Expression of Heat Shock Protein After Ischemic Preconditioning in Rabbit Hearts

Masaru Tanaka, MD; Hisayoshi Fujiwara, MD; Kenzo Yamasaki, MD; Ryoji Yokota, MD; Kiyoshi Doyama, MD; Tsukasa Inada, MD; Seiji Ohtani, MD; Takako Fujiwara, MD*; Shigetake Sasayama, MD

Previous studies have shown that preconditioning (PC) with a brief ischemic episode induces heat shock protein (HSP) in cardiac tissue. However, it is unclear when and where in the left ventricle HSP is expressed after PC. Hence, the expression of HSP was studied in rabbit hearts at various time intervals after PC using immunohistochemical methods. Rabbits were preconditioned four times with 5 min of occlusion and 5 min of reperfusion of the coronary artery and then were killed at 0, 3, 24, 48, 72 and 168 h after the PC (n=4, for each time interval). Samples were obtained from the subendocardium and subepicardium of the preconditioned and non preconditioned wall and were processed to 4 μm thick cryosections. The sections were immunolabelled with mouse monoclonal IgGs against HSP 72/73. Positive immunoreactivity was observed as early as 3 h after PC, persisting up to 72 h but not detected at 168 h. HSP was expressed not only in the preconditioned myocardium but also in the remote non preconditioned myocardium. There was a wide variation in expression among myocytes. Expression was dominant in myocytes compared with vessel walls. It was concluded that PC induced transient and inhomogeneous expression of HSP in rabbit hearts. (Jpn Circ J 1998; 62: 512–516)

Key Words: Heart; Heat shock protein; Immunohistochemistry; Ischemic preconditioning; Rabbits

Methods

The investigation conformed with the ‘Guide for the Care and Use of Laboratory Animals’ published by the US National Institutes of Health (NIH publication No85–23, revised 1985).

Animal Selection

Thirty-seven male Japanese white rabbits weighing 1.9–2.9 kg were used. None of the animals had clinically evident infection.

Experimental Design

A summary of the experimental protocol is shown in Fig 1. Rabbits underwent four episodes of 5 min of ischemia and 5 min of reperfusion and were killed at 0, 3, 24, 48, 72 and 168 h after PC (n=4, each group). Because the surgical intervention itself might also induce HSP, as 2 h, and were even more striking at 24 h, after PC. However, detailed information regarding when and where HSP70 is expressed in the left ventricle after PC ischemia is still unknown.

The present study used an immunohistochemical technique to evaluate the time course and left ventricular distribution of the expression of HSP72/73 after PC ischemia in rabbits. There are several different isoforms of HSP, such as HSP104, 90, 70, 60, 40 and 28, which work in both physiologic and pathologic conditions. Although the precise roles of each isoform are not well understood, HSP70 is the major isoform that is induced by various kinds of stresses and plays an important role in cytoprotection in pathologic conditions. Hence, we focused on this isoform of HSP.

M ost organisms react to hyperthermia and other environmental stresses by increasing the synthesis of a group of proteins called heat shock proteins (HSP), which work under nonstressed conditions in cells as ‘molecular chaperons’ for the proper folding and assembly of newly synthesized proteins or intracellular transport of peptides.1 But, more importantly, under stressed conditions in cells, HSP expression is enhanced in order to prevent aggregation of denatured proteins or to degrade abnormal proteins.1 Thus, enhanced HSP expression may serve to protect hearts against subsequent damage. In experimental ischemia and reperfusion models, when the animals are exposed to hyperthermia prior to ischemia, improved functional recovery2–3 and infarct size limitation4–5 have been reported.

