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Glutamine protects myocardial ischemia-reperfusion injury in rats through the PI3K/Akt signaling pathway

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Abstract. – OBJECTIVE: To study the protective mechanism of glutamine (Gln) on myocardial ischemia-reperfusion (IR) injury in rats through the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway.

MATERIALS AND METHODS: A total of 30 healthy SD rats weighing 200-300 g were used in this experiment. They were randomly divided into 3 groups: sham group (n=10), myocardial IR injury group (IR group, n=10), IR+Gln group (n=10). The protein expression levels of phosphorylated Akt (p-Akt), total Akt (t-Akt), phosphorylated mammalian target of rapamycin (p-mTOR), mTOR, proliferating cell nuclear antigen (PCNA), P21, and Tubulin were determined by Western blotting (WB). Quantitative Polymerase Chain Reaction (qPCR) was applied to detect the messenger ribonucleic acid (mRNA) levels of Akt and mTOR. 3-(4,5)-dimethylthiazol(-z-y1)-3,5-diphenyl tetrazolium bromide (MTT) test was utilized to examine the proliferation ability of cardiomyocytes *in vitro*. Besides, the contents of the inflammatory cytokines, tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) were measured *via* enzyme-linked immunosorbent assay (ELISA). Cell apoptosis in each group was examined through Hoechst staining.

RESULTS: Compared with those in the sham group, ratios of p-AKT/AKT, p-mTOR/mTOR, and the level of PCNA extremely significantly decreased, but the level of P21 notably increased in IR group ($p<0.01$). In comparison with those in the IR group, ratios of p-AKT/AKT, p-mTOR/mTOR, and the level of PCNA were remarkably raised, while the level of P21 was remarkably reduced in IR+Gln group ($p<0.05$). QRT-PCR results manifested that there were no significant differences in the mRNA levels of Akt and mTOR among the three groups [no significant difference (NS)]. Moreover, the cell proliferation ability in IR group was remarkably lower than that in the sham group ($p<0.01$), while it was enhanced in the IR+Gln group compared with that in the IR group ($p<0.05$). Additionally, IR group had significantly elevated expression levels of TNF-α and IL-6 compared with the sham group ($p<0.01$), whereas the IR+Gln group had notably decreased expression levels of TNF-α and IL-6 compared with IR group ($p<0.05$). In comparison with that in the sham group, the apoptosis in IR group was significantly raised ($p<0.01$), and compared with that in the IR group, the apoptosis in the IR+Gln group prominently decreased ($p<0.05$). The contents of the inflammatory cytokines, TNF-α, and IL-6 presented the same trends among the three groups.

CONCLUSIONS: Gln activates the PI3K/Akt signaling pathway by increasing the levels of p-AKT and p-mTOR. Gln can increase the PCNA level and reduce the P21 level, so as to enhance the proliferation ability of cardiomyocytes. Besides, Gln reduces the levels of inflammatory cytokines, TNF-α, and IL-6, and inhibits cell apoptosis. Finally, Gln can protect cells from myocardial IR injury by activating the PI3K/Akt signaling pathway.

Key Words:
Glutamine, PI3K/Akt signaling pathway, Myocardial ischemia-reperfusion injury.

Introduction

Phosphatidylinositol 3-kinase (PI3K) in the PI3K/protein kinase B (Akt) signaling pathway is composed of regulatory subunit p85 and catalytic subunit p110 and has the activities of serine/threonine (Ser/Thr) kinases and phosphatidylinositol kinase. Akt, which can be activated by PI3K, is able to activate the mammalian target of rapamycin (mTOR), thereby regulating glucose metabolism, inflammation, apoptosis, and other processes[1-3]. Some studies[4,5] have shown that the PI3K/Akt signaling pathway can play a protective role in myocardial ischemia-reperfusion (IR) injury in

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rats. Myocardial IR is a pathological process that ischemic myocardium is progressively aggravated following reperfusion of blood flow in osculated artery[6]. The pathogenesis of myocardial IR injury may be that ischemia-induced insufficient energy supply leads to myocardial tissue damage, cell proliferation inhibition, apoptosis or necrosis, etc. The PK3K/Akt signaling pathway plays a crucial role in glucose energy metabolism[7]. In this research, whether glutamine (Gln), an amino acid energy substance, can regulate intracellular energy metabolism and relieve reperfusion injury after IR injury by activating the PK3K/Akt signaling pathway was investigated.

