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Abstract

The cardioprotective effect of calmodulin antagonists, trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) was examined on the isolated rat heart exposed to hypothermic and ischemic conditions by measuring distribution of lysosomal enzymes in myocardial cells, and leakage of creatine kinase (CK) during reperfusion and postischemic recovery in myocardial systolic function. Experimental hearts were infused with 20 degrees C Krebs-Henseleit bicarbonate buffer (KHB) or KHB containing TFP or W-7 for 2min every 30min during hypothermic ischemia. After ischemia for 120min at 20 degrees C, rat hearts were reperfused at 37 degrees C for 30min. TFP and W-7 improved functional recovery and prevented CK release. In TFP treated hearts, leakage of lysosomal enzymes was reduced significantly, whereas stabilization of lysosomes by W-7 did not occur. These results suggest that calcium-calmodulin dependent enzymes may play an important role in the development of cellular damage of the myocardium during hypothermic ischemia, although levels of leakage of lysosomal enzymes may be unreliable predictors of functional recovery after hypothermic ischemia.

KEYWORDS: trifluoperazine, W-7, lysosomal enzyme, ischemic heart

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Protective Effects of Calmodulin Antagonists (Trifluoperazine and W-7) on Hypothermic Ischemic Rat Hearts

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The cardioprotective effect of calmodulin antagonists, trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) was examined on the isolated rat heart exposed to hypothermic and ischemic conditions by measuring distribution of lysosomal enzymes in myocardial cells, and leakage of creatine kinase (CK) during reperfusion and postischemic recovery in myocardial systolic function. Experimental hearts were infused with 20°C Krebs-Henseleit bicarbonate buffer (KHB) or KHB containing TFP or W-7 for 2 min every 30 min during hypothermic ischemia. After ischemia for 120 min at 20°C, rat hearts were reperfused at 37°C for 30 min. TFP and W-7 improved functional recovery and prevented CK release. In TFP treated hearts, leakage of lysosomal enzymes was reduced significantly, whereas stabilization of lysosomes by W-7 did not occur. These results suggest that calcium-calmodulin dependent enzymes may play an important role in the development of cellular damage of the myocardium during hypothermic ischemia, although levels of leakage of lysosomal enzymes may be unreliable predictors of functional recovery after hypothermic ischemia.

Key words : **trifluoperazine, W-7, lysosomal enzyme, ischemic heart**

Calmodulin is recognized as a multifunctional calcium-dependent regulatory protein and involved in the calcium-mediated enzymes. It may play an important role in the development of myocardial injury caused by ischemia/reperfusion (1, 2). Several studies have shown that some calmodulin antagonists improve metabolic and mechanical recovery of ischemic heart (3, 4).

Cold chemical cardioplegia has significantly improved myocardial protection during cardiac operation. However, the effectiveness of some cardioprotective agents were found to be temperature-dependent in an isolated rat heart model (5, 6).

Our present study was designed to test the myocardial preservative effects of trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W-7), calmodulin antagonists. Special attention was paid to the cardioprotective effects of these drugs under hypothermic and ischemic conditions.

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Materials and Methods

Experimental preparation. Male Wistar rats weighing 300 to 400 g were anesthetized with diethyl ether and given 200 units of heparin sodium intravenously via the femoral vein. The hearts were removed immediately, placed in iced heparinized saline, and then mounted on a perfusion apparatus with an aortic perfusion pressure of 100 cm H_2O. The perfusate consisted of modified Krebs-Henseleit buffer (KHB), aerated with 95% oxygen and 5% carbon dioxide. Buffer constituent concentrations (mM/L) were as follows: NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, glucose 11. The pulmonary artery was incised to ensure free drainage of coronary venous effluent. A water-filled, balloon-tipped catheter connected to a pressure transducer was inserted via the left atrium and placed in the left ventricle.

