, 5, 10 and 20 mM) on chronic hypoxic H9C2 cells *via* LDH. *$p<0.05$ *vs.* 0 mM NAM.

group. The results of Hoechst staining revealed that the proportion of apoptotic cells was significantly increased in the hypoxia group compared with that in the blank group (Figure 2A and 2B), and it markedly declined compared with that in the hypoxia group after NAM was added ($p<0.05$). The mRNA expression of apoptosis-related molecule caspase3 was detected *via* PCR. The PCR results showed that the caspase3 mRNA expression was remarkably increased in the hypoxia group compared with that in the blank group, and it declined compared with that in the hypoxia group after NAM was added. Those results proved that the apoptosis is markedly inhibited at the RNA level after the action of NAM (Figure 2C). According to the Western blotting, the expression of Bcl-2 protein expression was significantly decreased, while the Bax expression was remarkably increased after hypoxia. After the addition of NAM, the Bcl-2 protein expression was increased, while the Bax expression was decreased (Figure 2D and 2E). The Bcl-2/Bax ratio reflects the anti-apoptotic level of cells, and the results revealed that the Bcl-2/Bax ratio was significantly reduced in the hypoxia group compared with that in the blank group (Figure 2E, $p<0.05$), and it was significantly increased after NAM was added ($p<0.05$).

