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Effect of Heat Shock Protein 72 on the Generation of Reperfusion Arrhythmias

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The causal relationship between heat shock protein (HSP) and second window of cardioprotective effect is still undetermined. In the present study, we assessed whether HSP-producing substances, amphetamine and ketamine, afforded protection against reperfusion-induced ventricular fibrillation (VF) and these protective effect remained after the inhibition of HSP72 production by quercetin, a mitochondrial ATPase inhibitor. Adult mongreal male cats (n=60, 2.5~4 kg) were used in this study. Experimental animals were divided into five groups; control group (n=15), amphetamine ('A', n=11) group, ketamine ('K', n=9) group, amphetamine-ketamine ('AK', n=16) group and amphetamine-ketamine-quercetin ('AKQ', n=9) group. Twenty-four hours after the drug treatment, an episode of 20-min coronary artery occlusion was followed by 10-min reperfusion. The incidence of reperfusion-induced VF in the AK and AKQ groups was significantly lower than that in control group ($p<0.01$). After the ischemia/reperfusion procedure, western blot analysis of HSP72 expression in the myocardial tissues resected from each group was performed. HSP72 production in the AK group was marked, whereas HSP72 was not detected in the AKQ and control groups. These results suggest that the suppressive effect against reperfusion-induced VF induced by amphetamine and ketamine is not mediated by myocardial HSP72 production but by other mechanisms.

Key Words: Reperfusion arrhythmias, Reperfusion injury, Reperfusion-induced ventricular fibrillation, Heat shock protein 72, Amphetamine, Ketamine, Quercetin

INTRODUCTION

Reperfusion following ischemia adds further complexity to the ischemia-induced pathological changes (Jennings & Yellon, 1992). This spectrum of events includes reperfusion arrhythmias (Tennant & Wiggers, 1935; Goldberg et al, 1983; Tzivoni et al, 1983; Manning & Hearse, 1984; Hearse & Tosaki, 1987; Shiki & Hearse, 1987), myocardial stunning (Heyndrickx et al, 1975; Weiner et al, 1976; Kloner et al, 1983; Bolli et al, 1988) and accelerated necrosis (Hearse et al, 1978), which are collectively called reperfusion injury.

Since Murry et al (1986) first demonstrated that ischemic preconditioning of the myocardium provided the protection against reperfusion injury, large body of studies involving several types of preconditioning including ischemia (Shiki & Hearse, 1987; Li et al, 1992), hypoxia (Neely & Grotyohann, 1984), heat stress (Liu et al, 1992), and drugs such as amphetamine (Maulik et al, 1995) have been reported. Recently, preconditioning-induced protection was divided into two aspects according to the time course; first, classical preconditioning effect can be observed immediately but disappears rapidly: second, delayed effect, i.e., a second window of protection, appeared many hours after preconditioning (Yellon & Baxter, 1995). The underlying mechanisms leading to the second window of protection are known to be associated with multifactorial stress response. Especially, several lines of evidence suggested that stress proteins play a major role in the protection (Yellon & Latchman, 1992; Currie et al, 1993; Marber et al, 1993). For example, Das and his colleagues (Maulik et al, 1994) provided evidence that the improved postische-

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mic ventricular functional recovery by amphetamine was linked to its ability to induce heat shock protein (HSP). However, Thorton et al (1990) suggested that HSP72 produced by ischemic preconditioning was not likely to account for the protection against ischemia/reperfusion injury.

In the present study, using a cat model of regional cardiac ischemia, we assessed whether HSP afforded protection against ventricular fibrillation (VF) occurring during reperfusion after a 20-min coronary artery occlusion. If so, does this anti-arrhythmic effect remain after the inhibition of HSP72 production by quercetin, a mitochondrial ATPase inhibitor?

METHODS

Animal preparation

Male adult mongrel cats (n=60, 2.5~5 kg) were used in the present study. After inducing anesthesia by intramuscular injection of α-chloralose (60 mg/kg), a polyethylene catheter was inserted into the antecubital vein for administration of muscle relaxant (see below) or extra α-chloralose to maintain an appropriate level of anesthesia during the experiment. The trachea was cannulated for artificial respiration by a volume cycled respirator (Harvard, USA). Positive pressure respiration with room air was started immediately after muscle relaxant injection (pancuronium bromide, 1~2 mg). The stroke volume was ~10 ml/kg, and the rate was ~15~20 strokes/min. At this stroke volume and rate, the end-tidal pCO_2 was 29~36 mmHg. The electrocardiogram (ECG) was recorded (25 mm/sec chart speed) on a physiograph (Model 79E, Grass Inst. Co., USA) via the surface Lead II. Throughout the experiment, the core body temperature was maintained at $37\pm0.5^{\circ}C$ with the use of servo-controlled heating pad. Also, muscle relaxation was maintained by administering extra pancuronium bromide (0.5 mg/hr). The experimental procedures followed in this study were in accordance with the guidelines set by the Korea University College of Medicine Animal Research Policies Committee.

