

Research on protective mechanism of ibuprofen in myocardial ischemia-reperfusion injury in rats through the PI3K/Akt/mTOR signaling pathway

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Abstract. – OBJECTIVE: To study the protective mechanism of ibuprofen (Ib) in myocardial ischemia-reperfusion (I/R) injury in rats, and to analyze its regulatory effect on the phosphatidylinositol 3-hydroxy kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway.

MATERIALS AND METHODS: The rat model of myocardial I/R injury was established via ligation of the left main coronary artery (LCA) for 30 min and then reperfusion for 120 min. A total of 36 Sprague-Dawley (SD) rats were randomly divided into sham group (S group, n=12), model group (I/R group, n=12) and Ib group (n=12). The levels of serum creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in each group were detected. The rats were executed, the heart was isolated and the area of myocardial infarction was determined via 2,3,5-triphenyltetrazolium chloride (TTC) staining. The expression levels of vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1 (HIF-1) and apoptosis-related proteins in myocardial tissues in each group were detected via Western blotting. Moreover, the content of inflammatory factors in myocardial tissues in each group was detected using the enzyme-linked immunosorbent assay (ELISA) kit. The expression levels of related proteins in the PI3K/Akt/mTOR signaling pathway in myocardial tissues were further analyzed.

RESULTS: Compared with those in S group, the levels of CK-MB and LDH were significantly increased ($p<0.01$), the area of myocardial infarction was significantly increased ($p<0.01$), the VEGF, HIF-1 and Cleaved caspase-3 protein levels in myocardial tissues were increased ($p<0.01$), while Bcl-2/Bax declined ($p<0.01$), the content of interleukin-1 (IL-1), IL-6 and tumor necrosis factor- α (TNF- α) in myocardial tissues was increased ($p<0.01$), while the content of IL-

10 declined ($p<0.01$), and the expression levels of PI3K, p-Akt and p-mTOR proteins in myocardial tissues were significantly decreased ($p<0.01$) in I/R group. Compared with those in I/R group, the levels of CK-MB and LDH were significantly decreased ($p<0.01$), the area of myocardial infarction was significantly decreased ($p<0.01$), the VEGF, HIF-1 and Cleaved caspase-3 protein levels in myocardial tissues were decreased ($p<0.01$), while Bcl-2/Bax was increased ($p<0.01$), the content of IL-1, IL-6 and TNF- α in myocardial tissues declined ($p<0.01$), while the content of IL-10 was significantly increased ($p<0.01$), and the expression levels of PI3K, p-Akt and p-mTOR proteins in myocardial tissues were significantly increased ($p<0.01$) in Ib group.

CONCLUSIONS: Ib can activate the PI3K/Akt/mTOR signaling pathway, reduce the release of inflammatory factors and apoptosis, and alleviate the myocardial I/R injury in myocardial cells in rats.

Key Words:

Ibuprofen, Myocardial ischemia-reperfusion, PI3K/Akt/mTOR signaling pathway.

Introduction

Ischemic cardiomyopathy is one of the cardiovascular diseases with a very high mortality rate, and the cardiac blood reperfusion *via* coronary artery bypass grafting or stenting is an important treatment means for ischemic cardiomyopathy, which can significantly reduce the mortality rate of patients¹. In recent years, a large amount of research evidence strongly indi-

cates that myocardial ischemia-reperfusion (I/R) will further damage the myocardium, leading to significant structural, functional and metabolic injury in myocardial tissues, even causing heart failure and death in severe cases, so I/R injury is an important factor affecting the treatment of ischemic cardiomyopathy^{2,3}. With the progress in clinical research, a variety of drugs and endogenous substances have been proved to be able to significantly reduce the I/R injury in myocardial cells. Pang et al⁴ studied and showed that the intervention with tanshinone IIA in advance before myocardial I/R in dogs can effectively reduce the myocardial apoptosis level and alleviate the myocardial damage in dogs. Yu et al⁵ found that the transient I/R treatment before reperfusion for several times can effectively reduce the damage of reperfusion to myocardial cells. Li et al⁶ observed that the activation of the phosphatidylinositol 3-hydroxy kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway can act on nitric oxide synthases, increase the vascular permeability and promote the angiogenesis, and cyclooxygenase-2 (COX-2) can also affect Akt, thus affecting the angiogenesis. In clinic, the transient I/R for several times *via* repeated balloon dilatation before vascular opening often leads to plaque detachment in the coronary artery, greatly increasing the risk of thrombosis, while the drug intervention has no such effects^{7,8}. Therefore, reducing I/R injury during the treatment of ischemic cardiomyopathy *via* drug intervention in advance has become a research hotspot. Ibuprofen (Ib) is a non-steroidal anti-inflammatory drug that non-selectively inhibits COX, which, through affecting the release of lysosomes, inhibits COX from metabolizing arachidonic acid into prostaglandin, and suppresses the activity of leukocytes, thereby exerting antipyretic and analgesic effects, so it is applied in the basic treatment of acute lung injury and upper respiratory tract infection⁹. Marquez et al¹⁰ revealed that Ib can significantly reduce the content of serum inflammatory factors, including interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), and improve the oxygenation index in patients with acute lung injury. In the present study, the rat model of myocardial I/R injury was established *via* ligation of left main coronary artery (LCA) and then reperfusion, the protective effect of Ib on myocardial I/R injury in rats was studied, and whether the PI3K/Akt/mTOR signaling pathway is involved in the protective effect was explored.