HSP is induced in hearts not only by hyperthermia but also by other stimuli such as ischemia, hypoxia, hemodynamic overload and chemicals. Among them, ischemia is of great interest, because a transient ischemic episode markedly elevates cardiac tolerance to subsequent ischemia, so called ‘ischemic preconditioning’ (PC).6 Thus, enhanced HSP expression may mediate PC protection. An understanding of the time course and left ventricular distribution of the expression of HSP after PC may lead to a better understanding of the physiology of HSP in PC protection. Knowlton et al, using Western blot analysis, reported that changes in the amount of HSP70 were evident as early as 2 h, and were even more striking at 24 h, after PC. However, detailed information regarding when and where HSP70 is expressed in the left ventricle after PC ischemia is still unknown.

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Expression of Heat Shock Protein After Preconditioning


Based on the results from serial arterial blood gas analysis, bated and mechanically ventilated with room air supplement (30–40 mg/kg, iv) and additional doses were given when needed. A catheter was placed in the right carotid artery for blood gas and blood pressure monitoring. The rabbits were then systemically heparinized (500 u/kg). The chest was opened via a left thoracotomy, the pericardium was opened, and the heart was exposed. A 4–0 silk suture on a small curved needle was passed around an anterolateral branch of the coronary artery, and the ends of the suture were passed through a small vinyl tube to make a snare. Myocardial ischemia was confirmed by ST segment elevation on the electrocardiogram and regional cyanosis of the myocardial surface. Reperfusion was confirmed by myocardial blush over the risk area after release of the snare. All rabbits were given 20 min after the completion of the surgical preparation to reach steady state before experimental protocol was started.

Immunohistochemical Analysis

Immunohistochemical examination was performed by an indirect immunoperoxidase method. In the first step, intrinsic peroxidase activity was inhibited by the addition of 0.3% hydrogen peroxidase in 100% methyl alcohol, and nonspecific binding was blocked with normal goat serum. As the primary antibody, mouse monoclonal antibody against HSP 72/73 (Oncogene Science, Inc, Uniondale, NY, USA) was added to the sections for 24 h at 4°C. In the second step, the peroxidase-conjugated F(ab')2 fragment of the secondary antibody (goat anti-mouse IgG, Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA) was added for 40 min at room temperature. Then, the sections were stained with 40 mg 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.006% hydrogen peroxide in 100 ml Tris buffer solution (0.05 mol/L) for 30 min at room temperature. Between each step, the sections were washed four times (10 min each) with 0.1 mol/L phosphate buffered saline. Finally, nuclei were counterstained with hematoxylin. To confirm the specificity of the immunoreaction, serial sections from HSP-positive specimens were also stained using anti-HSP antibody after preincubation with HSP 72/73 (SPP-750) (StressGen Biotechologies Corp, Victoria, BC, Canada) to demonstrate that this resulted in loss of the immunoreaction.

The presence of immunoreactive HSP was assessed by light microscopy. Two trained, unbiased observers (M.T. & H.F.) reviewed the sections. With regard to the immunohistochemical analysis, we adopted a scoring system to semi-quantitatively evaluate the immunoreactivity of HSP in myocytes. We randomly observed 100 myocytes from each tissue sample, and thus 200 myocytes from either the subepicardial or subendocardial layer of each rabbit. Because we examined four rabbits in each group, we observed a total of 800 myocytes from either the subepicardial or subendocardial layer of each rabbit. We examined four rabbits in each group, and scored the immunoreactivity in four grades as follows: (i) score ++ represents groups in which more than 50% of myocytes observed showed positive immunoreactivity, (ii) score + represents groups in which more than 20% but less than 50% of myocytes observed showed positive, (iii) score ± represents groups in which less than 20% but more than 0% of myocytes showed positive, and (iv) score – represents groups in which no myocyte showed positive.

