Pretreatment with Tyrosine Kinase Inhibitor Attenuates the Reduction of Apoptosis 24 h after Ischemic Preconditioning

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Abstract: We investigated whether ischemic preconditioning (PC) attenuates ischemia/reperfusion-induced injury in part by decreasing apoptosis and whether tyrosine kinase (TK) can regulate the signaling pathway leading to apoptosis in delayed cardioprotection. Six groups of rabbits were studied in the early phase (EP) and in the delayed phase (DP): (1) sham-operated control animals were received vehicle only (Veh-sham); (2) rabbits that received I.V. genistein (a nonspecific TK inhibitor) 10 min before ischemia (Gen-sham); (3) rabbits that received I.V. daidzein (an inactive structural analog of genistein) 10 min before ischemia (Dzn-sham); (4) rabbits preconditioned with 4 cycles of 5-min occlusion of left anterior descending coronary artery (LAD) and 10-min reperfusion (PC); (5) rabbits that received I.V. genistein, 10 min before PC (Gen-PC); (6)

rabbits that received I.V. daidzein 10 min before PC (Dzn-PC). All rabbits underwent 30-min ischemia followed by 180-min reperfusion. Infarct size in the PC, Gen-PC, and Dzn-PC groups in the EP was significantly ($p < 0.0001$) reduced relative to controls Gen and Dzn. Delayed cardioprotection was blocked significantly ($p < 0.0001$) by genistein. In the EP, apoptosis was significantly ($p < 0.0001$) decreased in PC, Gen-PC, and Dzn-PC groups relative to controls Gen and Dzn. In the DP, a reduction of apoptosis was not seen in the Gen-PC group. This study suggests that PC reduces ischemic injury in part by decreasing apoptosis after ischemia/reperfusion and also that TK phosphorylation is involved in the signal transduction cascade leading to the decline of apoptosis in the DP. [The Japanese Journal of Physiology 54: 143–151, 2004]

Key words: ischemia/reperfusion, ischemic preconditioning, apoptosis, myocardial infarction, rabbit.

Ischemic preconditioning (PC) is the exposure of the myocardium to a brief episode of ischemia and reperfusion resulting in a reduction in the amount of tissue necrosis during a subsequent sustained ischemia [1]. The cardioprotective effect of PC is short-lived, subsiding within 2–3 h [1, 2]. However, preconditioning has been shown to induce protection that only appears after 24 h; this protection is described as the “delayed phase” or the “second window of protection” [3, 4]. The mechanism of the delayed phase of preconditioning is not clearly understood, though several receptors

[5] and intracellular signaling pathways such as protein kinase C (PKC) [6, 7] and tyrosine phosphorylation [8, 9] are known to be involved. Recent studies suggest that programmed cell death plays an important role in the death of myocytes following heart failure and ischemia/reperfusion. *In vivo* studies have demonstrated apoptosis of cardiomyocytes during postnatal maturation [10], spontaneous hypertension in rats [11], after rapid ventricular pacing [12], and microembolization-induced cardiac failure [13]. We hypothesized that an inhibition of apoptosis might be

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involved in both the early and delayed phases of PC. Since tyrosine kinase signaling has been identified as playing a key role in the cardioprotective effect in the delayed phase of preconditioning [8, 9], we further hypothesized that this pathway may also regulate apoptosis in preconditioning. The aims of the present study were to demonstrate that both early and delayed phases of PC are accompanied by an inhibition of apoptosis following ischemia/reperfusion injury and to show that tyrosine kinase signaling plays an important role in the regulation of apoptosis during preconditioning in the rabbit heart.

MATERIALS AND METHODS

Animals. This study involved 168 male New Zealand white rabbits (2.5–3.2 kg body weight) that were maintained on a standard diet. Ten rabbits were used for the measurement of the infarction and 6 for the observation of apoptosis in each group. The investigation was conducted in accordance with the “Guiding Principles for the Care and Use of Animals Approved by the Council of the Physiological Society of Japan.

Surgical preparation. The rabbits were anesthetized with intramuscular ketamine HCl (35 mg/kg) and xylazine (5 mg/kg).

Early phase. The rabbits were intubated through a tracheotomy and mechanically ventilated with room air by use of a positive pressure respirator. Catheters

were placed in the left carotid artery and jugular vein for blood pressure monitoring and drug infusion, respectively. Thoracotomy through the left fourth or fifth intercostal space exposed the heart. A 5-0 silk thread was passed around the left anterior descending coronary artery (LAD) and both ends of the ligature were threaded through a vinyl tube to make a snare.