Materials and Methods

Animal Feeding, Treatment, and Grouping

Sprague-Dawley (SD) rats purchased from Shanghai Bioraylab Co., Ltd. (Shanghai, China) were habituated in an SPF-grade animal room with 45% humidity and a 12 h/12 h light-dark cycle at 25°C. The rats had free access to food and water. One week later, SD rats were randomly divided into the sham group (n=10), myocardial IR injury group (IR group, n=10), and IR+ Gln (IR+Gln group, n=10). After myocardial IR surgery and treatment with Gln, the rats in the three groups were conventionally fed for 3 days and sacrificed by cervical dislocation to extract myocardial tissues. All operations for animals strictly were implemented following the regulations of the National Institute of Research on the Care and Use of Laboratory Animals. This study was approved by the Animal Ethics Committee of Affiliated Hulunbeir People's Hospital of Soochow University.

Establishment of the Rat Model of Myocardial IR

Rat trachea was connected with an artificial respiration machine. After skin disinfection, the chest cavity was exposed by creating a 2 cm incision. Then, ligation was conducted between the artery and the left atrial appendage to cause myocardial ischemia. A pair of hemostatic forceps was used to clamp the chest skin, muscle, and heart to prevent pneumothorax, and electrocardiograms changes were observed. Peaked or inverted T waves marked ischemia. If the changes in electrocardiograms were not evident, the pair of hemostatic forceps was loosened, and evident changes could be seen after the thread ends were tightened. During reperfusion, the pair of hemostatic forceps and the thread were loosened to induce reperfusion. At the end of modeling, suture, injection of solution, and disinfection were carried out sequentially.

Examination of the PI3K/Akt Signaling Pathway in Myocardial Tissues Via Western Blotting (WB)

Rat myocardial tissues were cut into pieces, homogenized, and centrifuged at 20,000 g, 4°C for 30 min. The total protein concentration was detected using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Then, the samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (IPVH00010, Millipore, Billerica, MA, USA). After that, the samples were incubated with phosphorylated Akt (p-Akt), Akt, phosphorylated mTOR (p-mTOR), mTOR, proliferating cell nuclear antigen (PCNA), and P21 antibodies purchased from CST Biotechnology Co., Ltd. (Danvers, MA, USA) at 4°C overnight. After PBS washing, they were incubated with the secondary antibodies conjugated with horseradish peroxidase (CST Biotechnology Co., Ltd., Danvers, MA, USA) for 1 h. Following the addition of enhanced chemiluminescence (ECL) mixture, the images were obtained using the fluorescence development technique.

Detection of the Messenger Ribonucleic acid (mRNA) Expression Levels of Akt and mTOR by Quantitative Polymerase Chain Reaction (qPCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to extract RNAs from the myocardial tissues, which were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) according to the instructions. Then, 2 μL of 5× PrimeScript RT Master Mix was added to 500 ng of RNAs, and the total volume of the reaction system was 10 μL. Subsequently, the amplification reaction was carried out in the PCR system, and the reaction system was added based on the instructions as follows: 2 μL of cDNAs were added with 10 μL of SYBR Premix Ex Taq II (Tli RNaseH Plus) (2×), 0.8 μL of forward primers, 0.8 μL of reverse primers and 0.4 μL of ROX Reference Dye II (50×). Ultimately, deionized water was added until the constant volume was 20 μL. The mRNA expression level was cal-

culated based on the amplification cycle number, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The primer sequences were shown in Table I.

Examination of Inflammatory Cells in Myocardial Tissues

Myocardial tissues extracted from rats were washed three times with high-pressure phosphate-buffered saline, and the lysate was added for lysing for 30 min, followed by centrifugation at 3000 rpm for 10 min at 4°C. The contents of cytokines, tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), in the supernatant were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) and quantified according to the protein concentration in the tissue supernatant.

Detection of Apoptosis Via Hoechst Staining

Myocardial cells were fixed with paraformaldehyde and stained with 1 μg/mL Hoechst 33342, a membrane permeable dye. Then, the cells were observed with an Olympus fluorescence microscope (Olympus, Tokyo, Japan). According to nuclear coagulation and fragmentation, apoptotic cells were identified, and their percentage in the total cells was calculated. At least 5 stained cell regions were randomly selected from each sample.

