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Original Article

Influence of proteasome inhibitor MG-132 on the expression of Hsp70 and myocardial apoptosis after myocardial infarction

Xin-Hua Wu[1], Zhang-Rong Chen[1], Wei Yang[1], Ying Yang[1], Yu Dong[1], Hong Liu[1], Shi-Quan Kuang[1], Kai-Liang Luo[2]

[1]*Department of Cardiology, The First Affiliated Hospital of Dali University, Dali 671000, Yunnan, China,* [2]*Department of Cardiology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing 404100, China*

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Abstract: Objective: This study aims to explore the influence of proteasome inhibitor MG-132 on the expression of Hsp70 and myocardial apoptosis after myocardial infarction. Methods: A myocardial infarction (MI) model was established. The animals used for this study were randomized into four groups: MI group (n=6), MG group (n=6), MG+quercetin (MG+Q) group (n=6), and sham-operation group (SH) (n=6). On the next day after surgery, the MG group was administered with MG-132 *via* intraperitoneal injection, the MG+Q group received MG-132 and quercetin *via* intraperitoneal injection, and MI and SH groups were administered with normal saline (NS) after the surgery for 28 days. Animals were sacrificed by inhalation of carbonic oxide, and the samples were collected. RT-PCR assay was used to detect the mRNA expression of heat shock protein 70 (Hsp70), and immunohistochemistry and western blot assays were performed to detect the protein expression of Hsp70 and caspase-3. TUNEL method was used to detect the myocardial apoptosis of tissues around the infarction area. Results: The apoptosis index of cells surrounding the non-infarction area in the SH, MI, MG and MG+Q groups was 0.89 ± 0.12, 21.31 ± 0.82, 5.27 ± 0.51 and 7.15 ± 0.41, respectively. Compared with the SH group, the apoptosis indexes in the MI, MG and MG+Q groups significantly increased (P<0.01). Compared with the MI group, the apoptosis indexes of myocardial cells in the MG and MG+Q groups significantly decreased (P<0.01), especially in the MG group (P<0.01). The difference in the mRNA and protein expression of Hsp70 between the SH and MI groups was not statistically significant (P>0.05). Compared with the SH and MI groups, the mRNA and protein expression of Hsp70 in the MG and MG+Q groups significantly increased (P<0.01), especially in the MG group (P<0.01). Compared with the SH group, the protein expression of caspase-3 in the MI, MG and MG+Q groups significantly increased (P<0.01). Compared with the MI group, the expression of caspase-3 in the MG and MG+Q groups significantly decreased (P<0.01), especially in the MG group (P<0.01). Conclusions: (1) A small dosage of proteasome inhibitor MG-132 reduced the apoptosis of myocardial cells in the non-infarction area after myocardial infarction. (2) MG-132 may inhibit the apoptosis of myocardial cells by increasing the expression of Hsp70 and inhibiting the expression of caspase-3.

Keywords: Myocardial infarction, myocardial remodeling, proteasome Inhibitor, HSP70, caspase-3

Introduction

In recent years, studies on myocardial remodeling have focused on the endogenous protective mechanism of the myocardium in addition to factors of myocardial damage, especially on heat shock proteins (HSPs). HSPs are a set of stress proteins that are important for protecting myocardial cells and inhibiting myocardial damage. Recent studies have shown that HSPs can improve myocardial remodeling [1]. HSPs are degraded by the ubiquitin-proteasome system (UPS). When UPS activity increases, HSPs become more degraded. Studies on the application of proteasome inhibitors in the treatment of tumors have indicated that proteasome inhibitors can induce the generation of HSPs, and that this increase in HSPs is the mechanism that tumor cells escape death [2, 3]. Meller [4] used MG-132 in cultured rat myocardial cells to induce the expression of HSPs, especially for the overexpression of Hsp70, which improves the ability of cells to resist hypoxia. However, at present, no literature has

reported on whether proteasome inhibitors can induce the generation of HSPs and reduce the apoptosis of myocardial cells. In the present study, this problem was investigated.