Materials and Methods

Experimental Animals and Grouping

A total of 36 male Sprague-Dawley (SD) rats weighing 250-300 g were purchased from the Laboratory Animal Center of Nanjing Medical University (Nanjing, China). The rats were fed adaptively for 1 week before the experiment under the humidity of (50 $\pm$ 5)%, temperature of (22 $\pm$ 2) $^{\circ}$ C and artificial lighting for 12 h every day, and they had free access to food and water. These 36 SD rats were randomly divided into sham group (S group, n=12), model group (I/R group, n=12) and Ib group (n=12). The chest was opened in S group, while the rat model of myocardial I/R injury was established *via* ligation of LCA for 30 min and then reperfusion for 120 min in I/R group and Ib group. In Ib group, Ib (20 mg/kg) was injected at one time into the external jugular vein at 20 min after ischemia. At 120 min after reperfusion, the rats in each group were immediately executed, the chest was quickly opened, and the heart was taken and placed in cold phosphate buffered saline (PBS). Then the blood was washed away, the water was sucked dry using the filter paper, the heart was cut open with a sharp blade, and the peripheral region of infarction was separated, placed in liquid nitrogen for 30 s and stored in a refrigerator at -80 $^{\circ}$ C for later use. All experimental operations in this experiment were performed in accordance with the relevant provisions in the Laboratory Animal Use Guidelines of the National Institutes of Health. The study protocol was reviewed and approved by the Laboratory Animal Ethics Committee.

Establishment of the Myocardial I/R Injury Model

After anesthesia *via* intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g), the rats were fixed on an experiment table, the cannula was inserted into the glottis, and the core was withdrawn then. The rodent ventilator was quickly connected for assisted ventilation (tidal volume: 5 mL, respiratory rate: 80 beats/min, and respiratory ratio: 5:4). The thoracic cavity was opened between the 3rd and 4th ribs, the pericardium was peeled off to expose the heart, and the pink LCA could be observed. LCA was reversibly ligated with the 7-0 silk thread at about 2 mm in the inferior margin of the left auricle. After compression ischemia for 30 min, instant partial whitening of the left ventricular anterior wall visible to the naked eyes indicated the successful ischemia. After that, the

silk thread was loosened for fluent coronary blood flow for reperfusion, and obvious hyperemia occurred in the left ventricle within a few seconds. After reperfusion for 120 min, the rats were executed for subsequent studies.

Determination of Serum Creatine Kinase-MB (CK-MB) and Lactate Dehydrogenase (LDH) Content

After I/R for 120 min, the femoral venous blood was drawn from rats in each group, placed at room temperature for 50 min, and centrifuged at 3000 rpm and 4°C for 10 min. Next, the upper-layer serum was taken to detect the content of serum CK-MB and LDH using a full-automatic biochemical analyzer (Olympus AU2700, Tokyo, Japan).

Determination of Area of Myocardial Infarction

After I/R for 120 min, the heart was taken from rats in each group, the blood was washed away with freshly-prepared PBS, and the heart was trimmed. The left ventricle was retained, weighed and sliced into 5 sections (1-2 mm thick), followed by staining with pre-heated 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution and incubation at 37°C for 15 min. After that, the sections were fixed with 4% paraformaldehyde for 30 min and photographed. The staining area was measured using the Image-Pro Plus image analysis software (Version X; Media Cybernetics, Silver Springs, MD, USA). Area of myocardial infarction (%) = (area of myocardial infarction in each section/myocardial area in the section *myocardial weight in the section) $\sum$ /left ventricular weight *100%.