Experimental protocol. The heart was aerobically perfused at 37°C for 20 min, during which period control values for developed pressure, and heart rate were recorded. After the 20-min control period, the aortic line was clamped and global ischemia maintained at 20°C for 120 min. At the beginning of the ischemic period, buffer was then infused for 3 min into the coronary vasculature through a side arm of the aortic cannula at a pressure of 75 cm H_2O. In addition, the hearts received multiple 2-min infusions of buffer at intervals of 30 min during the ischemic period. After hypothermic ischemia, hearts were reperfused with KHB at 37°C for 30 min (Fig. 1). Experimental hearts (n = 6 per group) were grouped as follows: Group I, infusion of KHB for 2 min every 30 min during hypothermic ischemia; Group II, infusion of KHB containing TFP (2.5×10^{-6} M/L); Group III, infusion of KHB containing W-7 (20×10^{-6} M/L). To determine functional recovery and levels of CK release, 6 hearts in each group were reperfused at 37°C after hypothermic ischemia. To assay the activities of lysosomal enzymes at the end of ischemia. The experiments were terminated, and 6 hearts in each group were immersed in a 0.9% saline solution at 4°C before reperfusion.

Hemodynamic measurements. Preischemic control values of the hemodynamic variables were measured after 20 min of perfusion. The intraventricular balloon was inflated to an end-diastolic pressure (EDP) of 10 mmHg and left ventricular developed pressure (DP) was calculated as the difference between left ventricular peak and end-diastolic pressure. Heart rate (HR) was obtained from the left ventricular pressure recording. At 15 and 30 min of reperfusion, the volume in the balloon was adjusted to produce an EDP of 10 mmHg and DP was measured. Recovery of systolic function was expressed as percentage of preischemic value.

Subcellular distribution of lysosomal enzymes. At the end of the preischemic and ischemic periods, the activities of lysosomal enzymes (N-acetyl-β-glucosaminidase, NAG and Cathepsin D) were assayed. According to the modified method of Ichihara *et al.* (7), and Ricutti (8), the myocardium was immersed in the extraction medium: sucrose 0.25 M/L, EDTA 0.1 mM/L, tris-HCl 10 mM/L, pH 7.4. The tissue was finely minced and homogenized with a Teflon pestle tissue grinder. The homogenates were centrifuged at 450 × g for 10 min at 4°C. The supernatant was centrifuged at 30,000 × g for 40 min at 4°C. The activity present in the 30,000 × g supernatant was considered non-sedimentable activity. The pellet, (the lysosome-containing fraction) was resuspended in the extraction medium containing 0.1% Triton X-100. The activity present in the lysosome containing fraction was considered sedimentable activity. The integrity of the lysosmal membrane was estimated by lysosomal enzyme distribution (LED)(7), where LED =

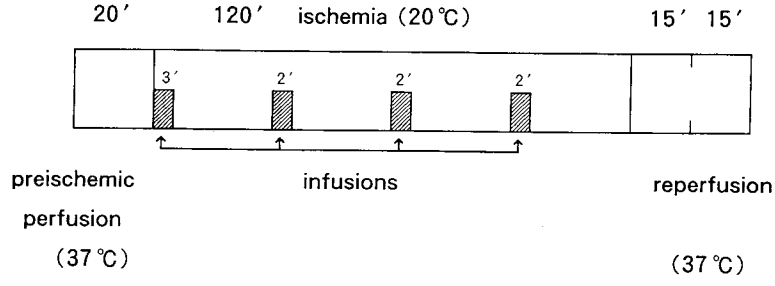

Fig. 1 Experimental protocol.

non-sedimentable enzyme activity × 100/(non-sedimentable + sedimentable) enzyme activity (%). NAG and Cathepsin D levels were determined using the method of Noto *et al.* (9) and Barrett *et al.* (10).

CK leakage. During the 15 min reperfusion period, coronary effluent was collected from each heart and its CK activity was assayed by the method of Rosalki (11). At the end of each experiment, hearts were heated to 110°C for 24 h for the determination of dry weight. Results were expressed as international units per g of dry weight.

Statistical analysis. All of measured values were expressed as means ± standard error of the mean. Differences between groups were evaluated by ANOVA. If a significant differfnce was detected by ANOVA, this was followed by a multiple-comparison (Fisher's PLSD test) to determine which individual comparisons were significant ($p < 0.05$).

Materials. W-7 was purchased from the Sigma Chemical Company, St Louis, MO, USA. TFP was kindly provided by Yoshitomi Pharmaceutical Corporation, Osaka, Japan.