Regional ischemia/reperfusion model

All cats were subjected to the ischemia/reperfusion injury, as our previous report (Na et al, 1996). In short, the chest was opened by a left thoracotomy, which involved removal of ribs 2~4. After construction of pericardial cradle, the left anterior descending (LAD) coronary artery was isolated at a distance <1 cm from the main trunk and a 6~0 silk thread was coiled (2 turns) loosely around the isolated artery. During the isolation procedure, care was taken to avoid damaging the pericoronary nerve coursing parallel to the LAD coronary artery. For the induction of regional ischemia, both ends of the silk thread were lifted and clamped to the skin with the use of hemostats. Reperfusion was induced by quickly removing the thread with scissors.

Experimental protocols

Five groups of cats were studied. Control group (n=15) was subjected to an episode of 20-min LAD coronary artery occlusion followed by 10-min reperfusion. 'A' group (n=11) was identical to control group except that amphetamine sulphate (10 mg/kg, i.p.) was injected 24 hours before LAD coronary artery occlusion. 'K' group (n=9) was identical to control group except that ketamine (60 mg/kg, i.m.) was injected between 21~24 hours before the LAD coronary artery occlusion. 'AK' group (n=16) was identical to the K group except that amphetamine sulphate (10 mg/kg, i.p.) was injected 24 hours before LAD coronary artery occlusion. 'AKQ' group (n=9) was identical to the AK group except that quercetin (3 mg/kg, i.v.) was injected 24 hours before LAD coronary artery occlusion. These experimental protocols are schematically illustrated in Fig. 1.

Classification and analysis of arrhythmias

The ventricular arrhythmias were classified into 3 types according to Lambeth convention (Riva et al, 1988): 1) discrete premature QRS complexes were defined as ventricular premature beats (VPB), 2) a run of four or more consecutive ventricular premature beats as ventricular tachycardia (VT) and 3) an electrical signal for which individual QRS deflections could not be identified and therefore the heart rate could not be determined as ventricular fibrillation (VF). We did not try to resuscitate the animals showing VF. When VF lasted for more than 5 minutes, the animal was considered dead, and the experiment was ended.

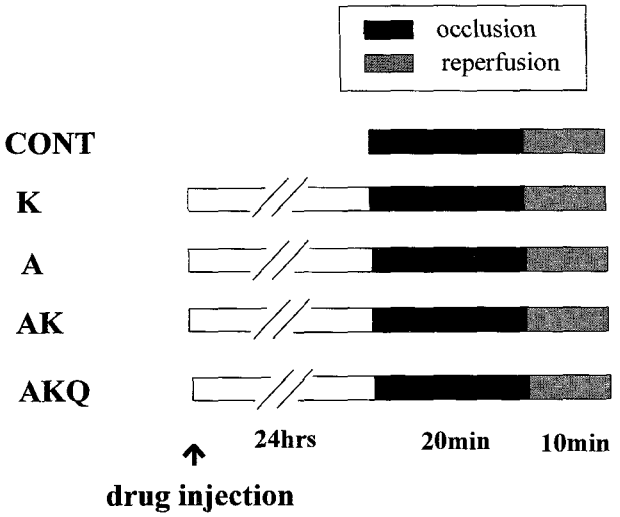

Fig. 1. Schematic diagram illustrating the experimental design. Animals in control group (CONT) were subjected to a 20-min episode of occlusion of the left anterior descending coronary artery followed by 10-min reperfusion. Animals in experimental group were subjected to the same ischemia/reperfusion procedure as control group, except that drugs were injected before ischemic reperfusion procedure (arrow). A group (n=11) was subjected to the injection of amphetamine sulphate (10 mg/kg, i.p.). K group (n=9) was subjected to the injection of ketamine (60 mg/kg, i.m.). AK group (n=16) was subjected to the combined injection of amphetamine sulphate (10 mg/kg, i.p.) and ketamine (60 mg/kg, i.m.). AKQ group (n=9) was subjected to the combined injection of amphetamine sulphate (10 mg/kg, i.p.), ketamine (60 mg/kg, i.m.) and quercetin (3 mg/kg, i.p.). Amphetamine sulphate and quercetin were injected 24 hours before the coronary occlusion, while ketamine was injected between 21~24 hours before the coronary occlusion.