Materials and methods

Modeling of animal myocardial infarction

A total of 68 Sprague-Dawley rats (female: 34; male: 34) were purchased from the Animal Experimental Center of Chongqing Medical University. These rats had a body mass of 200-250 g, and were within the national grade II experimental animal standards. The feeding and experimental process were strictly performed according to the requirements of the animal experiment outlines in the International Association for the Study of Pain (IASP) [5], and the myocardial infarction model was established based on a literature [6]. Rats that survived 24 hours after successful modeling were randomly divided into four groups: MI group (n=6), MG group (n=6), MG+quercetin (MG+Q) group (n=6) and sham-operation (SH) group (n=6). In addition, the same coronary arteries were only threaded but not ligatured.

Experiment grouping and administration methods

A total of 18 rats survived more than 24 hours after successful modeling. These rats were divided into four groups: MI group (n=6), MG group (n=6), PDTC group, and SH group (n=6). The same sites were only threaded but not ligatured. Rats were replaced if any of the rats died within 28 days. The intervention measures for animals in each group were as follows: on the next day after surgery, the MG group was administered with MG-132 (0.1 $mg \cdot kg^{-1} \cdot d^{-1}$) *via* intraperitoneal injection [7], MG+Q group received MG-132 (0.1 $mg \cdot kg^{-1} \cdot d^{-1}$) and quercetin (100 $mg \cdot kg^{-1} \cdot d^{-1}$) *via* intraperitoneal injection[7, 8], and MI and SH groups were administered with the same volume of normal saline (NS) until 28 days after the surgery. These rats were fed in separate cages and were continuously given 400,000 U of penicillin *via* intraperitoneal injection for three days.

Collection of specimens

The animals were sacrificed with carbon monoxide on day 28 after the operation. The hearts were taken out before cardiac arrest and washed with NS. Then, the hearts were divided into three sections along the long axis of the left ventricle. The cardiac base and cardiac apex were quickly frozen with liquid nitrogen and stored at -70°C for reverse transcription-polymerase chain reaction (RT-PCR) and western blot assays. The center section was cut into 3-mm thick sections, fixed in 4% paraformaldehyde solution for 24 hours, embedded in paraffin, and cut into 5 μm sections.

The in situ test and analysis of myocardial apoptosis cells

The DNA3'-OH of the cell nucleus was labeled by TdT-mediated dUTP nick end labeling (TUNEL), and the detailed procedures were as follows: The paraffin sections of myocardial tissue were dewaxed with xylene and placed in water at a ethanol gradient. The activity of endogenous POD was interdicted with a 3% hydrogen peroxide methanol solution at room temperature for 15 minutes, washed with distilled water, and the tissue was rinsed with phosphate buffered saline (PBS) for five minutes for a total of three times. Then, the tissues were digested with protease K (37°C, one hour), washed with PBS for five minutes for a total of three times, added with the right amount of TUNEL reaction mixture (Enzyme solution: label solution =1:9) at 37°C, and incubated for two hours. Next, the tissues were washed with PBS for five minutes for a total of times, incubated at 37°C with a Converter-POD for 30 minutes, washed with PBS for five minutes for a total of three times, colorized by DAB, re-dyed with hematoxylin, and the sections were sealed with balata. The sections observed under a light microscope, and the control experiment was set as the negative control: the sections were only incubated with the Label solution, but without the enzyme solution; and other procedures were the same as those included in the TUNEL assay. Positive control: The normal tissue sections were digested with DNase I to induce the DNA split, and the other procedures were the same as those included in the TUNEL assay. Each section was observed under a light microscope (× 400). Five non-overlapping visual fields were randomly selected to count the number of apoptotic cells and total cells in each visual field, and the mean value was obtained. Finally, the apoptosis index (AI) was calculated as AI = the number of apoptotic cells/the number of total cells × 100%.