Statistical Analysis

Data were statistically analyzed using GraphPad Prism 6.0 (La Jolla, CA, USA) and expressed as (x±s). The t-test was adopted, and $p<0.05$ represented that the difference was statistically significant.

Results

Effect of Gln on the PI3K/Akt Signaling Pathway

First, the protein levels of p-Akt, total Akt (t-Akt), p-mTOR, and mTOR were analyzed by WB. It was found that compared with those in the sham group, the ratios of p-Akt/Akt and p-mTOR/mTOR in the IR group were significantly lowered, and they were notably elevated in IR+Gln group compared with those in the IR group (Figure 1). It is indicated that IR can inhibit the PI3K/Akt signaling pathway, while Gln had an opposite effect.

MRNA Expression Levels of Akt/mTOR in Myocardial Tissues in Each Group

Next, the mRNA expression levels of AKT/mTOR in myocardial tissues were measured, and there were no significant differences in the mRNA levels of Akt/mTOR among the three groups according to qPCR detection results [no significant difference (NS)] (Figure 2). It can be seen that Gln activated the PI3K/Akt signaling pathway by raising the levels of p-Akt and p-mTOR, and this effect was not related to the mRNA expressions of Akt and mTOR.

Effects of Gln on the Expression Levels of Cell Proliferation-Related Proteins

Subsequently, the protein levels of PCNA and P21 in myocardial tissues were further determined. In comparison with those in the sham group, the level of PCNA was remarkably lowered, but the P21 level was markedly elevated in the IR group. Compared with the IR group, the IR+Gln group had a significantly increased PCNA level and an evidently decreased P21 level (Figure 3). The above results indicated that IR was able to downregulate PCNA and upregulate P21, thus suppressing cell proliferation. Gln prevented IR from inhibiting cell proliferation.

Impact of Gln on Cell Proliferation

Besides, the proliferation ability of myocardial cells in each group was examined using 3-(4,5)-dimethylthiazol(-z-yl)-3,5-diphenyl tetrazolium bromide (MTT) assay *in vitro*. It was discovered that the cell proliferation in the IR group was weakened compared with that in the sham group, and it was enhanced in the

Table I. Primer sequences of genes.

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
Akt	CTGCCCTTCTACAACCAGGA	GTGCTGCATGATCTCCTTGG
MTOR	ACAGGCCAGCATGCCATCGC	TAGTACTGCAGCACTTTGGGG
GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT

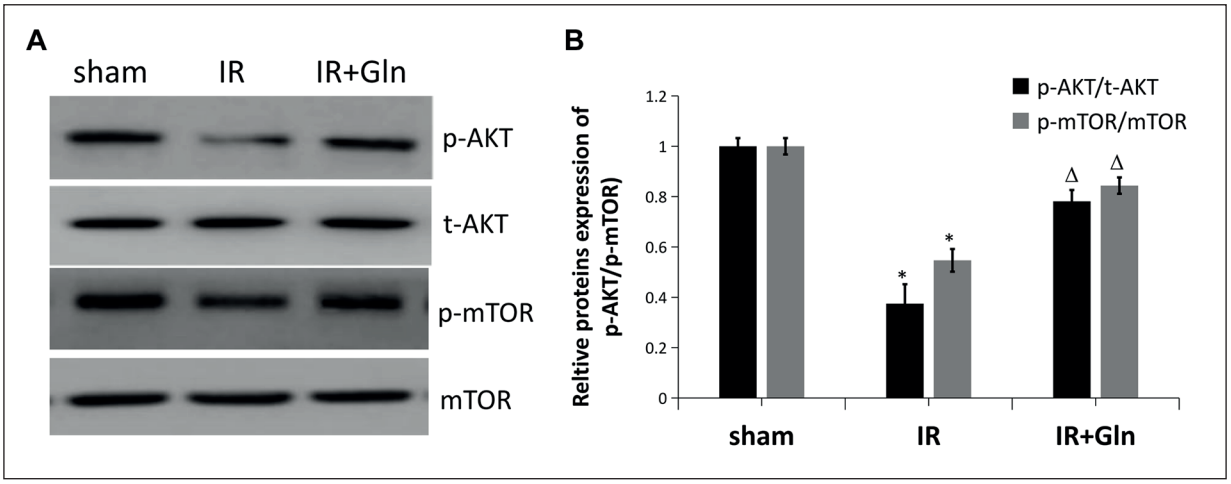

Figure 1. Effect of Gln on the PI3K/Akt signaling pathway. **A,** Protein levels of p-Akt, t-Akt, p-mTOR, and mTOR detected *via* WB. **B,** Quantification of the protein levels in Figure 1A: the protein level ratios of p-Akt/Akt and p-mTOR/mTOR are calculated. The values are expressed as mean ± standard error of the mean (SEM). *p<0.01: IR group *vs.* sham group. $^{\Delta}p$<0 .05: IR+Gln group *vs.* IR group.