Determination of Content of Inflammatory Factors

The content of inflammatory factors in myocardial tissues in each group was detected using the enzyme-linked immunosorbent assay (ELISA) kits of TNF- α , IL-1, IL-6 and IL-10 (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China): The heart tissues of rats in each group were homogenized using an ultrasonic homogenizer and centrifuged at 12000 rpm and 4°C. The supernatant was taken and stored for later use. After equilibrium of ELISA kits at room temperature for 20 min, the standard solution of TNF- α , IL-1, IL-6 and IL-10 at a concentration of 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 pg/mL was prepared using gradient dilution to plot the standard curve in strict accordance with the instruc-

tions of kits. Then, the stop buffer was added and mixed evenly, and the absorbance was measured at a wavelength of 450 nm using a microplate reader. The standard curve was plotted using CurveExpert 1.4 software, and the concentrations of TNF- α , IL-1, IL-6 and IL-10 in each sample were calculated.

Detection of Expression Levels of Related Proteins via Western Blotting

After I/R for 120 min, the myocardial tissues were taken and weighed. Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was added based on the mass/volume ratio, and 1% protease inhibitor and 1% phosphatase inhibitor were also added, followed by homogenization using the ultrasonic homogenizer under low temperature till there were no visible fragments of tissue, and centrifugation at 12000 rpm and 4°C for 10 min. The supernatant was collected, and the protein concentration in each group was detected using the bicinchoninic acid (BCA) protein quantification kit (Pierce, Rockford, IL, USA). An appropriate number of protein samples were taken and added with 2' sodium dodecyl sulphate (SDS) loading buffer to prepare the loading buffer system in an equal concentration, and the protein was inactivated at 95°C for 10 min. 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was prepared for electrophoresis under the constant voltage of 80 V. The protein was transferred onto a polyvinylidene difluoride (PVDF) membrane, the protein band was sealed with freshly-prepared 5% skim milk powder for 2 h and washed, and the target band was cut and incubated with the corresponding primary antibodies at 4°C overnight, including VEGF (1:1000, CST, Danvers, MA, USA), HIF-1 α (1:1000, CST, Danvers, MA, USA), PI3K (1:1000, CST, Danvers, MA, USA), p-Akt (1:1000, CST, Danvers, MA, USA), Akt (1:1000, CST, Danvers, MA, USA), p-mTOR (1:1000, CST, Danvers, MA, USA), mTOR (1:1000, CST, Danvers, MA, USA), caspase-3 (1:1000, CST, Danvers, MA, USA), Bcl-2 (1:1000, CST, Danvers, MA, USA), Bax (1:1000, CST, Danvers, MA, USA) and GAPDH (1:1000, CST, Danvers, MA, USA). The band was washed with Tris-buffered saline and Tween 20 (TBST) for 3 times, and incubated with the horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (1:5000, Shanghai Yihyson Biological Co., Ltd., Shanghai, China) at room temperature for 1 h. After the band was washed again with TBST for 3 times, the freshly-prepared enhanced chemilumi-

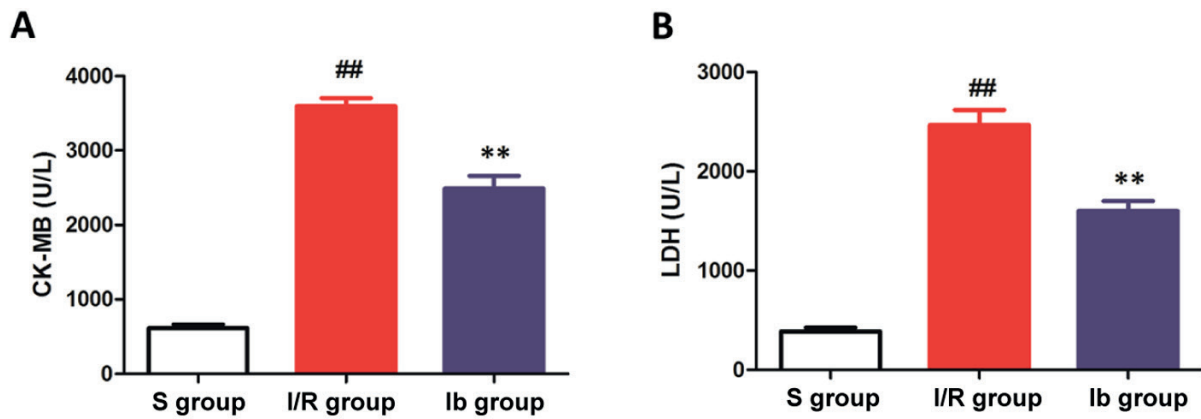


Figure 1. Serum CK-MB and LDH content in each group. **A**, Content of CK-MB, **B**, Content of LDH. The content of serum CK-MB and LDH is significantly higher in I/R group than that in S group, and it is significantly lower in Ib group than that in I/R group. ## $p < 0.01$ vs. S group, ** $p < 0.01$ vs. I/R group.

nescence (ECL) mixture was added, followed by color development and fixation using an automatic processor. The image was processed with ImageJ (NIH, Bethesda, MD, USA), and the expression levels of related proteins were calculated.