Table 1. Hsp70 mRNA expression level test by RT-PCR

Gene	Each sequence of the primer
β-actin (219 bp)	Upstream 5'-CAG CTT CTT CTA GTG CCG TTC C-3'
	Downstream 5'-GGA GTC AGG TGT TTC TGG TGG AG-3'
Hsp70 (503 bp)	Upstream 5'-TGT ATT GCG TAT CAT CAA CGA ACC-3'
	Downstream 5'-TCT CCT GGA TCT GGC CCT TG-3'

Hsp70 mRNA expression level test by RT-PCR

The total RNA of rat myocardial tissues was extracted using the TRIzol method. The primer was synthesized by Shanghai Boshang Biotechnology Co. Ltd., and each sequence of the primer is shown in **Table 1**. The RT-PCR reaction system was 50 μl, which was completed in two steps, according to kit instructions (Toyobo, Japan). The genetic number of the mRNA of the actin and Hsp70 of rats was respectively found in a genetic database of PubMed: NM_031144 and NM_021863. Shanghai Boshang Biotechnology Co. Ltd. was entrusted to design the relevant primer using primer 5.0 (**Table 1**). The image analysis was conducted using Quantity-one 4.6 to count the relative product amount of β-actin and Hsp70. The light absorption area integral ratio expressed the DNA content, and mRNA expression levels were assessed using the light absorption area integral ratio of the amplified β-actin and Hsp70 bands.

Hsp70 and caspase-3 protein expression test by immunohistochemistry

The procedures were conducted according to the instructions of SP kit, and Hsp70 expression was observed under a high-powered microscope (× 400). With reference to the study of Joki *et al.* [9], the semi-quantitative analysis method was used, as follows: the brown particles that appeared in the myocardial nuclei were considered as positive cells, and the positive index (PI) was counted as PI = the number of positive cells/the number of total cells in visual field × 100%. Five non-overlapping high power visual fields were randomly observed for each section to obtain the mean value. The procedures for β-actin were the same, and the concentration of the antibody was 1:400. The brown particles that appeared in the myocardial cytoplasm were considered as positive cells, and five non-overlapping high power visual fields were randomly observed for each section to count the integrated optical density (IOD) using the medical biological image analysis software. The brown particles that appeared in the myocardial cytoplasm were considered positive cells for the judgment of the result, and five × 400 visual fields were randomly selected for each section to count the IOD using the medical biological image analysis software.

Hsp70 and caspase-3 expression level test with western blot

In terms of Hsp70, rat myocardial tissue was used to extract myocardial total protein according to the instructions of RIPA reagent, and the protein content was measured using BCA-100. Then, 40 μg of protein was transferred onto the PVDF membrane after 10% SDS-PAGE electrophoresis, which was sealed with 5% skimmed milk for one hour, and respectively incubated with rabbit anti-rat Hsp70 antibody and caspase-3 antibody (Santa Cruz; diluted with Tris-buffered saline [TBS], the concentration was 1:200 and 1:400, respectively) at room temperature for two hours. Then, these were incubated with the secondary antibody (goat anti-rabbit IgG) at room temperature for one hour after the membrane was washed to develop with the chemiluminescence. Photos were taken and a scan analysis was conducted for the gel-imaging system.

Statistics analysis

All data were analyzed using statistical package SPSS 12.0. The measurement data was expressed as mean ± standard deviation ($\bar{x}$ ± SD), t-test was used for comparisons between two groups, and variance analysis was used for comparisons among three groups, $P<0.05$ was considered to indicate a statistically significant difference.