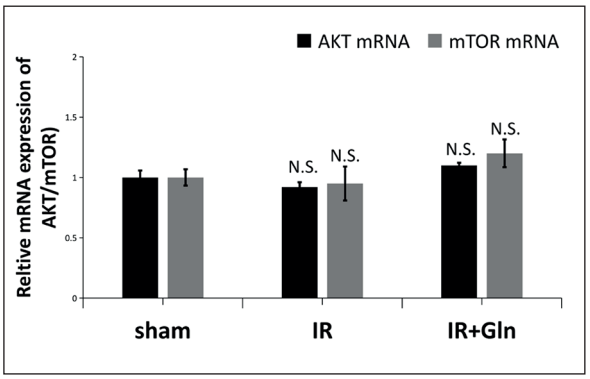

Figure 2. Detection of the mRNA expression levels of Akt and mTOR in myocardial tissues in each group. The values are expressed as mean ± SEM (n=6). NS represents no significant difference compared with the sham group.

IR+Gln group in comparison with that in the IR group (Figure 4). Thus, Gln can improve the proliferation ability of cardiomyocytes following IR injury.

Effects of Gln on the Levels of Inflammatory Cytokines (TNF-α and IL-6)

Further, ELISA results manifested that the contents of inflammatory cytokines in myocardial tissues were evidently higher than that in the sham group, and the expression levels of TNF-α and IL-6 in the IR group were significantly higher than those in the IR+Gln group. Compared with the IR group, IR+Gln group had remarkably decreased expression levels of TNF-α and

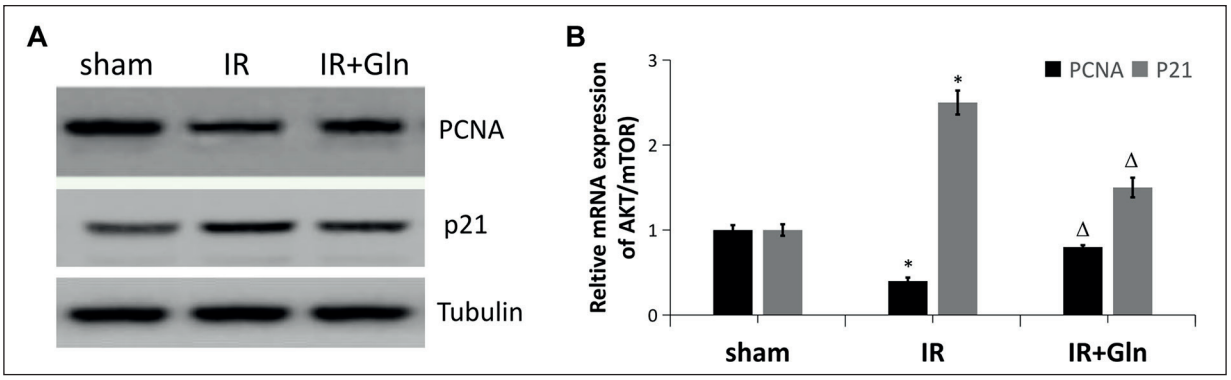

Figure 3. Effects of Gln on the expression levels of cell proliferation-related proteins. **A,** PCNA and P21 protein levels determined by WB. **B,** Quantification of the protein levels. The values are expressed as mean ± SEM. *p<0.01: IR group *vs.* sham group. $^{\Delta}p$<0 .05: IR+Gln group *vs.* IR group.