Statistical Analysis

The data in this study were expressed as mean $\pm$ standard deviation. Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Armonk, NY, USA) was used for the data processing. Analysis of variance was used for the comparison among groups. Bonferroni's method was adopted in the pairwise comparison in the case of homogeneity of variance, while Welch's method was adopted in the case of heterogeneity of variance. $p < 0.05$ suggested that the difference was statistically significant.

Results

Serum CK-MB and LDH Content

The content of serum CK-MB and LDH in each group was detected using the full-automatic biochemical analyzer. As shown in Figure 1, the content of serum CK-MB and LDH was significantly higher in I/R group than that in S group ($p < 0.01$), and it significantly declined in I/R rats after pretreatment with ibuprofen ($p < 0.01$).

Area of Myocardial Infarction

The influence of I/R on the area of myocardial infarction in each group was detected via TTC staining. As shown in Figure 2, there was no obvious myocardial infarction in S group, the area of myocardial infarction was significantly larger

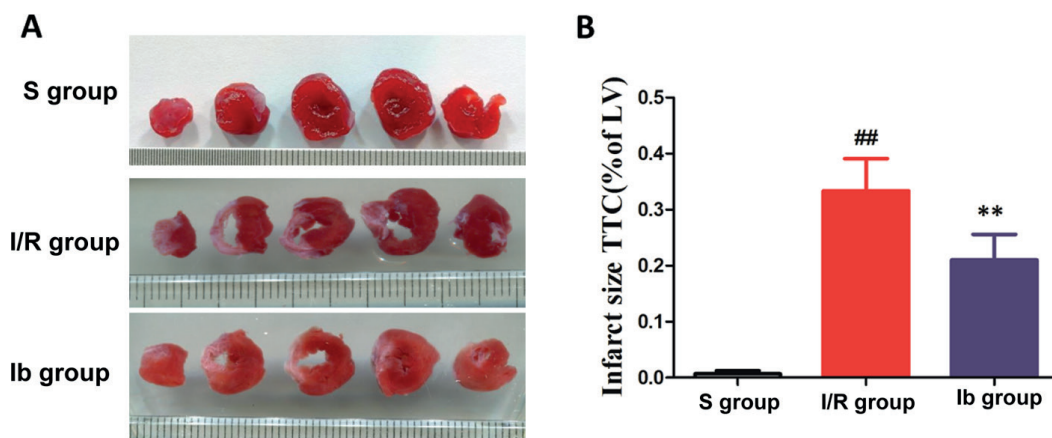


Figure 2. Area of myocardial infarction in each group detected via TTC staining. **A**, Photograph of TTC staining, **B**, Area of myocardial infarction. The area of myocardial infarction is significantly larger in I/R group than that in S group, and it is significantly smaller in Ib group than that in I/R group. ## $p < 0.01$ vs. S group, ** $p < 0.01$ vs. I/R group.

in I/R group than that in S group ($p<0.01$), and it significantly declined after treatment with Ib ($p<0.01$).

Protein Expression Levels in Myocardial Tissues

The expression levels of VEGF, HIF-1 α and apoptosis-related proteins in myocardial tissues in each group were detected *via* Western blotting. The results revealed that the VEGF, HIF-1 α and Cleaved caspase-3 protein levels in myocardial tissues were obviously increased ($p<0.01$), while Bcl-2/Bax significantly declined ($p<0.01$) in I/R group. The treatment with Ib could significantly reduce the VEGF, HIF-1 α and Cleaved caspase-3 protein levels in myocardial tissues ($p<0.01$) and increase Bcl-2/Bax ($p<0.01$) in Ib group (Figure 3-4).