Delayed phase. The rabbits were adequately anesthetized, orally intubated, and mechanically ventilated with room air on a positive pressure respirator. A catheter was placed in the ear artery for blood gas and blood pressure monitoring. A left thoracotomy was performed through the fourth or fifth intercostal space, and the pericardium was opened to expose the heart. A 5-0 silk thread was passed around the LAD, and the ends of the thread were passed through a vinyl tube to make a snare. The wound was closed with sutures, and the rabbit was returned to its cage for recovery. Twenty-four hours after surgery, the rabbit was anesthetized again, intubated by a tracheotomy, and placed on the ventilator. A catheter was inserted into the carotid artery to monitor blood pressure. A successful ligation was confirmed by a regional cyanosis of the myocardial surface with bulging and ST-T segment elevation on the electrocardiogram. Reperfusion was confirmed by myocardial blush over the risk area after the release of the snare. Hemodynamic parameters, including mean arterial pressure (MAP), systolic and diastolic arterial pressure, and heart rate (HR), were continuously recorded and a lead II electrocar-

Fig. 1. Experimental protocol. The vehicle-treated animals received 3 ml/kg vehicle (saline containing 2% dimethylsulfoxide adjusted to pH 9.0 with 1 N NaOH). The sham-operated animals underwent the same surgical procedure, including pericardiotomy and positioning the coronary artery suture, without coronary occlusion. Veh, vehicle; PC, preconditioning; Gen, genistein; Dzn, daidzein. Genistein (5 mg/kg) or daidzein (5 mg/kg) was given as an intravenous injection 10 min before the surgical procedure of ischemic preconditioning or 10 min before prolonged ischemia in the early phase. In the delayed phase, genistein (5 mg/kg) or daidzein (5 mg/kg) was given as an intravenous injection 10 min before the surgical procedure of ischemic preconditioning or 24 h before prolonged ischemia. A cross-hatched column showed the sham operation. PC induced by 4 cycles of 5-min occlusion of LAD (black column) and 10 min of reperfusion.

diagram (ECG) was recorded from subdermal electrodes throughout the procedures.

Experiment protocol. The experimental design is illustrated in Fig. 1. Six groups were studied in the early phase and in the delayed phase; all animals were subjected to a 30 min regional ischemia induced by an occlusion of the LAD followed by a 3 h reperfusion. They were divided into six groups in each phase. In the early phase these groups were as follows: (1) sham-operated control animals that received vehicle only (Veh-sham, $n=16$); (2) recipients of a nonspecific tyrosine kinase inhibitor, genistein (5 mg/kg) that was given as an intravenous injection 10 min before ischemia (Gen-sham, $n=10$); (3) recipients of an inactive structural analog of genistein, daidzein (5 mg/kg), that was given via intravenous injection 10 min before ischemia (Dzn-sham, $n=10$); (4) the PC groups that received the PC procedure of 4 cycles of 5 min regional ischemia/10-min reperfusion (PC, $n=16$); (5) recipients of a nonspecific tyrosine kinase inhibitor, genistein (5 mg/kg) that was given as an intravenous injection 10 min before the PC procedure (Gen-PC, $n=16$); and (6) recipients of an inactive structural analog of genistein, daidzein (5 mg/kg) that was given via intravenous injection 10 minutes before the PC procedure (Dzn-PC; $n=16$).

In the delayed phase, the six groups consisted of (1) sham-operated control animals that were received vehicle only (Veh-sham, $n=16$); (2) recipients of a nonspecific tyrosine kinase inhibitor, genistein (5 mg/kg) that was given as an intravenous injection 24 h before ischemia (Gen-sham, $n=10$); (3) recipients of an inactive structural analog of genistein, daidzein (5 mg/kg) that was given via intravenous injection 24 h before ischemia (Dzn-sham, $n=10$); (4) PC groups that received the PC procedure of 4 cycles of 5 min regional ischemia/10-min reperfusion (PC, $n=16$); (5) recipients of a nonspecific tyrosine kinase inhibitor, genistein (5 mg/kg), that was given via intravenous injection 10 min before the PC procedure (Gen-PC, $n=16$); and (6) recipients of an inactive structural analog of genistein, daidzein (5 mg/kg), that was given via intravenous bolus injection 10 min before the PC procedure (Dzn-PC, $n=16$).

