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Hypothermic Preconditioning Lowers the Incidence of Hypothermic Arrest in Neonatal Rat

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This study was performed to examine 1) Whether hypothermic cardiac arrest produces myocardial HSP72 expression; 2) And if, whether it serves to protect the heart against the subsequent hypothermic arrest. In the present study, neonatal rats were placed in an icebath to induce hypothermia. To determine whether hypothermic cardiac arrest produces myocardial HSP72, experimental animals were subjected to 10-min hypothermic insult before the extraction of the heart. The intervals between the insult and extraction were 1 (1 HR), 4 (4 HR), 8 (8 HR), 24 (24 HR) or 72 (72HR) hours. A minimal amount of HSP72 was detected in control, 1 HR and 72 HR groups. In contrast, 8 HR and 24 HR groups showed a significant level of HSP72 expressions. To assess the cardioprotective effect of HSP72 against hypothermic cardiac arrest, we compared the proportion of recovery from the arrest between control and preconditioned (PREC) animals. Control animals were subjected to 20-min hypothermic insult, while PREC group was preconditioned by 10-min hypothermic insult 8 hours before the 20-min test hypothermic insult. Resuscitation rate from cardiac arrest induced by the 20-min hypothermic insult in PREC group was significantly higher than that in controls. These results suggest that the cardioprotective effect of hypothermic preconditioning is associated with an increase in HSP72 expression.

Key Words: Hypothermia, Heat shock protein72, Neonatal rat

INTRODUCTION

Hypothermic treatment has been applied in heart and brain surgery and to the preservation of human organs for transplantation. In 1950, Bigelow et al introduced the idea that whole body hypothermia by surface cooling might be useful in total circulatory arrest for cardiac surgery. Lewis & Taufic (1953), and Swan et al (1953) also reported successful results in a number of patients treated by cardiac surgery under surface cooling. Recently, the cardiac surgery under hypothermia has steadily advanced along with the development of surgical methods. However, little is known concerning the underlying mechanisms of hypothermic treatment.

A broad category of stressful stimuli, such as ischemia (Currie, 1987), hypoxia (Howard & Geoghegan, 1986), heavy metals (Low et al, 1989) and hyperthermia (Currie & Whith, 1983), has been shown to increase synthesis of heat shock proteins (HSPs) by various organs, while decreasing the levels of other proteins. It is believed, thus, that HSPs play a protective role by responding to stress (Lindquist & Craig, 1988). Similarly, several studies reported that elevated HSPs following brief episode of ischemia or hyperthermic insult, i.e., preconditioning, may provide a protective effect against subsequent insult (Currie et al, 1988; Currie et al, 1993). In hibernating animals, prehibernation prior to hibernation probably confers protection against cell damage during hibernation (Wang, 1989). However, unlike the other types of preconditioning, it is uncertain whether the development of hypothermic protection is accompanied by the induction of HSPs (Glofcheski et al, 1993). Furthermore, although ischemia/reperfusion as a result of reversible hypothermia-induced cardiac arrest ex-

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presses myocardial HSPs, hypothermia itself is known to diminish the ischemic induction of HSPs (Lanks, 1990). In addition, Chopp et al (1992) suggested that HSP72 expression is not a mechanism by which hypothermia serves the protective effect against ischemia/reperfusion injury.

Therefore, the present study was designed to solve several questions using a neonatal rat which is more tolerable to hypothermia than the adult one (Bove & Strammers, 1986). The first question addressed was whether the hypothermic insult produces myocardial HSP72 expression. Second was, if so, whether HSP72 expression makes the neonatal heart recover easily from cardiac arrest induced by the subsequent hypothermia? The results from this experiment suggest that the cardioprotective effect of hypothermic preconditioning is associated with an increase in HSP72 expression.

METHODS

Neonatal rats (3~5 days after birth) were used in the present study. To induce whole-body hypothermia, the neonatal rats were placed in an icebath and then rewarmed with thermoblanket. To determine whether the hypothermic insult produces myocardial HSP72 expression, experimental animals were subjected to 10-min hypothermic insult before the extraction of the heart. The intervals between the end of insult and the extraction were 1, 4, 8, 24 or 72 hours. According to the interval differences, experimental animals were divided into "1 HR", "4 HR", "8 HR", "24 HR" and "72 HR" groups. These experimental protocols are schematically illustrated in Fig. 1.