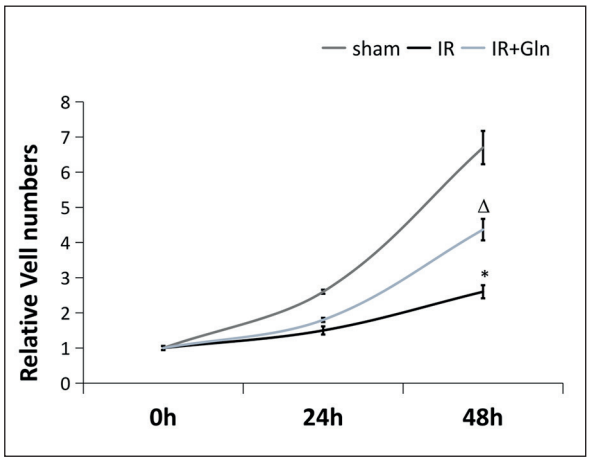

Figure 4. Impact of Gln on cell proliferation. The proliferation ability of myocardial cells in each group is tested by MTT assay. The values are expressed as mean ± SEM. $^{*}p<0.01$: IR group *vs.* sham group. $^{\Delta}p<0$ .05: IR+Gln group *vs.* IR group.

IL-6 (Figure 5). The above findings suggested that Gln was capable of reducing the expressions of inflammatory cytokines (TNF-α and IL-6) and may inhibit cell apoptosis.

Effect of Gln on the Apoptosis of Myocardial Tissue Cells in Each Group

Hoechst staining assay was employed to detect the cell apoptosis, and the results demonstrated that compared with that in the sham group, the cell apoptosis in IR group was markedly elevated, and it was remarkably reduced in the IR+Gln group compared with that in the IR group (Figure 6), displaying that Gln can suppress the apoptosis of myocardial cells.

Gln Protected Myocardial IR Injury in Rats Via the PI3K/Akt Signaling Pathway

Finally, the mechanism of Gln on protecting myocardial IR injury in rats through the PI3K/Akt signaling pathway was mapped. According to the map, Gln could raise the level of p-Akt and further increase the level of p-mTOR, thus activating the PI3K/Akt signaling pathway. Besides, it was also able to increase PCNA and decrease the P21 level, thus facilitating cell proliferation. Gln reduced the expressions of the inflammatory cytokines (TNF-α and IL-6) and suppressed cell apoptosis, so as to protect myocardial tissues from IR injury (Figure 7).

Discussion

Gln is a class of crucial amino acids in organisms. The high-level Gln in the blood provides a carbon and nitrogen source to support cell biosynthesis, energy metabolism, intracellular homeostasis, and cell proliferation[8-10]. Over-activated PI3K/Akt/mTOR signaling pathway metabolizes glutamic acid to α-ketoglutaric acid by glutamate enzymes. α-ketoglutaric acid enters the tricarboxylic acid cycle and can provide energy for cells, thus resisting apoptosis and coping with adverse external stimuli. Recently, it has been reported that glutamine protects the body from intestinal IR injury and oxidative stress in rats[11-13].

The activation of the PI3K/Akt signaling pathway, a vital metabolic pathway in organisms, can activate or suppress a series of its downstream substrates, thereby regulating

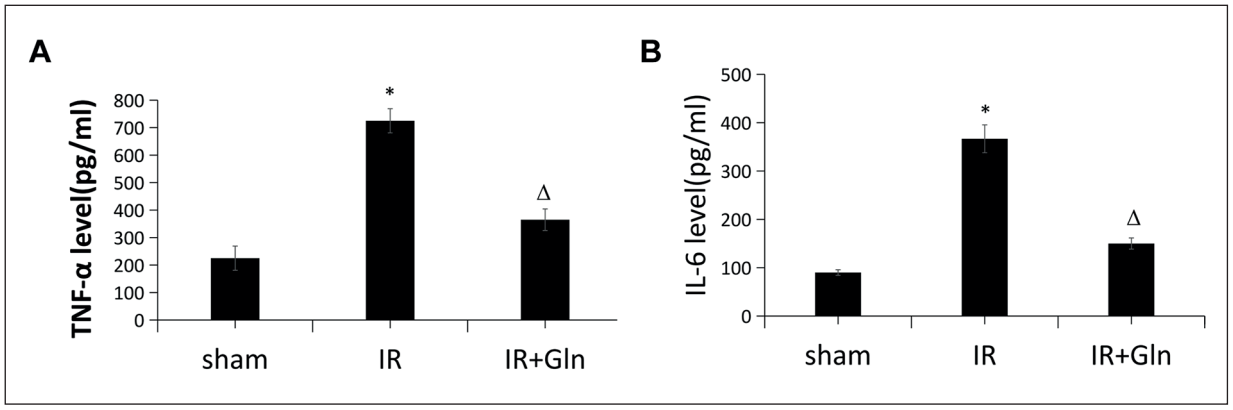

Figure 5. Effects of Gln on the levels of inflammatory cytokines (TNF-α and IL-6). The values are expressed as mean ± SEM. $^{*}p<0.01$: IR group *vs.* sham group. $^{\Delta}p<0$ .05: IR+Gln group *vs.* IR group.