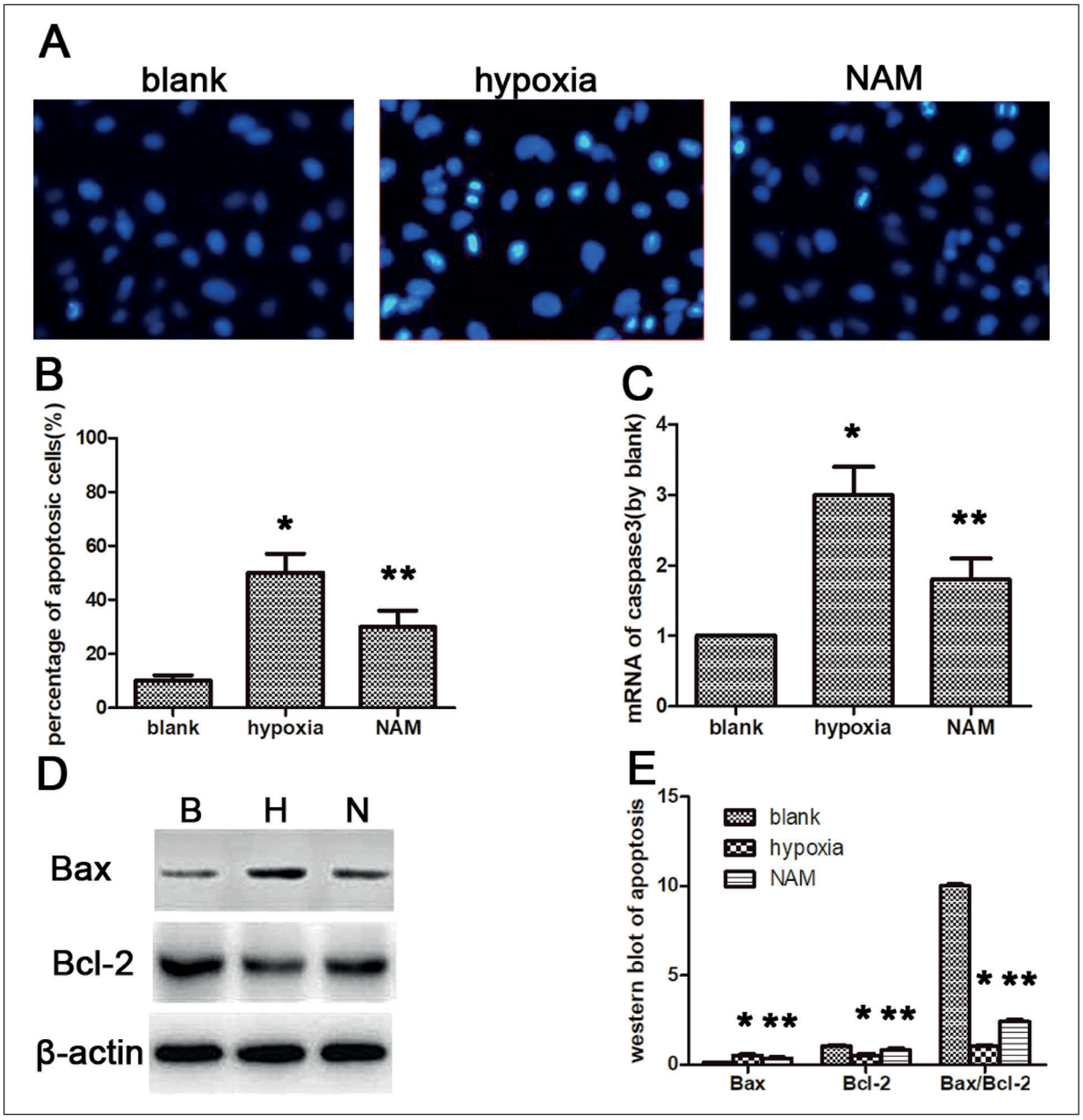

Figure 2. NAM inhibited apoptosis of hypoxic myocardial cells. ***A,*** Detection of apoptosis level in blank group, hypoxia group and NAM group *via* Hoechst staining. ***B,*** Data statistics of apoptosis ratio in each group. ***C,*** Detection of the expression of caspase3 mRNA in blank group, hypoxia group and NAM group *via* PCR. ***D,*** Detection of the expression of Bcl-2 and Bax in blank group, hypoxia group and NAM group *via* Western blotting. ***E,*** Gray analysis of the expression of Bcl-2 and Bax expression and Bcl-2/Bax ratio in each group. **p*<0.05 *vs.* blank group, ***p*<0.05 *vs.* hypoxia group.

NAM Induced Autophagy of Chronic Hypoxic Myocardial Cells

The formation of autophagosomes in cells was detected *via* MDC staining. There was almost no fluorescence accumulation point in cells in the blank group, and fluorescence accumulation points appeared in cells after hypoxia, which was markedly increased compared with that in the blank group. After NAM was added, the fluorescence accumulation points were further increased (Figure 3A). The results of flow cytometry manifested that the proportion of MDC-positive cells was (3.78 ± 1.02)% in the blank group, (24.76 ± 2.04)% in hypoxia group and (44.15