Heat Shock protein analysis (Western blotting)

After the ischemia/reperfusion procedure, a small sample of myocardial tissue (200~300 mg) was taken from the ischemic zone of 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 particles were no longer visible. After being strained through a 27-gauge needle, the sample solution was 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 into Laemmli sample buffer solution (0.0625 M Tris, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue, 10 mM EGTA, pH 6.8). Equal total protein loads of 45 mg were placed into lanes of 12.5% polyacrylamide gels. Electrophoresis was then performed to separate proteins in the sample on the resolving gel. After the proteins were transferred onto nitrocellulose paper, equal total protein loads were confirmed with Coomasie blue staining of untransferred gels. Blots were incubated on 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 HSP antibody (code RPN. 1197; Amersham, Mississauga, Ont.) and then a 1 : 500 dilution of a peroxidase-conjugated goat anti-mouse IgG. ECL solution (code RPN. 2106; Amersham, Mississauga, Ont.) was used for visualization of the results of the immunoreaction.

Statistical tests

Pearson chi-square test was used for comparison of data obtained from different experimental groups with those from control group. $P<0.05$ was considered significant.

RESULTS

Arrhythmias occurring during reperfusion phase

Control group

Out of 15 control cats which were subjected to the 20-min ischemia without any pretreatment, 11 (73.3%) exhibited VF during the subsequent reperfusion phase, and 12 (80%) and 14 (93.3%) exhibited VT and VPB, respectively. These arrhythmias emerged within a few to several tens of seconds of reperfusion (Fig. 2).

A group

Out of 11 cats which were received amphetamine sulphate, 4 (36.3%), 11 (100%) and 11 (100%) exhibited VF, VT and VPB during the subsequent reperfusion phase, respectively. The proportion of cats that exhibited VF in the A group was smaller than that in control group, but it was not significantly

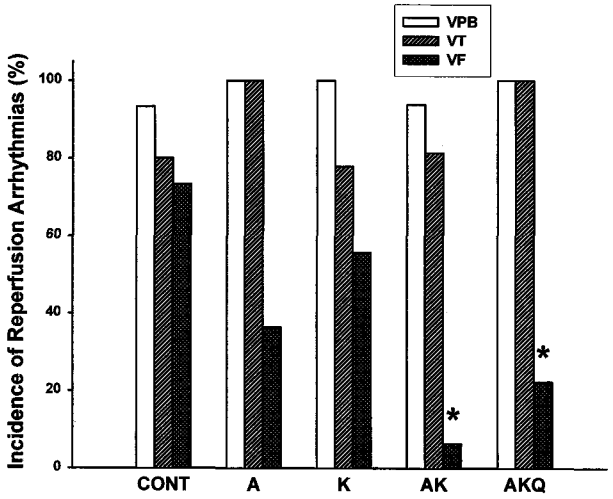

Fig. 2. Incidence of different types of reperfusion-induced ventricular premature beats (VPB), ventricular tachycardia (VT) or ventricular fibrillation (VF) in each experimental condition. Asterisks indicate significant differences between scores labeled with these symbols and their counterparts in control group ($p<0.05$ by the Pearson chi-square test). Abbreviations are the same as in Fig. 1.

different (Fig. 2).

K group

Of 9 cats, 5 (55.6%), 7 (77.8%) and 9 (100%) exhibited VF, VT and VPB during the subsequent reperfusion phase, respectively. The incidence of VF in the K group was smaller than that in control group, but it was not significantly different (Fig. 2).

AK group

Out of 16 cats which were received amphetamine along with ketamine, 1 (6.3%), 13 (81.3%) and 5 (93.8%) exhibited VF, VT and VPB within a few to several tens of seconds of reperfusion, respectively. The proportion of cats that showed VF in the AK group was significantly smaller than that in control group ($p<0.05$, Fig. 2).

AKQ group

Out of 9 cats which were received amphetamine along with ketamine and quercetin, 2 (22.2%), 9 (100%) and 9 (100%) exhibited VF, VT and VPB within a few to several tens of seconds of reperfusion, respectively. The incidence of VF in the AKQ group was significantly smaller than that in control group ($p<0.05$, Fig. 2).