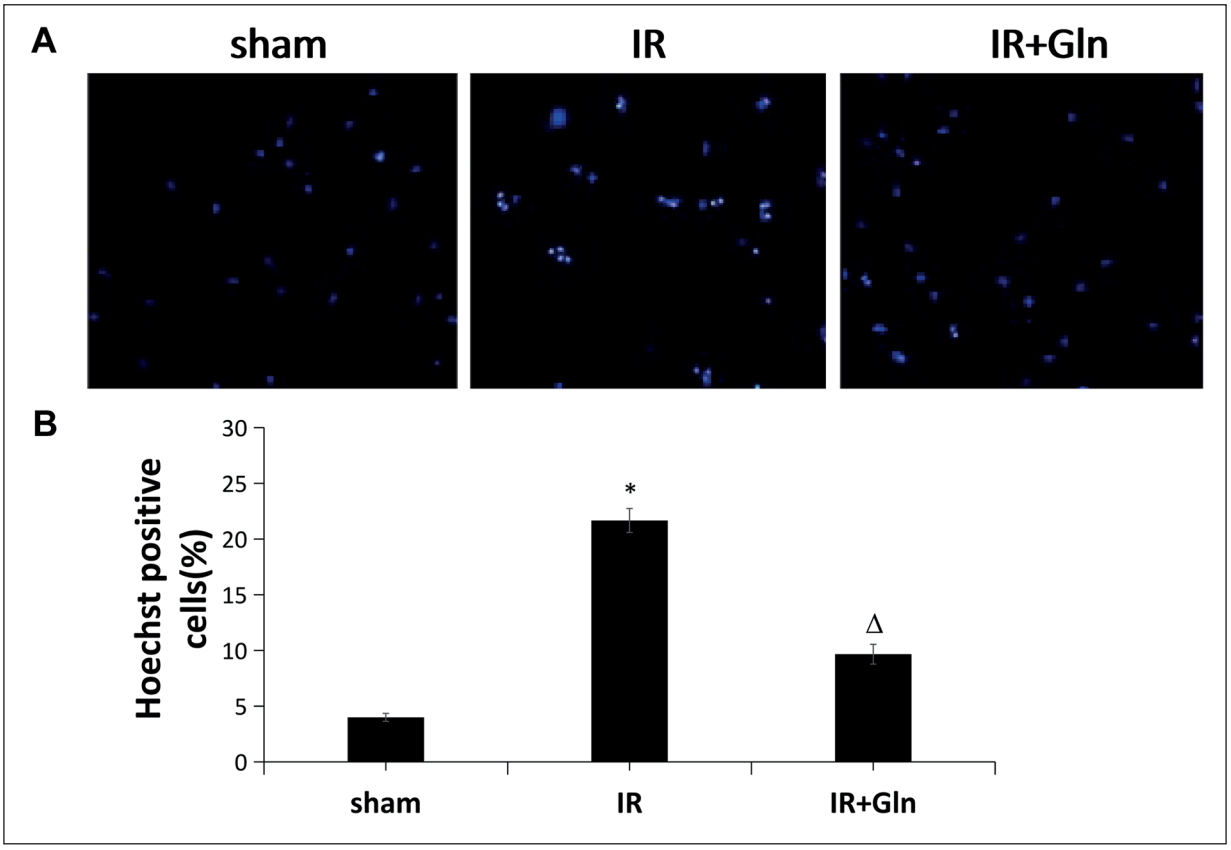

Figure 6. Effect of Gln on the apoptosis of cells in each group. **A,** Hoechst staining results of the cell apoptosis (40X). **B,** Quantification of the staining results in Figure 1A. The values are expressed as mean ± SEM. *p<0.01: IR group *vs.* sham group. $^{\Delta}p$<0.05: IR+Gln group *vs.* IR group.