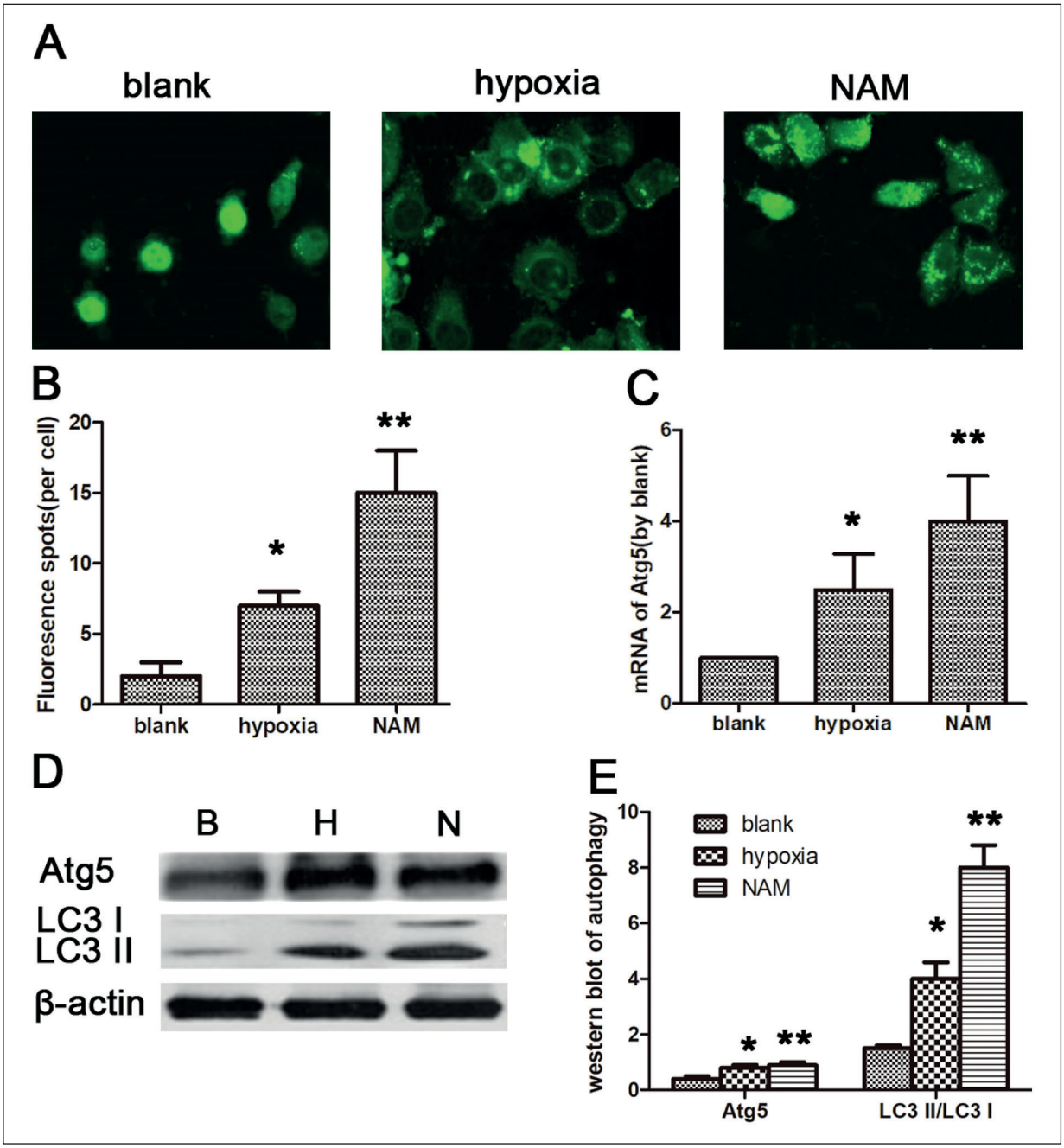

Figure 3. NAM induced autophagy of hypoxic myocardial cells. ***A,*** Detection of autophagy level in blank group, hypoxia group and NAM group *via* MDC staining. ***B,*** Analysis of MDC fluorescence spots per cell in each group. ***C,*** Detection of the expression of ATG5 mRNA in blank group, hypoxia group and NAM group *via* PCR. ***D,*** Detection of expression of autophagy-related proteins (ATG5, LC3I and LC3II) in blank group, hypoxia group and NAM group *via* Western blotting. ***E,*** Gray analysis of ATG5 expression and LC3II/LC3I ratio in each group. **p*<0.05 *vs.* blank group, ***p*<0.05 *vs.* hypoxia group.

± 2.04)% in NAM group. It could be seen that NAM remarkably increased the autophagy level of hypoxic myocardial cells (Figure 3B). The mRNA expression of the autophagy-related molecule was detected *via* PCR. PCR results showed that the autophagy-associated gene 5 (ATG5) mRNA expression was markedly increased in hypoxia group compared with that in the blank group, and it was further raised after NAM was added. Those results proved that the autophagy is substantially increased at the RNA level after the action of NAM (Figure 3C). The expression of

the autophagy-related proteins ATG5, LC3I and LC3II was detected *via* Western blotting. The results manifested that the expression of ATG5 and LC3II/LC3I ratio were markedly increased in the hypoxia group compared with those in the blank group, and they were further increased after the NAM was added. All those results demonstrated that the autophagy is significantly induced at the protein level after the action of NAM (Figure 3D, 3E and 3F).