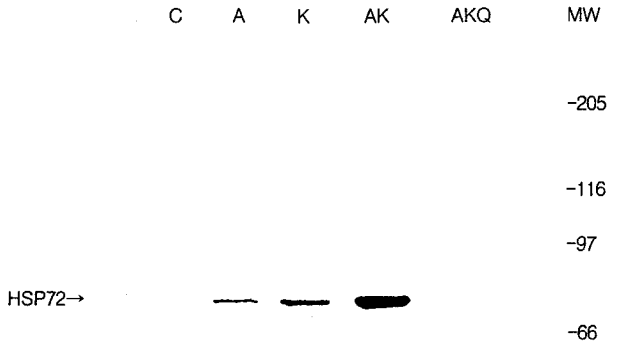

Fig. 3. Western blot analysis of HSP72 expression in the myocardial tissues that were previously subjected to 20-min ischemia/10-min reperfusion with or without pretreatment. HSP72 was not detected in control and AKQ groups and only a small amount of HSP72 was detected in A and K groups. In contrast, HSP72 expression in AK group was marked. Arrow indicates HSP72 band. MW: molecular weight. Abbreviations are the same as in Fig. 1.

Synthesis of myocardial HSP

Fig. 3 is a typical picture of the western blotting, which shows the expression of HSP72 in each experimental condition. As illustrated in this figure, HSP72 was not detected in the myocardial tissues taken from the AKQ and control groups and only a small amount of HSP72 was detected in tissues taken from the A and K groups. In contrast, HSP72 expression in the AK group was marked.

DISCUSSION

Recent studies have suggested that stress proteins (e.g., HSP) provide cardioprotection against myocardial reperfusion injury. Maulik et al (1994, 1995) showed that pretreatment of amphetamine, an agent known to increase body temperature by enhancing lipolysis, improved postischemic ventricular recovery after cardiopulmonary bypass and that this amphetamine effect was linked to its ability to induce heat shock. Also, Steare & Yellon (1993) demonstrated that preconditioning by heat stress provided cardioprotection against reperfusion arrhythmias.

In the present study, we tested the hypothesis that the expression of HSP in the myocardial tissue is a critical part of the mechanism of cardioprotection afforded by preconditioning. In this study, it was noted that amphetamine and ketamine, when applied alone, induced the expression of HSP only slightly

and were not effective in preventing the reperfusion-induced arrhythmias. Since the lack of cardioprotective effect of these drugs might be due to the insufficient HSP expression, we took the strategy of administering the drugs together. To our expectation, the co-administration of amphetamine and ketamine led to a marked expression of HSP in the myocardial tissues and reduced significantly the incidence of reperfusion-induced ventricular fibrillation. These results are in line with those of previous studies. Steare & Yellon (1993) reported that the protection against reperfusion arrhythmias induced by heat stress was associated with expression of the inducible HSP70. In addition, Maulik et al (1994, 1995) suggested that amphtamine improved postischemic ventricular recovery might be linked with its ability to induce heat shock.

The results obtained with quercetin, however, indicate that HSP expression may not be a key part of the mechanism underlying the cardioprotection offered by the drug-preconditioning. Because, quercetin did not significantly alter the anti-fibrillation effect of co-administered amphetamine and ketamine, although it completely blocked the expression of HSP.

The notion that HSP has no causal relationship with preconditioning-afforded cardioprotection is not new. Thorton et al (1990) already showed that neither cycloheximide nor actinomycin D blocked the cardioprotection against myocardial stunning offered by preconditioning. However, their effects on the protection afforded by preconditioning against arrhythmias were not examined. In this respect, the present study provides for the first time the evidence that HSP expression is not related to the anti-fibrillation effect of preconditioning.

The results of the present study do not exclude the possibility that HSP has a cardioprotective effect against other forms of reperfusion injury than ventricular fibrillation, such as myocardial infarction (Hutter et al, 1994) and stunning (Marber et al, 1994), since these types of reperfusion injury are produced by mechanism(s) potentially different from those underlying ventricular fibrillation. Whether this is true or not remains to be elucidated.

CONCLUSION

Employing a cat model of regional cadiac ischmia, we demonstrated that drug-preconditioning (i.e., combined administration of amphetamine and ketamine 24 hours prior to ischemia-reperfusion insult) afforded cardioprotection against reperfusion-induced ventricular fibrillation. In addition, we showed that quercetin did not significantly alter the anti-fibrillation effect of the drug-preconditioning despite its complete blockade of the myocardial HSP expression. From these results, we concluded that HSP might not be critical in the preconditioning-afforded cardioprotection against reperfusion-induced ventricular fibrillation.

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