Content of Inflammatory Factors in Myocardial Tissues

The content of inflammatory factors in myocardial tissues in each group was detected using the ELISA kits. Compared with those in S group, the content of IL-1, IL-6 and TNF- α in myocardial tissues was significantly increased

($p<0.01$), while the IL-10 content was significantly decreased ($p<0.01$) in I/R group. The treatment with Ib could remarkably lower the content of IL-1, IL-6 and TNF- α in myocardial tissues ($p<0.01$), and increase the IL-10 content in Ib group ($p<0.01$) (Figure 5).

Expression Levels of PI3K/Akt/mTOR Signaling Pathway-Related Proteins

The expression levels of PI3K/Akt/mTOR signaling pathway-related proteins in myocardial tissues in each group were detected *via* Western blotting. The results showed that the PI3K, p-Akt and p-mTOR protein expression levels in myocardial tissues in I/R group were remarkably lower than those in S group ($p<0.01$). The treatment with ibuprofen could significantly raise the PI3K, p-Akt and p-mTOR protein expression levels in myocardial tissues in Ib group ($p<0.01$) (Figure 6).

Discussion

Increasingly more research evidence suggests that I/R is an important cause of poor therapeutic

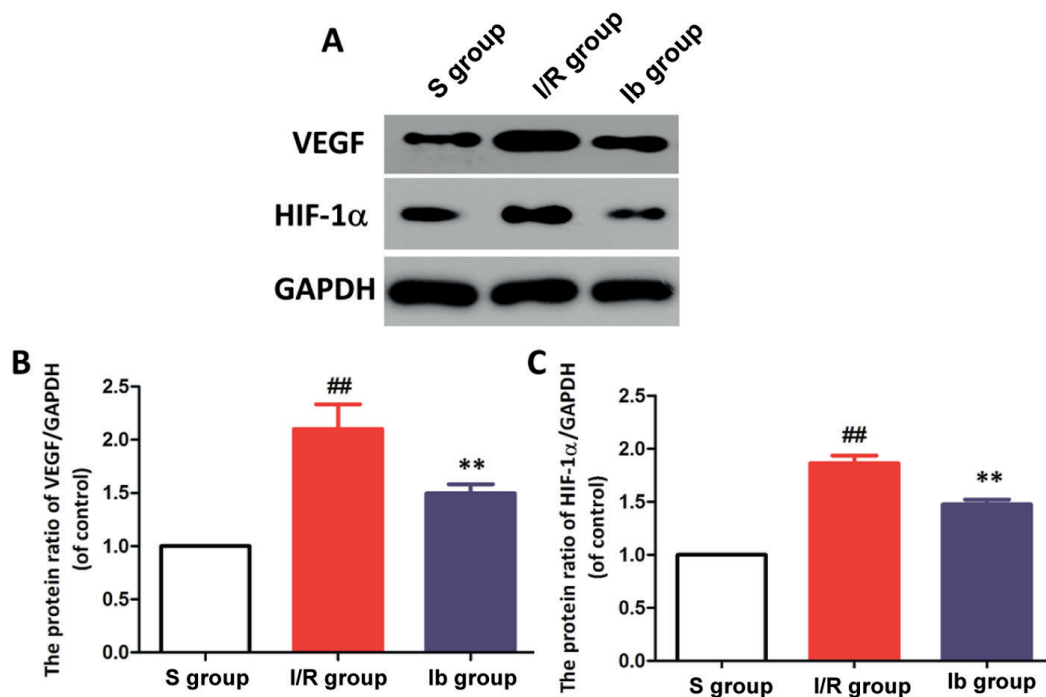


Figure 3. Protein expression levels in myocardial tissues in each group. **A**, Protein band, **B**, VEGF protein, **C**, HIF-1 α . The VEGF and HIF-1 α levels in myocardial tissues are significantly higher in I/R group than those in S group, while they are significantly lower in Ib group than those in I/R group. ^{##} $p<0.01$ vs. S group, ^{**} $p<0.01$ vs. I/R group.