To examine the induction of HSP72, western blot analysis was performed with a monoclonal anti-72 KD heat shock protein antibody in extracted myocardial samples after the hypothermia/rewarming procedure.

Heat shock protein analysis (Western blot)

After the hypothermia/rewarming procedure, an entire heart was taken from each animal. The sample was minced with a razor blade and placed immediately in a tissue dounce homogenizer (Wheaton, USA) filled with 1 ml lysis buffer (5% SDS/1% 2-mercaptoethanol). Then, it was homogenized and boiled until the tissue particles were no longer visible. The samples were quickly frozen in liquid nitrogen and kept in freezer (−20°C) until analyzed.

On a later day, frozen samples were thawed and centrifuged, and precipitates were removed. The overall protein concentration was determined for each sample using a modified Lowry procedure (Lowry et al, 1951). Protein samples were diluted in Laemmli sample buffer solution (0.0625 M Tris, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue, and 10 mM EDTA, pH 6.8). An equal amount of total protein loads was placed into lanes of 12% polyacrylamide gels. Electrophoresis was then performed to separate proteins in the samples. After the proteins were transferred onto nitrocellulose paper, an equal amount of total protein loads was confirmed with Coomasie blue staining of transferred gels. Blots were incubated in phosphate-buffered saline containing 5% skim milk powder to block non-specific binding sites on the membranes. Immunoreaction was started first with a 1 : 500 dilution of monoclonal anti-72 kD heat shock protein antibody (code RPN. 1197; Amersham, Mississauga, Ont.) and then with a 1 : 1000 dilution of a peroxidase-conjugated goat anti-mouse IgG. ECL solution (code

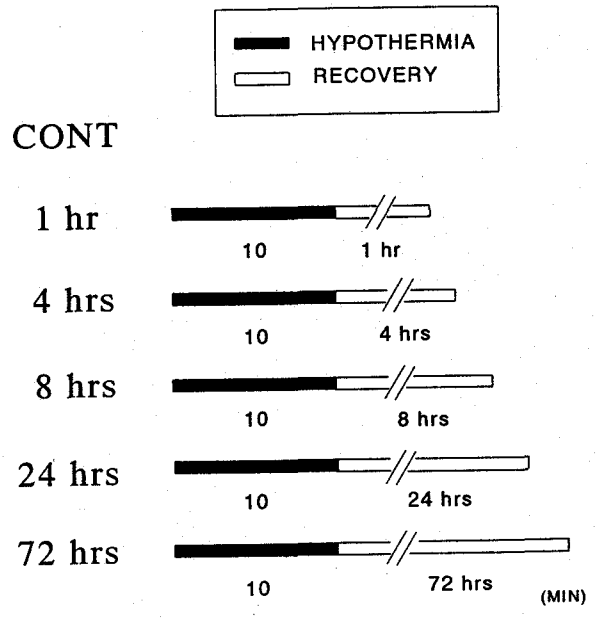

Fig. 1. Schematic diagram illustrating the experimental design to assess whether hypothemia induces HSP72. Animals in experimental group were subjected to hypothermic insult for 10 min. Following the insult, animals were allowed to recover for 1 (1 HR), 4 (4 HR), 8 (8 HR), 24 (24 HR) or 72 (72 HR) hours, respectively. Controls did not receive any insult.

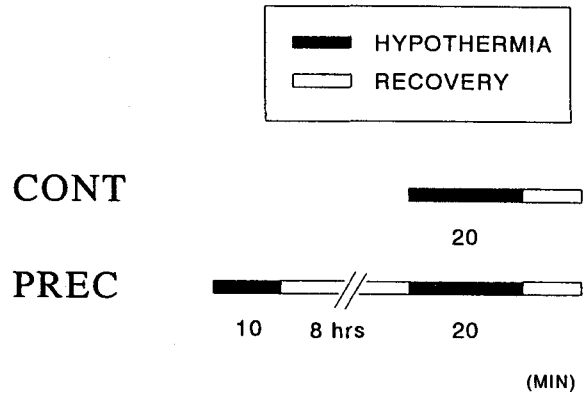

Fig. 2. Schematic diagram illustrating the experimental design for the assessment of cardioprotective effect of HSP72 induced by hypothermia. Animals in control group (CONT) were subjected to a 20-min episode of hypothermic insult. Animals in preconditioned group (PREC) were received a 10-min hypothermic insult 8 hours prior to the 20-min insult.