Surgical Preparation

Rabbits were anesthetized with sodium pentobarbital (30–40 mg/kg, iv) and additional doses were given when required throughout the experiment. They were orally intubated and mechanically ventilated with room air supplemented with a low flow of oxygen (tidal volume 25–35 ml, respiration rate 20–30/min). The respirator was adjusted, based on the results from serial arterial blood gas analysis, to keep the arterial blood gases within the physiological range. The standard limb leads of the electrocardiogram were monitored. Surgery was done under sterile conditions. A catheter was placed in the right carotid artery for blood gas and blood pressure monitoring. The rabbits were then systemically heparinized (500 u/kg). The chest was opened via a left thoracotomy, the pericardium was opened, and the heart was exposed. A 4–0 silk suture on a small curved needle was passed around an anterolateral branch of the coronary artery, and the ends of the suture were passed through a small vinyl tube to make a snare. Myocardial ischemia was confirmed by ST segment elevation on the electrocardiogram and regional cyanosis of the myocardial surface. Reperfusion was confirmed by myocardial blush over the risk area after release of the snare. All rabbits were given 20 min after the completion of the surgical preparation to reach steady state before experimental protocol was started.
Results

Time Course of the Expression of HSP72/73 (Table 1)
Immediately after PC left ventricular myocytes did not show positive immunoreactivity for HSP 72/73. At 3 h after PC, immunoreactive cells were evident. This positive immunoreactivity persisted up to 72 h after PC, although it started to decay from 48 h after PC. At 1 week after PC, no immunoreactivity was observed. Specimens from sham-operated rabbits or control rabbits did not show positive immunoreactivity.

Left Ventricular Distribution of Immunoreactive Cells (Table 1) (Fig 2)
Between 3 and 72 h after PC, immunoreactivity was observed not only in the preconditioned myocardium but also in the remote nonpreconditioned myocardium. Immunoreactivity was similar between the subepicardial and subendocardial zones. Immunoreactivity varied greatly from cell to cell and was evident only in myocytes, not in vascular cells.

Specificity of Immunoreaction
In order to evaluate the specificity of the immunoreaction, three serial sections of preconditioned myocardium were obtained from a rabbit 3 h after PC. Positive immunoreactivity was observed in the section stained regularly. Another section stained with anti-HSP antibody, which was preincubated and absorbed with HSP 72/73, did not show positive immunoreactivity. (Fig 2) The third section, stained with nonimmune mouse serum as a primary antibody, did not show positive immunoreactivity either (data not shown).

Discussion
The present study revealed that PC induces transient and inhomogeneous expression of HSP72 not only in the preconditioned myocardium but also in the remote nonpreconditioned myocardium.

Methodological Consideration
We used monoclonal antibody, which recognizes both
the inducible (HSP72) and constitutive form (HSP73) of HSP70. Because the constitutive form is expressed in cells under physiological conditions to work as a 'molecular chaperon', all cells should express this isoform to some extent. However, immunoreactivity was not observed in the myocardium from control rabbits. This is due to the relatively low sensitivity of the immunohistochemical technique to small amount of peptides. Thus, negative immunoreactivity does not mean that HSP is absent but that it does not reach the threshold that is detectable by immunohistochemistry. Similarly, we can not exclude the possibility that a small amount of HSP72 is expressed even later than 72 h after PC. Thus, the immunohistochemical technique is semi-quantitative and has these limitations. However, we used this method because it was essential for evaluating the spatial distribution of HSP in the heart, including the cell-to-cell difference, which was the main goal of this study and is not achievable by other quantitative methods such as Western blot analysis.

**Time Course of HSP Expression**

Ischemia has been shown to induce HSP in myocardial tissue. Such expression does not occur immediately after ischemia but is delayed. Knowlton et al reported that, using Western blot analysis, the expression of HSP70 was evident as early as 2 h and was more prominent at 24 h after a single 5-min ischemic episode in rabbits? Donnelly et al also used Western blot analysis and reported that 20-min ischemia and 8-h reperfusion induced HSP72 in rats. The present study confirmed these data and the expression of HSP72 was evident as early as 3 h after PC. Moreover, it was also revealed that its expression decays from 48 h after PC and is not detectable 1 week later.