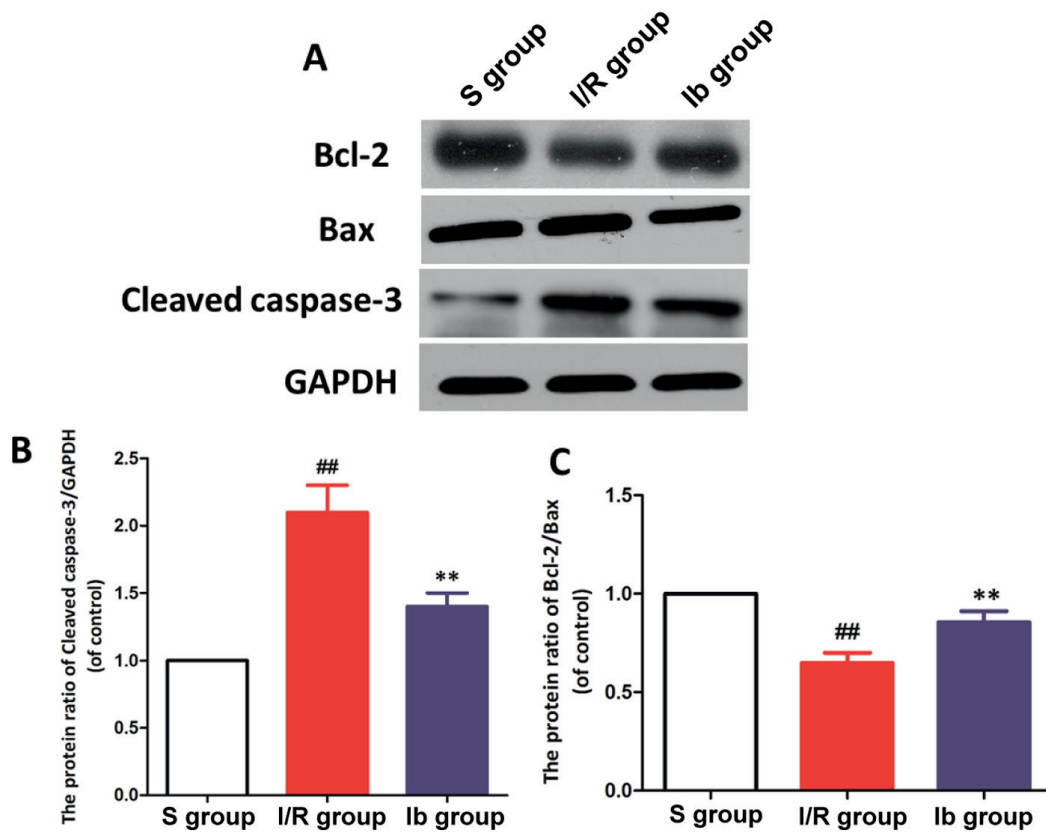


Figure 4. Expression levels of apoptosis-related proteins in myocardial tissues in each group. **A**, protein band, **B**, Cleaved caspase-3 protein, **C**, Bcl-2/Bax. The Cleaved caspase-3 level in myocardial tissues is significantly higher in I/R group than that in S group, while Bcl-2/Bax is significantly lower than that in S group. The Cleaved caspase-3 level in myocardial tissues is significantly lower in Ib group than that in I/R group, while Bcl-2/Bax is significantly higher than that in I/R group. ^{##} $p < 0.01$ vs. S group, ^{**} $p < 0.01$ vs. I/R group.

tic effect and prognosis of patients with cerebral ischemia. The stress response, Ca^{2+} metabolic disorder, acid-base balance disorder and inflammatory response during reperfusion are important factors leading to structural changes in heart tissues and abnormalities in function and metabolism, as well as the culprits of myocardial apoptosis¹¹⁻¹³. In the present study, the rat model of myocardial I/R injury was established, and it was found that myocardial I/R significantly increased the area of myocardial infarction in the left ventricle, increased the serum CK-MB and LDH levels, raised the expressions of apoptosis-related proteins in myocardial tissues and promoted the release of inflammatory factors. The stress can lead to the increased level of reactive oxygen species in myocardial cells, promote the mitochondrial DNA oxidation, cause the intracellular Ca^{2+} overload and

ATP metabolism disorder, activate the release of apoptosis-related protein Cleaved caspase-3 in myocardial tissues^{14,15}, further activate the inflammatory factors and increase the release of pro-inflammatory factors. Koeppen et al¹⁶ studied and found that the expression levels of serum inflammatory factors in patients with myocardial infarction are significantly higher than those in normal people, which is an important factor affecting the progression of disease. The PI3K/Akt/mTOR signaling pathway is an important regulatory pathway for protein synthesis in the body, and it is closely related to the intracellular mitochondrial oxidation and reduction¹⁷. Very et al¹⁸ studied and found that *in-vitro* and *in-vivo* stress can result in the increased phosphorylation level of receptor tyrosine in the body, thus activating PI3K, further promoting the phosphorylation of downstream protein