Results

Recovery of systolic function after 120 min of hypothermic global ischemia is displayed in Table 1. The recovery of DP was significantly better in Groups II and III than in Group I ($p < 0.05$ for Group I *vs.* Group II ; $p < 0.05$ for Group I *vs.* Group III). These findings indicate that TFP and W-7 improved functional recovery significantly. No difference in recovery of DP was observed between hearts treated with TFP and those receiving W-7. There was no difference in HR among the three experimental groups.

The mean postischemic CK leakage for Groups I, II and III was as follows: 65.3 ± 9.7, 32.5 ± 5.2, and 20.0 ± 4.6 IU/15min/g dry weight, respectively (Fig. 2). CK release was significantly lower in Groups II and III than in

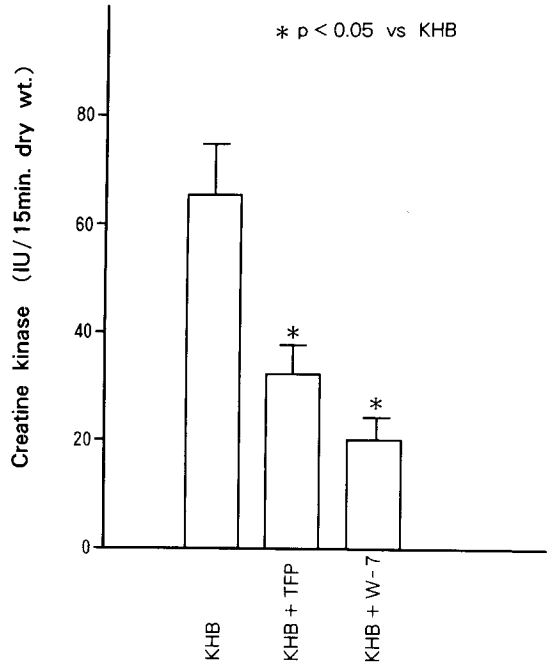

Fig. 2 Effects of TFP and W-7 on CK release. Vertical bars indicate SEM.

Table 1 Recovery of systolic function after 120 min of global hypothermic ischemia.

Group	n	Preischemic value		15-Min reperfusion		30-Min reperfusion	
		DP (mmHg)	HR (beats/min)	DP (%)	HR (%)	DP (%)	HR (%)
I. (KHB)	6	106 ± 4	223 ± 7	30.2 ± 4.9	95.0 ± 4.0	41.7 ± 5.8	92.7 ± 2.4
II. (KHB + TFP)	6	104 ± 6	228 ± 6	66.3 ± 5.6*	97.7 ± 0.8	78.2 ± 2.3*	96.8 ± 2.5
III. (KHB + W-7)	6	103 ± 6	232 ± 8	69.2 ± 6.4*	94.7 ± 7.1	75.0 ± 4.4*	93.2 ± 5.6

* $p < 0.05$ compared with Group I

Measurements were made at an end-diastolic pressure of 10 mmHg and are expressed as percent of preischemic control.
Legend: Data are means ± standard error of the mean. DP, Developed pressure. HR, Heart rate. KHB, Krebs-Henseleit bicarbanate buffer. TFP, Trifluoperazine.

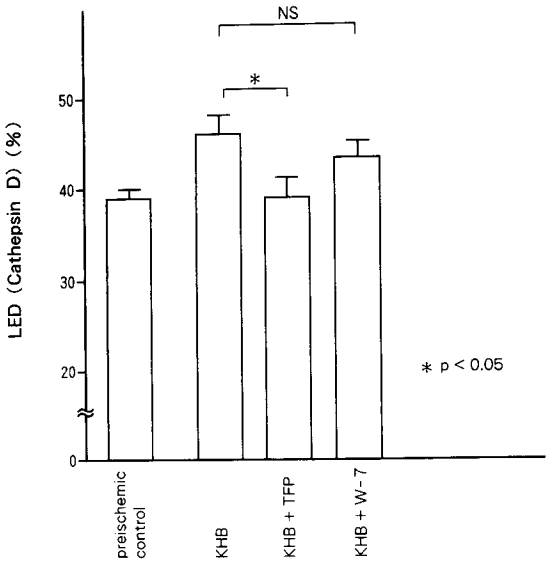

Fig. 3 Effects of TFP and W-7 on lysosomal enzyme (N-acetyl-β-glucosaminidase) distribution.