Twenty-four hours later, all rabbits underwent prolonged occlusion for 30 min followed by 3 h of reperfusion. The sham-operated animals underwent the same surgical procedure, including pericardiotomy and positioning the coronary artery suture, without coronary occlusion. The vehicle-treated group received 3 ml/kg vehicle (saline containing 2% dimethylsulfoxide adjusted to pH 9.0 with 1 N NaOH). Four rabbits in each group were sacrificed to deter-

mine the percentage of cardiomyocytes with DNA fragmentation.

The samples were excluded from the study if any of the following criteria resulted. (1) Coronary artery occlusion did not produce severe ischemia (the area at risk was less than 20% of the left ventricle). (2) Intractable ventricular fibrillation occurred during ischemia and/or reperfusion. (3) Death of the animal occurred during the surgical procedure, and the protocol was not completed.

Measurement of infarct size. At the end of each experiment, the LAD was reoccluded, and 50% Unisperse blue dye solution was injected into the right jugular vein until the eyes turned blue. This was to distinguish between perfused and non-perfused (risk area) regions of the myocardium: the Unisperse blue solution stains the perfused myocardium, but the occluded vascular bed remains uncolored. The animal was then sacrificed and the heart was removed. Heart slices were incubated at room temperature for 20 min in a 1% solution of TTC. After staining, the infarct area (TTC staining-negative) and risk area (area non-sustained by Unisperse blue) were determined by computer morphometry using Bioquant imaging software, followed by a calculation of the percentage of infarcted and risk areas.

In situ identification of nuclear DNA fragmentation. After deparaffinization, the tissue sections were stained by use of the TUNEL method, and *in situ* apoptosis-induced nuclear DNA fragmentation was visualized with a fluorescence assay [14–16]. Apoptotic cardiomyocyte nuclei were examined under a fluorescence microscope after 30 min ischemia followed by a 3 h reperfusion. The percentage of apoptotic cardiomyocyte nuclei was determined microscopically by use of an eyepiece grid. A quantitative analysis of DNA fragmentation was performed with the TUNEL method at the single-cell level. The percentage of TUNEL-positive cardiomyocyte nuclei was determined microscopically by use of an eyepiece grid (200 $\times$ magnification). Five microscopic fields per section were selected from within the ischemic region. In each field, the cells were counted in the ischemic region, and the percentage of apoptotic cardiomyocytes was calculated.

Genomic DNA extraction and agarose gel electrophoresis. The procedures were based on a previously published methodology [17]. For a determination of genomic DNA fragmentation, rabbit hearts were rapidly removed after the prolonged ischemia and reperfusion and tissue samples from the LV were homogenized. The ApoAlert™ ligation-mediated PCR ladder assay kit (Clontech Laboratories,

Fig. 2. Hemodynamic data. The heart rate, mean arterial blood pressure, and rate pressure product during the experimental protocol in the early and delayed phase. There were no significant differences between the groups throughout the experiment. Data were shown the means $\pm$ SEM. Veh, vehicle; PC, preconditioning; Gen, genistein; Dzn, daidzein. Genistein (5 mg/kg) or daidzein (5 mg/kg) was given as an intravenous injection 10 min before the surgical procedure of ischemic preconditioning or 10 min before prolonged ischemia in the early phase. In the delayed phase, genistein (5 mg/kg) or daidzein (5 mg/kg) was given as an intravenous injection 10 min before the surgical procedure of ischemic preconditioning or 24 h before prolonged ischemia. PC induced by 4 cycles of 5-min occlusion of LAD and 10 min of reperfusion.

Inc., CA, USA) was used, and DNA electrophoresis was conducted on 3% agarose.

Drugs and treatment. Genistein, daidzein, and triphenyl tetrazolium chloride (TTC) were purchased from Sigma Chemical Co. Ketamine hydrochloride was obtained from Sankyo, Inc. Xylazine was obtained from Fuji Chemical, Inc.

Statistical analysis. Values are expressed as mean $\pm$ SEM. Comparisons of means were made by use of a Student's *t*-test for unpaired values. The statistical significance was determined by ANOVA with repeated measurements. If a significant *F* value was found, the Scheffé test for multiple comparisons was used to identify differences among groups. The values were considered significant when $p < 0.05$.