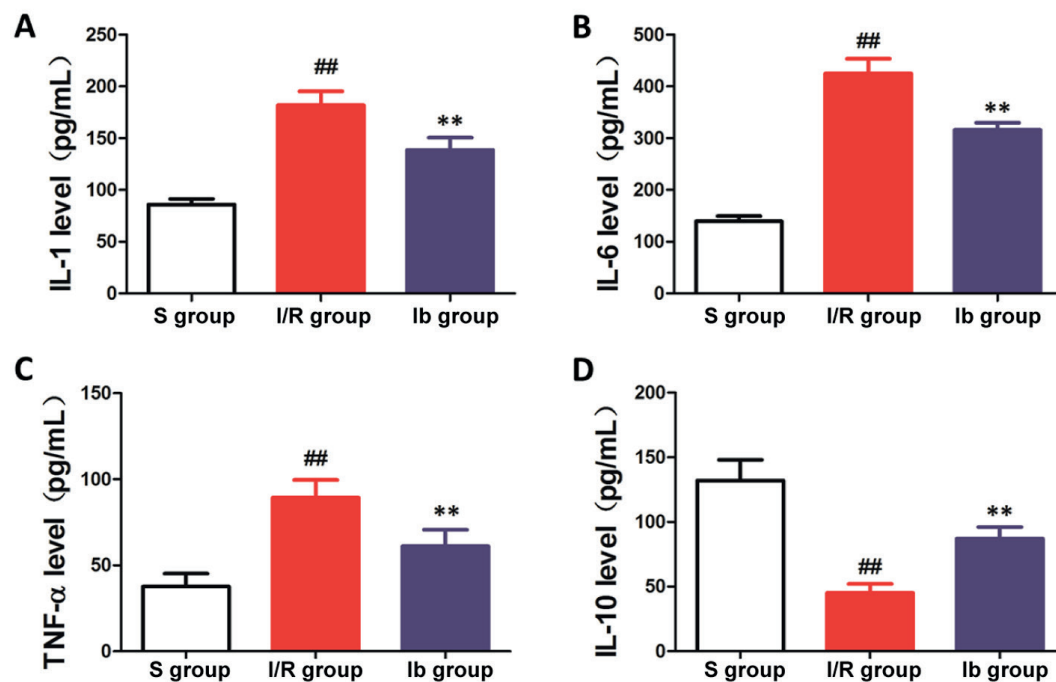


Figure 5. Content of inflammatory factors in myocardial tissues in each group detected *via* ELISA. **A**, Content of IL-1, **B**, Content of IL-6, **C**, Content of TNF- α , **D**, Content of IL-10. The content of IL-1, IL-6 and TNF- α in myocardial tissues is significantly higher in I/R group than that in S group, while the content of IL-10 is significantly lower than that in S group. The content of IL-1, IL-6 and TNF- α in myocardial tissues is significantly lower in Ib group than that in I/R group, while the content of IL-10 is significantly higher than that in I/R group. ## $p < 0.01$ vs. S group, ** $p < 0.01$ vs. I/R group.

Akt, increasing the p-mTOR level, inhibiting the expression of apoptosis protein Cleaved caspase-3, and promoting the expression of anti-apoptotic factor Bcl-2. Zhang et al¹⁹ detected that the PI3K/Akt/mTOR signaling pathway is suppressed in myocardial cells of rats with myocardial infarction, leading to the significant increase in myocardial apoptosis. It was found in the present study that the expression levels of PI3K, p-Akt and p-mTOR in myocardial cells of myocardial I/R rats were significantly lower than those in normal rats. After I/R injury, the expression levels of Cleaved caspase-3 and Bax were significantly increased in myocardial cells, while the expression level of Bcl-2 significantly declined, which are consistent with the results in a large number of literature reports. The above research results strongly indicate that the myocardial apoptosis and myocardial tissue damage caused by myocardial I/R injury in rats are closely related to the PI3K/Akt/mTOR signaling pathway. Markworth et al²⁰ observed that Ib, a classic COX-2 inhibitor, can act on the PI3K/Akt/mTOR signaling pathway in liver

cancer cells, and act on mTOR through affecting TSC1/2, thereby affecting the release of matrix metalloproteinase and the cell proliferation and migration. In the present study, intervention with ibuprofen in I/R rats before reperfusion could significantly reduce the area of myocardial infarction, lower the levels of serum CK-MB and LDH, activate the PI3K/Akt/mTOR signaling pathway, decrease the expression levels of apoptosis proteins and promote the release of anti-apoptotic factors, thereby reducing the levels of inflammatory factors.

Conclusions

We showed that Ib can activate the PI3K/Akt/mTOR signaling pathway, reduce the release of inflammatory factors and apoptosis, and alleviate the myocardial I/R injury in myocardial cells in rats.