Results

TUNEL results of myocardial apoptosis index

*Myocardial apoptosis in non-infarcted areas of rats in each group (**Figure 1**):* Some sporadic positive cells could be observed in the myocardial tissue in the SH group, and positive cells significantly increased in the MI, MG and MG+Q

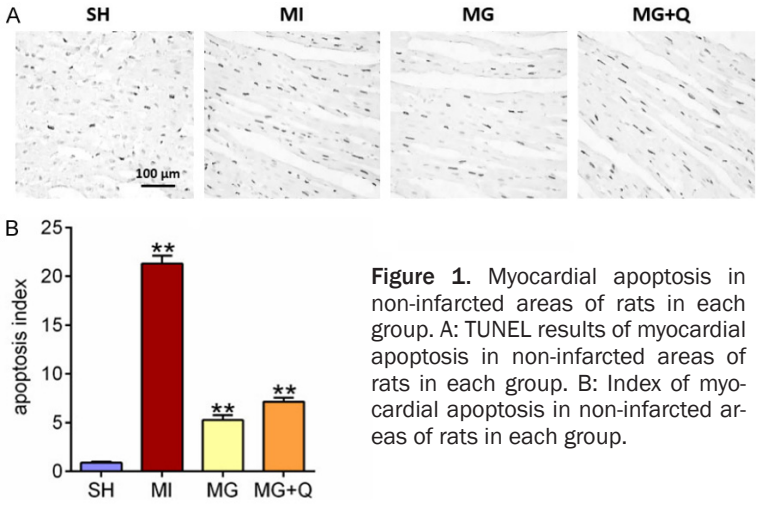

Figure 1. Myocardial apoptosis in non-infarcted areas of rats in each group. A: TUNEL results of myocardial apoptosis in non-infarcted areas of rats in each group. B: Index of myocardial apoptosis in non-infarcted areas of rats in each group.

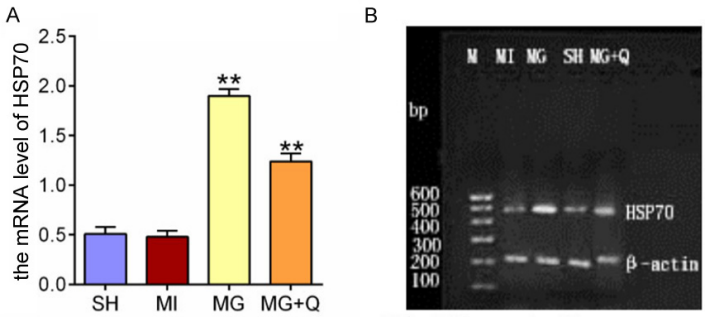

Figure 2. Hsp70 mRNA expression of rat myocardial tissues in each group. A: HSP70 mRNA expression (M represents gene mark) in all group by RT-PCR. B: HSP70 mRNA expression (M represents gene mark) in all groups.

groups. Positive cells in MG and MG+Q groups significantly decreased compared with that in the MI group. Positive cells in MG group decreased, compared with the MG+Q group. The apoptosis index of cells in the SH, MI, MG and MG+Q groups was 0.89 ± 0.12, 21.31 ± 0.82, 5.27 ± 0.51 and 7.15 ± 0.41, respectively. The myocardial apoptosis indexes of non-infarcted areas in the latter three groups significantly increased, compared with SH group (P<0.01); and the index in the MI group was the most significant. The myocardial apoptosis index in the MG and MG+Q groups significantly decreased, compared with that in MI group (P<0.01). The myocardial apoptosis index in the MG group was more significantly decreased compared with that in the MG+Q group (P<0.01).

Detection of Hsp70 mRNA expression level by RT-PCR

*Hsp70 mRNA expression of rat myocardial tissues in each group (**Figure 2**)*: Distinct bands could be observed at 503 bp in rats in each group. The Hsp70 electrophoresis results were shown in **Figure 2B**. The IOD of the mRNA in the SH, MI, MG and MG+Q groups was 0.51 ± 0.07, 0.48 ± 0.06, 1.90 ± 0.07 and 1.24 ± 0.08, respectively. The difference in the mRNA expression of Hsp70 between the SH and MI groups was not statistically significant (P>0.05). Compared with the MI and SH groups, this expression significantly increased in the MG and MG+Q groups (P<0.01); and the highest expression was observed in the MG group (P<0.01). The expression level in the MG group significantly increased compared with that in the MG+Q group (P<0.01).