RESULTS

Hemodynamic data

Values for heart rate (HR), mean arterial blood pressure (MAP), and rate pressure product (RPP) during the experiment are given in Fig. 2. During ischemia and reperfusion, these values were similar among all the experimental groups ($p = \text{NS}$) in the early and delayed phases. Thus all groups were comparable with regard to major determinants of infarct size such as hemodynamic parameters.

Infarct size

Figure 3 shows the infarct size in the early phase. The mean percentage of risk area in the left ventricle (%RA/LV) was $46.0 \pm 1.5\%$ in the Veh-sham group, $48.9 \pm 3.6\%$ in the Gen-sham group, $46.5 \pm 4.3\%$ in the Dzn-sham group, $44.0 \pm 1.8\%$ in the PC group, $46.0 \pm 2.2\%$ in the Gen-PC group, and $48.4 \pm 2.1\%$ in the Dzn-PC group. No significant difference in the risk area was observed among all groups ($p = \text{NS}$). Thus they all were comparable with regard to major determinants of infarct size such as the risk area. Infarct size expressed as a percentage of risk area (%IS/RA) was $16.8 \pm 1.6\%$ in the PC group, $19.2 \pm 2.1\%$ in the Gen-PC group, and $19.0 \pm 0.9\%$ in the Dzn-PC group, all values of which were significantly smaller than $48.9 \pm 1.0\%$ in the Veh-sham group, $46.2 \pm 5.5\%$ in the Gen-sham group, and $49.1 \pm 3.8\%$ in the Dzn-sham group ($p < 0.0001$). The infarct size (%IS/RA) in the delayed phase (Fig. 4) averaged $48.1 \pm 2.2\%$ in the Veh-sham group, $46.5 \pm 4.2\%$ in the Gen-sham group, $45.5 \pm 3.9\%$ in the Dzn-sham group, $19.9 \pm 0.9\%$ in the PC group, $49.5 \pm 2.1\%$ in the Gen-PC group, and $19.9 \pm 1.2\%$ in the Dzn-PC group. These values for the PC group and the Dzn-PC group represented significant reductions in infarct size relative to the Veh-sham group ($p < 0.0001$), whereas there were no such significant differences between the Veh-sham I and the Gen-PC group.

Fig. 3. In the early phase, the risk area (% of left ventricle [LV]) and infarct size expressed as % of area at risk and % of LV. In the control group, hearts were subjected to 30 min ischemia followed by 3 h reperfusion. In the PC group, animals received preconditioning with four 5 min coronary artery occlusions, each followed by a 10 min reperfusion. In the Gen-PC group, preconditioned animals received Gen (5 mg/kg, i.v.) 10 min before procedure of ischemic preconditioning. In the Dzn-PC group, preconditioned animals were treated with Dzn (i.v.) 10 min before the procedure of ischemic preconditioning. Each bar represents mean $\pm$ SEM. Veh, vehicle; PC, ischemic preconditioning; Gen, genistein; Dzn, daidzein. ^a $p < 0.0001$ vs. Vehicle-sham. ^b $p < 0.0001$ vs. Gen-sham. ^c $p < 0.0001$ vs. Dzn-sham.

Fig. 4. In the delayed phase, risk area (% of left ventricle [LV]) and infarct size expressed as % of area at risk and % of LV. In the PC group, animals received preconditioning with four 5 min coronary artery occlusions, each followed by a 10 min reperfusion. In the Gen-PC group, preconditioned animals received Gen (5 mg/kg, i.v.) 10 min before procedure of ischemic preconditioning. In the Dzn-PC group, preconditioned animals were treated with Dzn (i.v.) 10 min before the procedure of ischemic preconditioning. Twenty-four hours later, all rabbits underwent prolonged occlusion for 30 min followed by 3 h of reperfusion. Each bar represents mean $\pm$ SEM. Veh, vehicle; PC, ischemic preconditioning; Gen, genistein; Dzn, daidzein. ^a $p < 0.0001$ vs. Vehicle-sham. ^b $p < 0.0001$ vs. Gen-sham. ^c $p < 0.0001$ vs. Dzn-sham.