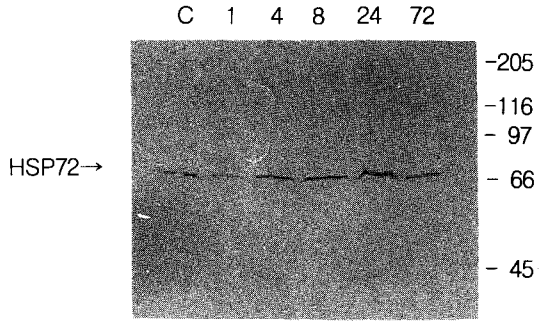

Fig. 3. Western blot analysis of HSP72 expression in the neonatal rat heart. 1, 4, 8, 24 or 72: tissues from experimental animals that were allowed to recover for 1, 4, 8, 24 or 72 hours following the 10-min hypothermic insult, respectively. C: tissues from control animals did not receive any insult. Arrow indicates HSP72 band.

RPN. 2106; Amersham, Mississauga, Ont.) was used for the visualization of the results of immunoreaction.

Assessment of cardioprotective effect of HSP72

To assess the cardioprotective effect of HSP72 against hypothermic cardiac arrest, we compared the proportion of spontaneous recovery from the arrest between the control and preconditioned (PREC) animals. Control animals (n=28) were subjected to 20-min hypothermic insult followed by the rewarming with thermoblanket. PREC group (n=26) was subjected to an episode of 10-min hypothermia (preconditioning) prior to 20-min hypothermia. The interval between preconditioning and 20-min hypothermia was 8 hours. These experimental protocols are schematically illustrated in Fig. 2.

The electrocardiogram (ECG) was recorded (25 mm/sec chart speed) from the end of 20-min hypothermia to a return to normal sinus rhythm on a physiograph (Model 79E, Grass Inst. Co., USA) via the surface Lead II.

Statistical tests

Pearson chi-square test was used for the comparison of the proportion of spontaneous recovery between the control and PREC groups. $P<0.05$ was considered significant.

RESULTS

Synthesis of myocardial heat shock protein

Fig. 3. is a typical picture of the western blotting which shows an expression of HSP72 in each experimental condition. Experimental animals were subjected to 10-min hypothermic insult before the extraction of the heart. The intervals between the end of insult and the extraction were 1 (1 HR), 4 (4 HR), 8 (8 HR), 24 (24 HR) or 72 (72 HR) hours. As illustrated in this figure, only a minimal amount of HSP72 was detected in the myocardial tissues resected from control, 1HR, and 72 HR groups. In contrast, HSP72 expression in 8 and 24 HRs groups was significant.

Assessment of cardioprotective effect of HSP72

Fig. 4. illustrates electrocardiogram findings recorded from a newborn rat heart from the end of 20-min hypothermic insult to a return to the sinus rhythm. First trace shows electrocardiogram findings of the hypothermic cardiac arrest at the end of insult. Second trace displays the findings that the atria and ventricles beat independently of one another (complete AV block) 1 min after the end of insult. Third trace shows bradycardia evoked 2 min after the end of insult. Two large waves were made by deep inspiration. Fourth trace, recorded 4 min after the end of insult, shows bradycardia faster than third trace. Large waves are also more frequent than those in third trace.