Detection of Hsp70 and caspase-3 protein expression by immunohistochemistry

*Detection of Hsp70 and caspase-3 protein expression by immunohistochemistry (**Figure 3**):* (A) Some pale brown positive cells could be seen in the myocardial cytoplasm of rats in each group, the myocardial cell staining in the MG group was the deepest, staining in the MG+Q group was the second deepest, and staining in the MI and SH groups were pale. (B) Some pale brown positive cells could be observed in the myocardial cytoplasm of rats in each group, and the myocardial cell staining in the MG group was the deepest, followed by the MG+Q, MI and SH groups, which were the most pale. (C) The IOD of Hsp70 in SH, MI, MG and MG+Q groups was 5.14 ± 0.78, 4.95 ± 0.41, 13.89 ± 1.03 and 9.28 ± 1.22, respectively. The difference in Hsp70 protein expression between the SH and MI groups was not statistically significant (P>0.05). Compared with the MI and SH groups, this expression significantly increased in the MG and MG+Q groups (P<0.01); and the highest was in the MG group (P<0.01). The expression level in MG group was significantly increased compared with that in MG+Q group (P<0.01). (D) The IOD in the SH, MI, MG and MG+Q groups was 10.96 ± 0.76,

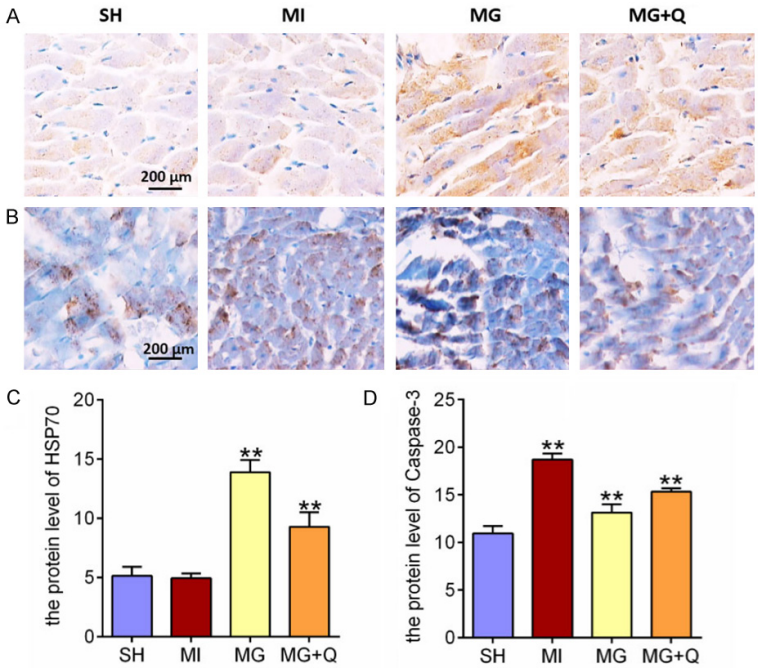

Figure 3. Detection of Hsp70 and caspase-3 protein expression by immunohistochemistry. A: TUNEL results of HSP70 expression in all group by immunohistochemical staining. B: TUNEL results of Caspase-3 expression in all group by immunohistochemical staining. C: Protein expression of HSP70 in all group by immunohistochemical staining. D: Protein expression of Caspase-3 in all groups by immunohistochemical staining.