NAM Regulated mTOR Pathway in Chronic Hypoxic Myocardial Cells

The activation level of the mTOR pathway in myocardial cells in each group was determined. The results revealed that the expression of mTOR mRNA in the hypoxia group declined compared with that in the blank group, and it was further decreased after NAM was added (Figure 4A, $p<0.05$). The protein levels of mTOR and p-mTOR were also measured *via* Western blotting; it was found that the p-mTOR expression remarkably declined after hypoxia compared with that in the blank group, and it was further decreased after the addition of NAM (Figure 4B and 4C, $p<0.05$).

Discussion

The autophagy mechanism is highly conserved in evolution and widely exists in eukaryotic cells. In recent years, the study on autophagy has attracted extensive attention, and it has been found that the autophagy mechanism exerts an important effect on the pain, cardiovascular diseases and endocrine diseases. The regulation on the autophagy process is mediated by ATG, in which both

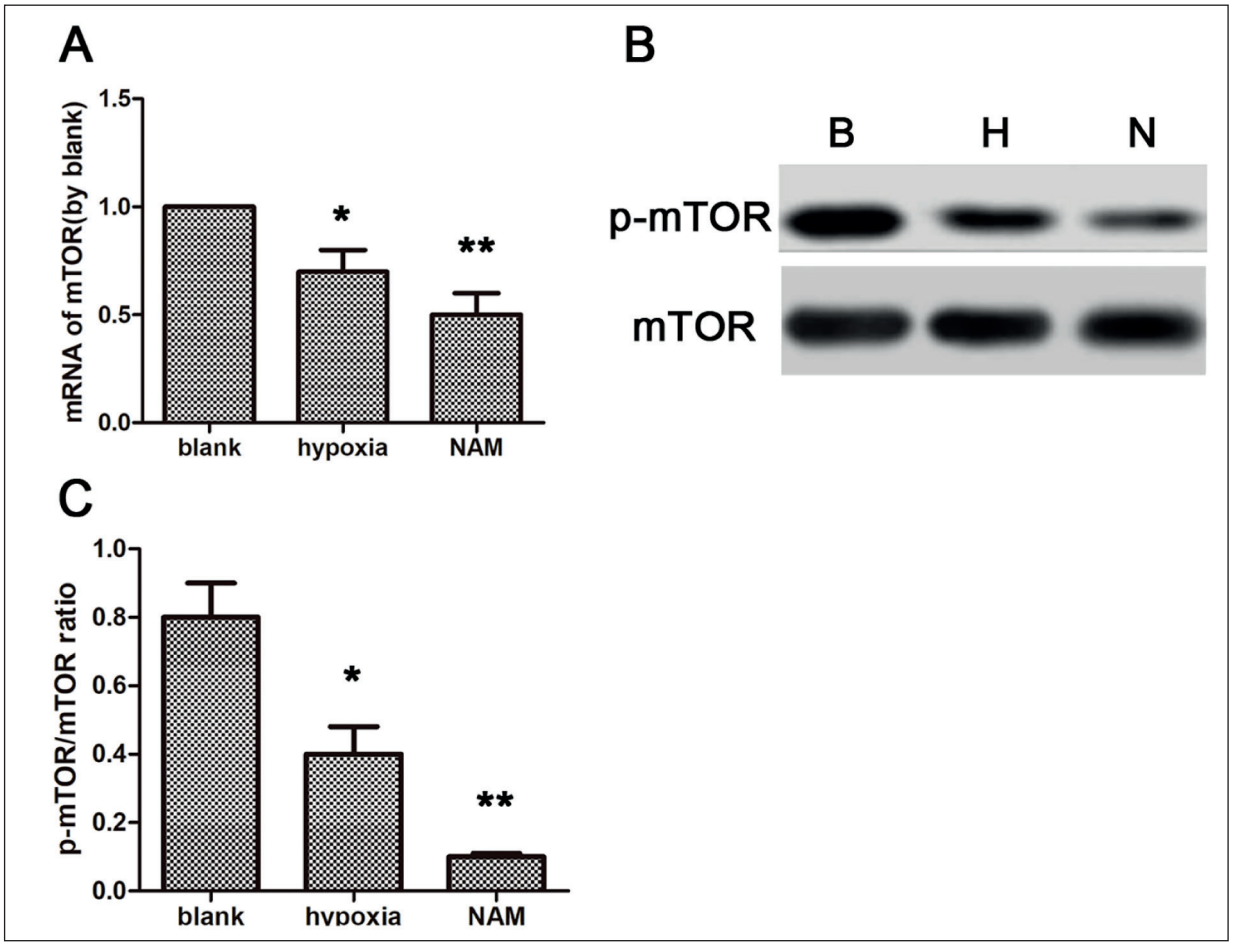

Figure 4. NAM inhibited mTOR pathway activity in hypoxic myocardial cells. ***A,*** Detection of the expression of mTOR mRNA in blank group, hypoxia group and NAM group *via* PCR. ***B,*** Detection of the expression of mTOR and p-mTOR in blank group, hypoxia group and NAM group *via* Western blotting. ***C,*** Gray analysis of mTOR and p-mTOR expression and p-mTOR/mTOR ratio in each group. *$p<0.05$ *vs.* blank group, **$p<0.05$ *vs.* hypoxia group.

Beclin1 and LC3 are important autophagy-related genes[12]. The cell viability in diseases and other stress states can be maintained by autophagy initiated[13]. In the early stage of acute myocardial hypoxia, inducing the increased autophagy level can protect hypoxic myocardial cells from apoptosis[14]. However, the research results on the effect of autophagy in chronic hypoxic myocardial cells are inconsistent. In this work, chronic hypoxic H9C2 cells, were used as object of study to explore the autophagy level.