Percentage of cells with DNA fragmentation

The typical TUNEL-positive cardiomyocyte nuclei at magnifications of 200 $\times$ and 1,000 $\times$ are shown in Fig. 5. A quantitative analysis of DNA fragmentation was performed by using the TUNEL method at the

single-cell level (data shown in Fig. 6). The mean percentage of DNA fragmentation was $3.5 \pm 1.0\%$ in the PC group, $3.3 \pm 0.6\%$ in the Gen-PC group, and $2.8 \pm 0.5\%$ in the Dzn-PC group, these values being significantly lower than the values of $11.2 \pm 1.0\%$ seen

Fig. 5. *In situ* identification of nuclear DNA fragmentation. Tissue sections were labeled using the terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) method with detection of *in situ* apoptosis-induced nuclear DNA fragmentation showing by a fluorescence assay. Rabbit hearts were subjected to 30 min ischemia followed by 3 h reperfusion. dUTP-labeling is depicted in several cardiomyocyte nuclei (arrows). Original magnification, **A**: 200 $\times$; **B**: 1,000 $\times$.

Fig. 6. Quantitative analysis of DNA fragmentation. The percentage of apoptotic cardiomyocytes was calculated for the ischemic region. DNA fragmentation was significantly ($p < 0.001$) reduced in the PC, Gen-PC, and Dzn-PC group compared with the vehicle-sham group, Gen-sham group, and Dzn-sham group in early phase. However, a reduction

of TUNEL-positive cardiomyocytes were significantly blocked by genistein in delayed phase. Veh, vehicle; PC, ischemic preconditioning; Gen, genistein; Dzn, daidzein. Values are mean $\pm$ SEM. ^a $p < 0.001$ vs. Vehicle-sham. ^b $p < 0.001$ vs. Gen-sham. ^c $p < 0.001$ vs. Dzn-sham.

in the Veh-sham group ($p < 0.001$) in the early phase. There were no significant differences among the Veh-sham group, Gen-sham group ($10.2 \pm 2.3\%$), or Dzn-sham group ($9.7 \pm 3.1\%$). The percentage of DNA fragmentation in the delayed phase averaged $12.3 \pm 1.5\%$ in the Veh-sham group, $10.6 \pm 3.2\%$ in the Gen-sham group, $11.5 \pm 2.8\%$ in the Dzn-sham group, $3.5 \pm 1.2\%$ in the PC group, $14.0 \pm 2.1\%$ in the Gen-PC group, and $3.7 \pm 1.8\%$ in the Dzn-PC group. These values for the PC group and the Dzn-PC group represented significant reductions in apoptosis formation relative to the Veh-sham group, the Gen-sham group, and the Dzn-sham group ($p < 0.001$), whereas there were no such significant differences between the Veh-sham group and the Gen-PC group.

Detection of genomic DNA fragmentation

DNA fragmentation was evaluated by agarose gel electrophoresis of ligation-mediated polymerase chain reaction (PCR) with a DNA ladder assay (Fig. 7). For

the detection and qualitative evaluation of DNA fragmentation, we examined whether genomic DNA extracted from ischemic hearts produced a typical "ladder" pattern. DNA fragmentation was not observed in the myocytes of rabbits in the PC, Gen-PC, and Dzn-PC groups in the early phase. However, ischemic LV regions obtained from the Veh-sham, Gen-sham, Dzn-sham, and genistein-treated rabbits before the PC group that subjected to 30 min of LAD occlusion and 180 min of reperfusion showed a typical DNA electrophoretic pattern characterized by mononucleosomal and oligonucleosomal DNA fragmentation in the delayed phase.

DISCUSSION

We examined whether ischemic preconditioning attenuates irreversible ischemia/reperfusion injury in part by decreasing apoptosis and whether tyrosine kinase can regulate the signaling pathway leading to apopto-

Fig. 7. DNA electrophoresis. DNA extraction and electrophoresis were carried out according to the ligation-mediated PCR method. Electrophoretic analysis was performed on cardiac tissue samples from control, PC, Gen-treated and Dzn-treated before PC groups in early and delayed phase. M, DNA ladder markers; Veh, vehicle; PC, preconditioning; Gen, genistein; Dzn, daidzein. Genistein (5 mg/kg) or daidzein (5 mg/kg) was given as an intravenous injection

10 min before the surgical procedure of ischemic preconditioning or 10 min before prolonged ischemia in the early phase. In the delayed phase, genistein (5 mg/kg) or daidzein (5 mg/kg) was given as an intravenous injection 10 min before the surgical procedure of ischemic preconditioning or 24 h before prolonged ischemia. PC induced by 4 cycles of 5-min occlusion of LAD and 10 min of reperfusion.

sis formation in delayed cardioprotection. When present during ischemic preconditioning, genistein failed to block the reduction in infarction and apoptosis in the early phase.