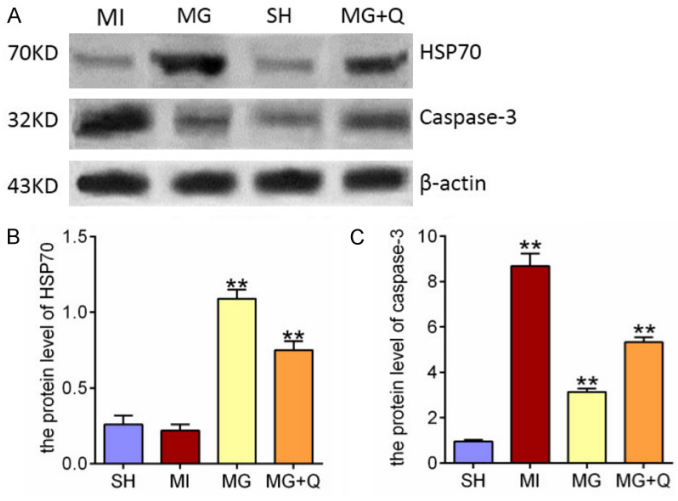

Figure 4. Detection of Hsp70 and caspase-3 expression in rat myocardial tissues in each group by western blot. A: Protein expression of HSP70 and Caspase-3 in all group by Western blot. B: Protein expression of HSP70 in all groups by Western blot. C: Caspase-3 protein expression of Caspase-3 in all groups by Western blot.

18.69 ± 0.65, 13.13 ± 0.86 and 15.33 ± 0.36, respectively. Compared with the SH group, the IOD values of caspase-3 in the latter three groups significantly increased (P<0.01). However, IOD values in the MG and MG+Q groups were significantly lower than in the MI group (P<0.01), while that in the MG group was the lowest, and the difference was statistically significant when compared with the MG+Q group (P<0.01).

Detection of Hsp70 and caspase-3 expression level by western blot

*Detection of Hsp70 and caspase-3 expression in rat myocardial tissues in each group by western blot (**Figure 4**):* (A) Distinct bands could be seen at 70 KD and 32 KD with electrophoresis results. (B) Hsp70 protein expression in rat myocardial tissues: The protein expression IOD values of myocardial tissues in the SH, MI, MG and MG+Q groups was 0.26 ± 0.06, 0.22 ± 0.04, 1.09 ± 0.07 and 0.75 ± 0.06, respectively. The difference in Hsp70 protein expression between the SH and MI groups was not statistically significant (P>0.05). Compared with the MI and SH group, this expression significantly increased in the MG and MG+Q groups (P<0.01); and the highest expression was in the MG group (P<0.01). The expression level significantly increased in the MG group, compared with that in the MG+Q group (P<0.01). (C) The protein expression of caspase-3 in rat myocardial tissues: The mRNA IOD in the SH, MI, MG and MG+Q groups was 0.96 ± 0.06, 8.69 ± 0.54, 3.13 ± 0.16, and 5.33 ± 0.21, respectively. Compared with the SH group, the protein expression of caspase-3 increased in the MI, MG and MG+Q groups (P<0.01). Especially in the MI group, this expression was significantly higher than that in the MG and MG+Q groups (P<0.01),

while the protein expression level of caspase-3 was higher in the MG+Q group was than that in the MG group.

Discussion

Myocardial apoptosis in non-infarcted areas after myocardial infarction plays an important role in the myocardial remodeling process. Joki Y *et al.* [9] found that within 1-6 months after myocardial infarction, the progressive expansion of the left ventricle and progressive decrease of the pressure peak in the left ventricle were related to myocardial apoptosis distant from the infarcted area. Furthermore, through a rat MI model, Palojoki E *et al.* [10] discovered that apoptotic cells surrounding the infarcted area continuously increased at 12 weeks after myocardial infarction, and the number of apoptotic cells distant from the infarcted area was positively correlated with the increase in left ventricular end-diastolic diameter. This indicates that apoptosis participates and plays an important role in the ventricular remodeling process.