Conflict of interest

The authors declare no conflicts of interest.

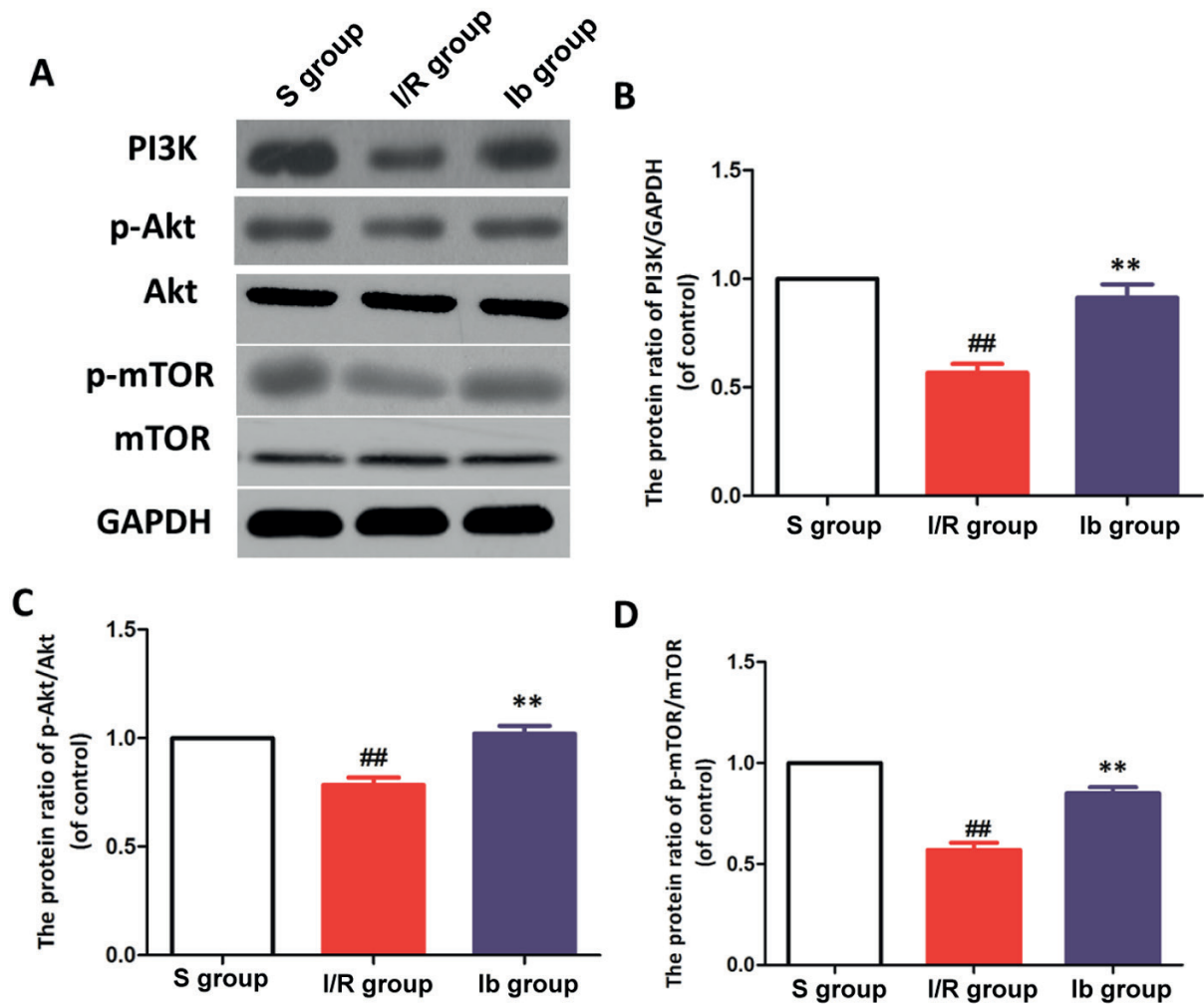


Figure 6. Expression levels of PI3K/Akt/mTOR signaling pathway-related proteins in myocardial tissues, **A**, Protein band, **B**, PI3K Protein, **C**, p-Akt protein, **D**, p-mTOR protein. The PI3K, p-Akt and p-mTOR protein expression levels in myocardial tissues in I/R group are remarkably lower than those in S group, while they are remarkably higher in Ib group than those in I/R group. ^{##} $p < 0.01$ vs. S group, ^{**} $p < 0.01$ vs. I/R group.

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