In terms of supporting data, a previous study reported that the protective action of ischemic preconditioning was not affected when genistein was present during preconditioning ischemia, but it was completely eliminated when the inhibitor was present during the long ischemia [18]. Moreover, the anti-infarct effect and the reduction of apoptosis formation attributable to ischemic preconditioning was attenuated completely by genistein in the delayed phase.

It is now clear that the cardioprotective mechanism of PC differs between the early and delayed phases. There are several proposed mechanisms for preconditioning: (1) various endogenous mediators (adenosine [19], opioid [20], bradykinin [21], active oxygen [22], nitric oxide [23], and others); (2) energy conservation; (3) new gene transduction mediated by the transcription of such factors as Mn-superoxide dismutase (SOD) [24] and heat shock protein (HSP) [25, 26]; (4) opening of the mitochondrial K_{ATP} channel; (5) various signal transduction processes, including G protein, protein kinase C, tyrosine kinase, and mitogen-activated kinase (MAP kinase).

Ever since Fliss and colleagues [27] reported that apoptosis was induced by subjecting rat hearts to ischemia followed by reperfusion, it has generally been accepted that there is a close relationship between

apoptosis and ischemia followed by reperfusion. Furthermore, Piot and colleagues [28] induced PC by subjecting rat hearts to 30 min of ischemia followed by 180 min of reperfusion. They reported that the size of infarcts decreased and the number of TUNEL-positive cells decreased significantly in preconditioned rats relative to non-preconditioned rats. As a result, they concluded that PC reduces the extent of irreversible ischemic injury by decreasing apoptosis after prolonged ischemia and reperfusion.

In this study, we used ischemic and reperfused rabbit myocardium models to investigate tyrosine kinase-dependent mechanisms by administering genistein, a tyrosine kinase receptor inhibitor. During the early phase, PC reduced the infarct size and suppressed apoptosis, and these cardioprotective effects of PC were observed even when genistein was administered. However, during the delayed phase, the infarct size was reduced and apoptosis was suppressed in the PC and Dzn groups, but when genistein was administered, the infarct size and the extent of apoptosis were comparable to those in the control group. Furthermore, genistein or daidzein by itself without PC did not contribute to the reduction of infarct size or to apoptotic cardiomyocyte. These results indicate that the role of the tyrosine kinase signal pathway differs between the early and delayed phases. In other words, during the early phase, the tyrosine kinase signal pathway is not involved in reducing the infarct size and suppressing apoptosis. However, during the delayed phase, this

pathway is very important in enabling PC to exhibit its cardioprotective effects.

Various pathways are thought to be involved in apoptosis. Nevertheless, of the various apoptosis effectors, caspase-3 is the most important one in the caspase family [29–31].

It has been suggested that a blockage of the caspase proteolysis cascade by HSP70 or interference with the stress-activated protein kinase (SAPK)/Jun N-terminal kinase (JNK) signaling pathway could suppress the activation of caspase-3 or the SAPK/JNK signaling pathway [32, 33].

We previously reported that the administration of genistein suppressed the expression of HSP70 and eliminated the reduction of infarct size by PC. We thus concluded that in the delayed phase, the tyrosine kinase signal pathway was closely involved in the infarct size-reduction effect and that the production of HSP70 had an influence on this mechanism [8]. These findings show that HSP70, produced by PC during the delayed phase, suppresses the activity of caspase-3, thereby preventing apoptosis. Therefore, as shown in Fig. 3, during the early phase, even when genistein is administered, apoptosis is suppressed by ischemic preconditioning regardless of HSP70. The infarct size as observed by TTC staining was significantly decreased because of the suppression of necrosis and apoptosis. On the other hand, the delayed cardioprotective effect of IPC was blocked by genistein because the production of HSP70 was suppressed and the activity of caspase-3 was elevated, consequently accelerating apoptosis.

These findings suggest that tyrosine phosphorylation plays an important role in the delayed cardioprotective effect of IPC.

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