HSPs have an important effect on cell growth regulation and apoptosis. According to researches, several members of the HSP family, such as Hsp90, Hsp70, Hsp60 and Hsp27, can control the apoptosis process by playing a role in many links, such as Fas death receptor levels and caspase levels [11-13]. Foreign scholars have found that Hsp70 can restrain mitochondria from releasing cytochrome C induced by heat stress and inhibit the activation of caspase through directly bonding with the apoptotic protease structure domain of apoptosis protein activating factor-1 (Apaf-1), thereby reducing the apoptosis [11, 14]. Hsp70, as the most conservative and primary in HSPs, can be induced to occur under stress conditions, and has a protective effect against cellular damage. The degradation of HSPs is controlled by the ubiquitin proteasome system. That is, when ubiquitin proteasome activity increases, the degradation of HSPs is intensified; otherwise, it is declined. Proteasome inhibitors can be adopted to reduce ubiquitin proteasome activity, and thereby induce the increase in HSP expression. Du *et al.* [15] found that MG-132 could induce the expression of Hsp70 and fight against INFγ-induced apoptosis in lens epithelial cells. Experiments *in vitro* have revealed that MG-132 can induce Hsp70 and Hsp32 expression in myocardial cells via the P38, and play a protective effect on the myocardium [16]. However, it has not been reported whether the *in vivo* application of proteasome inhibitors can induce HSP and reduce myocardial apoptosis.

The results of the present study revealed that the mRNA and protein expression of Hsp70 increased in the MG and MG+Q groups, which was consistent with the results of the *in vitro* experiment conducted by Meller *et al.* [4]. This indicates that the proteasome inhibitor of MG-132 can induce the generation of Hsp70. After the application of the proteasome inhibitor, proteasome activity decreased. Hence, the degradation of Hsp70 also decreased. On the other hand, the decrease in proteasome activity increased abnormal protein levels, stimulated the production of heat shock transcription factor 2 (HSF-2) and the binding of HSF-2 with heat shock elements (HSE), and facilitated the activation of Hsp70 genes; thereby increasing Hsp70 expression [17]. Compared with the MG+Q group, Hsp70 expression significantly increased in the MG group, which reveals the weakening effect of quercetin on Hsp70 expression activation induced by MG-132. This was similar to the observation results reported by Henryk Drege *et al.* [16] Quercetin is a kind of five-peptide hydroxyl biological flavonoid, which can inhibit the transcription of HSPs by inhibiting the phosphorylation of the heat shock transcription factor [18] and further partly weakening the activation effect of MG-132 on Hsp70 generation. In the present study, the difference in Hsp70 expression between the MI and SH groups was not statistically significant, which is similar to that reported by Marunouchi T *et al.* [19]. Marunouchi T [19] reported that at two weeks and eight weeks after rat myocardial infarction, there was a difference in Hsp72 expression when compared with that in the SH group. However, when high heat stimulation was administered at a corresponding time point, the Hsp72 expression in the MI group significantly decreased compared with that in the SH group. Under stress conditions, the expression of Hsp70 is related to the physiological status of cells, and Hsp70 mRNA and the protein synthesis and function will decreased to some extent with organism aging and cell senescence. These experiments revealed that Hsp70 expression in myocardial cells of young rats sig-

nificantly increased compared with aged rats, while such expression in hypertrophic myocardial cells decreased compared with normal myocardial cells.