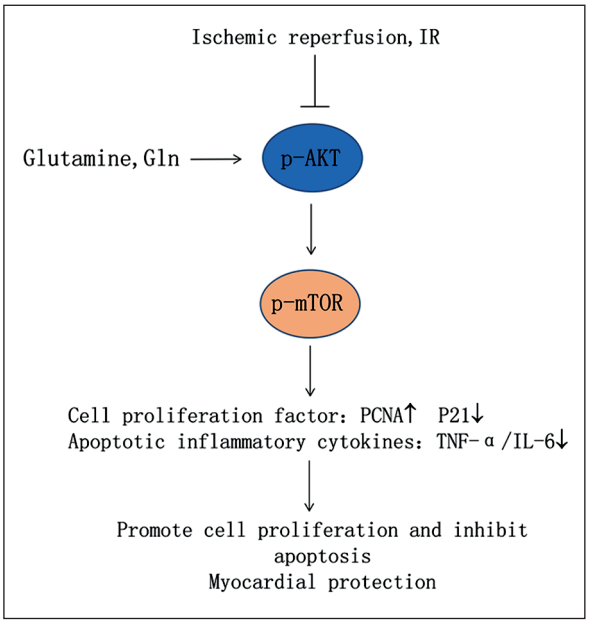

Figure 7. Mechanism map of Gln on myocardial IR injury in rats *via* the PI3K/Akt signaling pathway.

cell proliferation, differentiation, apoptosis, and migration[14-16]. Akt, also called PKB, exerts a pivotal role in controlling survival and apoptosis, and it is activated by insulin and various factors associated with growth and survival. P-Akt has Thr308 and Ser473 phosphorylation sites, and they present different biological roles. Akt inhibits apoptosis through phosphorylation and inactivation of several targets (Bad, c-Raf, and caspase-9), thus promoting cell survival[17-19]. It also plays a crucial role in cell growth by directly phosphorylating mTOR in raptor-containing rapamycin-sensitive complexes. More importantly, Akt phosphorylates and inactivates TSC2 protein (an inhibitor of mTOR in the mTOR-raptor complex), thereby activating the mTOR signals. The activation of Akt/mTOR signaling is capable of suppressing the NF-κB signaling pathway, thereafter, reducing secretion and expressions of inflammatory cytokines, such as TNF-α and IL-6[20]. It is reported that

Gln can prevent hypoxia-induced myocardial injury by activating the PI3K/Akt signaling pathway. Therefore, it was speculated that Gln can protect myocardial IR injury in rats through the PI3K/Akt pathway.

In this study, SD rats were divided into the sham group, IR group, and IR+Gln group. In the first place, WB detection results revealed that compared with those in the sham group, the ratios of p-Akt/Akt and p-mTOR/mTOR in IR group were significantly reduced, and they were notably elevated in the IR+Gln group compared with those in the IR. It is indicated that Gln can activate the PI3K/Akt signaling pathway. The results of qPCR showed that there were no significant differences in the mRNA levels of Akt and mTOR among the three groups (NS). Furthermore, the protein levels of PCNA and P21 in myocardial tissues were measured. PCNA and P21 were the marker proteins reflecting cell proliferation levels. In comparison with those in the sham group, the level of PCNA was remarkably lowered, but the P21 level was markedly elevated in the IR group. Compared with the IR group, the IR+Gln group had a remarkably increased PCNA level and an evidently decreased P21 level, suggesting that Gln enhanced cell proliferation ability. In addition, MTT results were consistent with WB findings. According to ELISA detection results, the expression levels of TNF-α and IL-6 in IR group were significantly higher than those in the IR+Gln group. Compared with the IR group, IR+Gln group had remarkably decreased expression levels of TNF-α and IL-6. It can be seen that Gln was capable of reducing the expressions of inflammatory cytokines (TNF-α and IL-6). Ultimately, the Hoechst staining method was adopted to detect apoptosis, and it was discovered that Gln could inhibit myocardial cell apoptosis.

Conclusions

In summary, Gln activates the PI3K/Akt signaling pathway by raising the levels of p-Akt and p-mTOR. Gln can increase the PCNA level and reduce the P21 level, so as to enhance the proliferation ability of myocardial cells. Besides, it reduces the levels of inflammatory cytokines, TNF-α, and IL-6 to inhibit cell apoptosis. Eventually, Gln can protect cells from injury caused by myocardial IR in rats by activating the PI3K/Akt signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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