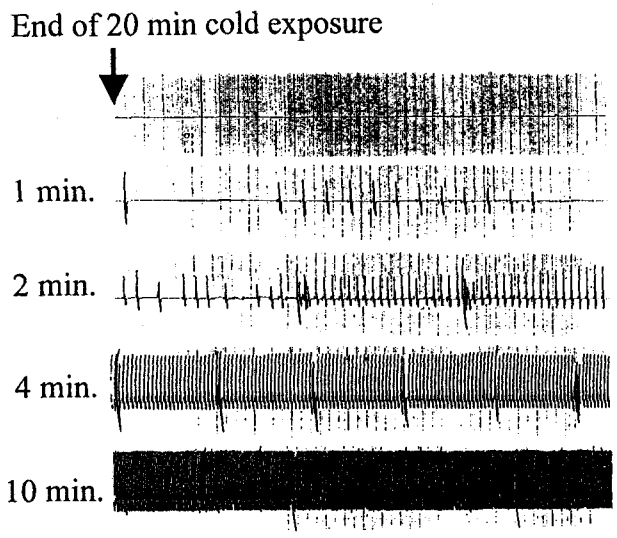

Fig. 4. Electrocardigram findings recorded from a neonatal rat heart subjected to the 20-min hypothermic insult. First trace displays electrocardiographic findings of the hypothermic cardiac arrest the end of the 20-min hypothermic insult. Second trace displays similar to third degree block 1 min after the end of insult. Third trace displays bradycardia elicited 2 min after the end of insult. Two large waves were evoked by deep inspiration. Fourth trace shows bradycardia faster than third trace. Last trace displays normal sinus rhythm 10 min after the end of insult.

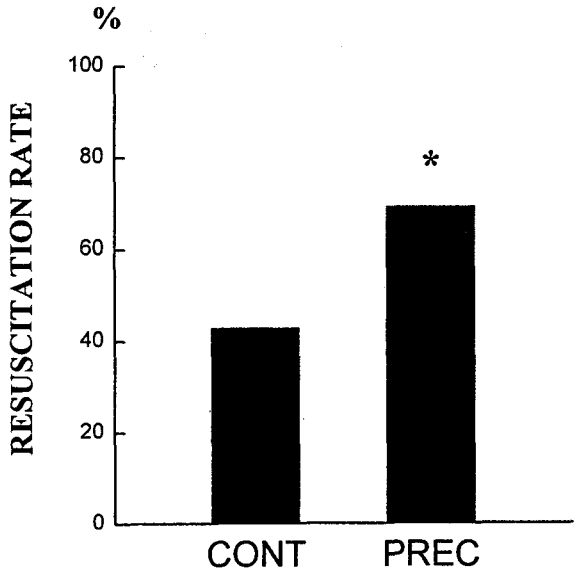

Fig. 5. Histogram for the proportion of resuscitation from cardiac arrest induced by the 20-min hypothermic insult. The proportion of resuscitation of preconditioned group (PREC) is significantly higher than that of control group. PREC; a 10-min episode of hypothermic preconditioning 8 hours before the 20-min insult, CONT; 20-min hypothermic insult without preconditioning. *, significantly different between two groups ($p<0.05$ by Pearson chi-square test).

Last trace illustrates normal sinus rhythm. All animals which survived the experimental challenge, i.e., hypothermic insult, regained normal respiration and sinus rhythm within about 10 to 20 min.

Fig. 5. illustrates the histogram for the proportion of resuscitation from cardiac arrest which was induced by the 20-min hypothermic insult. Of 26 rats in PREC group, 18 (69.2%) were resuscitated from cardiac arrest. In contrast, out of 28 control animals, 12 (42.8%) survived the arrest. The proportion of spontaneous recovery was significantly higher in PREC group than in control group ($p<0.05$, by Pearson chi-square test).

These results suggest that hypothermic preconditioning increases HSP72 expression and induces a state of tolerance to the tissue injury by subsequent hypothermic insult in a neonatal rat heart.

DISCUSSION

The purpose of present study was to determine whether hypothermic insult produces myocardial HSP72 expression and whether expressed HSP72 helps the heart to recover easily from the arrest induced by the subsequent hypothermia.

Hypothermic treatment has been widely employed in cardiac surgery to lessen detrimental effects during mandatory ischemic arrest (Kirklin et al, 1961; Kern et al, 1991). Hypothermia is known to reduce oxygen demand throughout the arrest period and thus render normal structure and function to return after restoration of blood flow (Ferrari et al, 1990). Although many experimental and clinical reports concerning the profound hypothermia and total circulatory arrest have been introduced (Belsey et al, 1968; Bove et al, 1987; Bellinger et al, 1991), to our knowledge, the underlying mechanisms of hypothermic treatment still remain largely as a matter of speculation.