Caspase has an important effect on the apoptosis process. In the present study, caspase-3 was the core apoptosis executor. According to the results of the present study, compared with the SH group, caspase-3 expression increased in the MI group, which was consistent with results reported in a literature [20]. Since ischemia, anoxia and oxygen radical generation are increased and the mitochondrial membrane is destroyed after myocardial infarction, some active apoptosis-related substances originally located in the mitochondrion such as cytochrome C, as well as apoptosis-inducing factor (AIF), would be released, which generally occurs when the mitochondrial membrane permeability transition (PT) hole is open, thereby increasing the expression of caspase-3. Compared with the MI group, caspase-3 expression significantly decreased in the MG group; indicating that MG-132 can inhibit the expression of caspase-3. However, it remains to be determined whether MG-132 inhibits the expression of caspase-3 by inducing the generation of Hsp70. In the present study, quercetin, the inhibitor of Hsp70, was adopted and combined with MG-132 to carry out the intervention. Results revealed that the expression of caspase-3 significantly increased in the MG+Q group compared with the MG group, and this is similar with the results of the *in vitro* experiment conducted by Henryk Drege *et al.* [16]. In a research on MG-132 resistance against lens epithelial cell apoptosis, Henryk Drege *et al.* [16] found that MG-132 inhibited the expression of caspase-3 by inducing the generation of Hsp70. The present study indicated that the *in vivo* application of MG-132 inhibited the expression of caspase-3 by inducing the increased Hsp70 expression. In addition to Apaf-1, HSP can directly bind with AIF released by the mitochondria, and inhibit the caspase-independent apoptosis mediated by it [21].

After caspase-3 activation was inhibited, apoptosis is decreased. In the present study, the myocardial apoptosis condition in non-infarcted areas after 28 days was evaluated, and results indicated that at four weeks after myocardial infarction, myocardial apoptosis in the non-infarcted area was significantly higher in the MI group than in the SH group. This reveals that myocardial apoptosis is the main method of myocardial cell death in non-infarcted areas after myocardial infarction. Schwarz K *et al.* [22] reported that four weeks after myocardial infarction, myocardial apoptosis in the non-infarcted area continued to increase, and no myocardial necrosis was observed in these areas; indicating that the occurrence of apoptosis is independent of necrosis, and the only mechanism of myocardial apoptosis in these non-infarcted areas after myocardial infarction. After a small dosage of proteasome inhibitor MG-132 as administered *via* intraperitoneal injection, myocardial apoptosis in the non-infarcted area significantly decreased. This shows that a small dosage of MG-132 can relieve myocardial apoptosis in non-infarcted areas. Dai Cuilian *et al.* [23] carried out a research on myocardial ischemia reperfusion, and discovered that MG-132 could reduce the apoptosis of myocardial cells. However, when Vijayakumar A K *et al.* [24] treated the myocardial cells of neonatal rats with MG-132 (0.5 μmol/L) in a research, they found that the increase in MG-132 could increase the expression of pro-apoptotic proteins (such as P53 and Bax), but reduce the expression of anti-apoptotic proteins (bcl-2 and bcl-xl). Therefore, myocardial apoptosis increased. The reason for such difference may be related to the dosage of the proteasome inhibitor. MJ Li *et al.* [7] applied MG-132 at different concentrations to rat myocardial fibroblasts, and found that MG-132 could inhibit the expression of matrix metalloproteinase at different concentrations, while high concentrations of MG-132 (0.5-1.0 μmol/L) could increase cell death; hence, cell death was dependent to the dosage. However, low concentrations had no effect on the survival of cells. Therefore, the proteasome inhibitor has dual effects on the apoptosis process, that is, it can not only facilitate the apoptosis, but also resist against apoptosis. The effect of the proteasome inhibitor on the apoptosis depends on the dosage of the proteasome inhibitor, cell type, and the strength of stimulus signals [25-27]. In the present study, myocardial apoptosis increased in the MG+Q group compared with that in the MG group; and the increase in Hsp70 expression had the anti-apoptosis effect. Henryk Dreger *et al.* [16] found that MG-132 could reduce the INFγ-

induced apoptosis in lens epithelial cells by inducing the generation of Hsp70.

Disclosure of conflict of interest

None.

Address correspondence to: Zhang-Rong Chen, Department of Cardiology, The First Affiliated Hospital of Dali University, No. 32, Carlsberg Avenue, Dali 671000, Yunnan, China. Tel: +86 18687286038; E-mail: chenzhangrong1013@21cn.com

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