Niacinamide, also known as NAM, is an amide compound of the B-vitamin niacin, which is a component of coenzyme I and coenzyme II and involved in cell respiration and energy synthesis. NAM possesses the pharmacological effects of anti-inflammation, anti-aging and promotion of metabolism in the body, which has no adverse side-effects in the digestive system, such as the gastrointestinal tract, liver and biliary tract[15]. Clinically, NAM is mainly used in the treatment of cardiac conduction block and viral myocarditis, obtaining significant efficacy. Wang et al[16] have found that NAM regulates the cellular energy metabolism by adjusting the AMPK metabolic pathway. The mTOR is an upstream regulatory protein of the autophagy pathway, and the increased activity of the mTOR can inhibit the downstream autophagy signaling protein, thus inhibiting the autophagy level[17].

In this work, it was found that both autophagy and apoptosis levels in chronic hypoxic myocardial cells were markedly increased. After application of NAM, the apoptosis level declined and the autophagy level was further increased. These findings proved that autophagy has a protective effect on chronic hypoxic myocardial cells, and NAM can induce autophagy of chronic hypoxic myocardial cells to protect cells from apoptosis. The further study on its regulatory mechanism manifested that NAM can inhibit the activity of the mTOR pathway in hypoxic cells, and induce the increased autophagy level by inhibiting the mTOR phosphorylation, thus protecting hypoxic myocardial cells.

Conclusions

We found that NAM can protect chronic hypoxic myocardial cells, reduce apoptosis and induce an increased autophagy level. The above regulatory effects are realized by the regulation of the mTOR pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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