Various stressful stimuli to the heart, including hyperthermia (Currie & Whith, 1983), hypoxia (Howard & Geoghegan, 1986) and ischemia, (Currie, 1987) raise the transcription of several genes and induce preferential translation of these mRNAs into myocar-

dial HSPs (Kukreja et al, 1994), which give the protective effect against subsequent damage. Hypothermic cardiac arrest gives inevitable ischemia/reperfusion injury to the heart, so it also probably produces HSPs. However, Shaver et al (1995) reported that, comparable to ischemia at 23°C, deep hypothermia (15°C) reduced the ischemic-induction of HSP72 mRNA in piglet brain. Moreover, Lanks (1990) demonstrated that hypothermia decreased the magnitude of arsenite-induced HSP72 expression with a sharp threshold between 30°C and 33°C, suggesting that HSP72 expression is regulated by a temperature-dependent mechanism. In the present study, we could not confirm the inhibitory effect of hypothermia on HSP72 expression due to the methodological difficulties in the induction of the arrest under normothermia in neonates. However, this study clearly showed that the hypothermic cardiac arrest expressed myocardial HSP72 8 and 24 HRs after the hypothermia, whereas only a small amount of HSP72 was detected 1 HR and 72 HRs after the hypothermia.

At present, we have no clear explanation for the mechanism of HSP72 expression by the hypothermic cardiac arrest, but several possibilities exist. First, it has been known that stress-induced protein denaturation leads to increase in HSP72 (Anathan et al, 1986). Furthermore, the microinjection of denatured proteins has triggered the activation of heat shock transcription factor (Kozutsumi et al, 1988). It is thus possible that increases in HSP72 expression may have been due to the denaturation of proteins induced by hypothermic cardiac arrest. Second, perfused or reperfusion-induced oxygen radicals may trigger decrease in intracellular ATP content (Abd-Elfattah et al, 1990; Ytrehus et al, 1986), which contributes to the induction of HSP72 (Beckmann et al, 1992). In this study, it is postulated that denatured proteins and/or decrease in ATP content under hypothermic cardiac arrest trigger(s) the induction of HSP72.

To date, although there is no direct evidence for a causal relationship between the HSPs expression and cardioprotective effect, some interesting implications have been proposed that HSPs play a pivotal role in resistance to stress (Pelham, 1986). For example, free radicals generated during brief periods of ischemia, i.e., ischemic preconditioning, may actually contribute to the induction of HSP72. This implication is based on the results that superoxide dismutase reduces ischemia/reperfusion-induced increase in HSP72 mRNA (Kukreja et al, 1994). In addition, Marber et al (1993) reported that HSP72 elevation, induced by four 5-minute episodes of coronary ligation or whole-body temperature elevation to 42°C for 15 minutes, was partially responsible for the myocardial protection, i.e., the reduction of infarct size after the 30-minute ligation and 120-minutes of reperfusion. In addition, the administration of noradrenaline 24 hrs prior to ischemia has been shown to result in the alleviation of reperfusion-induced arrhythmias, enhancement of recovery of contractile function during ischemia/reperfusion in rat isolated hearts (Meng et al, 1993) and marked increases in c-fos, c-jun and hsp gene expression (Meng et al, 1993). In the present study, the PREC animals were recovered easier from the hypothermic cardiac arrest than controls. These results suggest that myocardial HSP72 induced by the hypothermic preconditioning confers the cardioprotective effects on neonatal rat heart. Although speculative, it is possible that free radicals or denatured proteins, generated by mandatory ischemia/reperfusion injury during hypothermic cardiac arrest, lead to myocardial HSP72 expression which could contribute to the recovery from the arrest. These results are not in line with those obtained from Wistar rat studies (Chopp et al, 1992), showing that less damaged neurons and less HSP72 were detected in transient forebrain ischemia under hypothermia than in the ischemia under normothermia. They suggested that hypothermic protection against ischemia/reperfusion injury was not correlated with HSPs expression. The inconsistency between these two results may be due to the difference in species and/or organ. Alternatively, another mechanism for protective effects of hypothermia may exist in the study performed with Wistar rat forebrain. In conclusion, cardioprotective effect of hypothermic preconditioning may be related to an increase in HSP72 expression.

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