**Left Ventricular Distribution of HSP**

The present study revealed that PC induced HSP expression not only in the preconditioned myocardium but also in the nonpreconditioned myocardium. This observation was consistent with the data from Knowlton et al that 20-min ischemia and 8-h reperfusion induced HSP in the nonischemic wall as well as in the ischemic wall. Such a mode of expression was also evident with Mn-SOD, another cardioprotective protein. Hoshida et al reported that in a canine model the tissue content of Mn-SOD, an endogenous antioxidant increased not only in the preconditioned but also in the nonpreconditioned myocardium more than 12 h after PC. Thus, the time course and tissue distribution of expression are similar for these peptides. This suggests that HSP and Mn-SOD may share some common inducing mechanisms, and may cooperate with each other to protect the heart during ischemic preconditioning, especially in the late phase (see later). The mechanism responsible for the induction of HSP in the nonischemic myocardium remains unclear but could be due to one of the following. First, ischemia enhances the tissue levels of hormonal factors such as catecholamine and angiotensin, which have been reported to induce HSP expression. These humoral factors may affect the nonischemic myocardium, resulting in HSP expression. Second, ischemia causes stunning in the preconditioned myocardium, which induces compensatory hypercontraction of the nonpreconditioned myocardium. Such an increased workload, or an elevated left ventricular end-diastolic pressure, may result in the modest increase in HSP expression in the nonpreconditioned myocardium similar to a pressure overload, which reportedly increases HSP expression.

The present study also revealed that positive immunoreactivity was observed only in myocytes, not in microvascular cells. It is well known that during myocardial ischemia and reperfusion the microvascular cellular damage slowly follows the damage to the myocytes which suggests an ischemic episode is more stressful to myocytes than to microvascular cells. This may explain the potentiation of HSP expression in myocytes relative to microvascular cells.

Rabbits lack collateral channels and coronary occlusion produces severe transmural homogeneous ischemia. This may explain the similar HSP expression between the subepicardial and sub-endocardial wall. Although it is known that wall stress is higher in the subendocardium than in the subepicardium, this apparently seemed not to cause the difference in HSP expression between these walls. Immunoreactivity varied considerably even among neighboring myocytes. This may be due either to the difference in ischemia due to the variable distance from the adjacent capillaries or to the difference in the sensitivity to ischemia among myocytes. Further examination is needed to clarify these issues.

**HSP Expression and Myocardial Protection**

It is well established that PC immediately and markedly elevates cardiac tolerance to subsequent ischemia. However, this tolerance wanes rapidly within 2 h. The present study revealed that the time course of HSP expression is not consistent with such a 'rapid' PC effect. However, it has been recently proposed that PC may also induce a slower form of tolerance, resulting in a restoration of cardioprotection after the rapid PC effect has worn off; that is, there may be a second window of protection (SWOP). Kuzuya et al and Marber et al reported SWOP in terms of infarct size limitation in a canine and a rabbit model, respectively. However, we and Schaper et al did not observe SWOP in a rabbit and a porcine model, respectively. Thus, the concept of SWOP in terms of infarct size limitation remains controversial. The present study suggested that the elevated tissue level of HSP more than 3 h after PC could possibly protect the heart against a subsequent ischemic insult in terms of still undefined endpoints. Previously, Przyklenk et al reported that a transient left anterior descending artery occlusion could precondition the remote left circumflex artery myocardium in a canine model. Although this was an acute effect, the present study suggested that SWOP may also occur in the remote nonpreconditioned myocardium, due to the elevated HSP expression there.

Morphologically, infarct size limitation in preconditioned myocardium represents small patchy necrosis, which may indicate that the acute PC effect varies among myocytes. The present study indicated that HSP expression varied greatly among myocytes, which may cause different grades of SWOP against ischemia among myocytes.

**Clinical Implications**

PC markedly elevates cardiac tolerance to subsequent ischemia and its infarct size limiting power is far beyond any previously reported interventions. However, the potential shortcoming of PC for its clinical application is that the effect wanes rapidly. The delayed expression of HSP by PC and its pharmacological potentiation may offer cardiologists a new strategy for the treatment of ischemic heart disease.
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