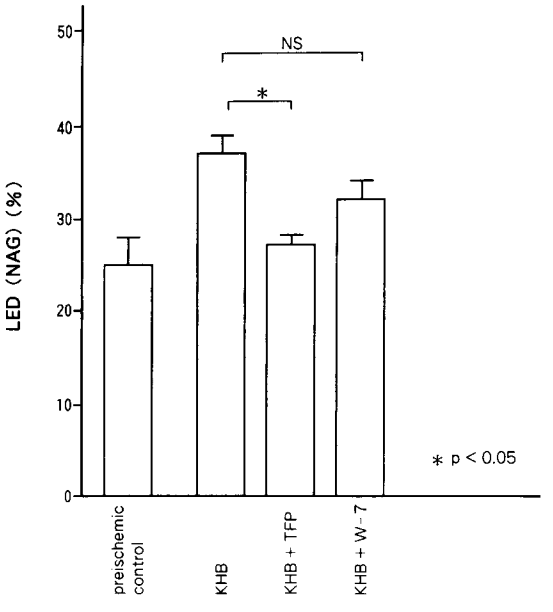

Fig. 4 Effects of TFP and W-7 on lysosomal enzyme (Cathepsin D) distribution.

Group I ($p < 0.05$ for Group I *vs.* Group II; $p < 0.05$ for Group I *vs.* Group III).

LED of NAG for Groups I, II, and III was as follows: 37.2 ± 2.1, 27.1 ± 1.1, and 32.3 ± 1.3%, respectively. LED of Cathepsin D for Groups I, II, and III was as follows: 46.2 ± 2.2, 39.1 ± 1.9, and 43.4 ± 2.3%, respectively (Figs. 3, 4). TFP prevented a significant rise in LED of NAG and Cathepsin D, which was observed in the untreated hearts ($p < 0.05$). LED of NAG and Cathepsin D in hearts receiving W-7 was lower than that in untreated hearts, but the difference was not statistically significant.

Discussion

Calmodulin has been reported to be involved in several calcium-activated processes in the myocardial cell including deteriorating ones (1). Several studies have shown that calmodulin antagonists improve metabolic and mechanical recovery of ischemic hearts under conditions of normothermic ischemic arrest (12–14). Although some cardioprotective agents failed to afford tissue protection under hypothermic conditions, present study showed that TFP and W-7 dramatically improved myocardial recovery even after hypothermic ischemia, and reduced CK release during reperfusion. These effects of TFP and W-7 against myocardial injury induced by ischemia and reperfusion suggest that calcium-calmodulin dependent enzymes may play an important role in the development of cellular damage of the myocardium during hypothermic ischemia.

The exact mechanism by which calcium-calmodulin dependent intracellular, metabolic processes produce myocardial injury is still unclear. Sodium-calcium exchange may be one of the major routes by which calcium overloading occurs during postischemic reperfusion (15). Preservation of myocardial membranes and prevention of influx of excessive amounts of calcium during ischemia and reperfusion should increase tissue viability. Otani *et al.* speculate that calmodulin antagonists inhibit the activation of

sodium-calcium exchange through inhibition of calcium-calmodulin dependent protein kinase, and thereby prevent intracellular calcium accumulation (16, 17).

Degenerative changes of membrane phospholipids induced by ischemia have been considered to be caused by activation of membrane-bound phospholipase (18). Several studies suggest that calmodulin antagonists may maintain membrane integrity by inhibiting the calcium-calmodulin dependent phospholipase A_2 (19, 20).

A definitive mechanism for the protective effects of these drugs cannot be established from the present study due to the non-specific nature of the effects of these calmodulin antagonists. Also, it would be useful to include investigations of the role of protein kinase C, because TFP and W-7 also inhibit protein kinase C (21).

Disruption of lysosomal membranes is one of the earliest changes in ischemic hearts, and leakage of lysosomal enzymes into the cytoplasm plays a role in the development of irreversible injury since the lysosomal system contains acid hydrolases capable of degrading cellular constituents (22, 23). Several investigators have suggested that stabilizing the lysosomal membrane may lead to maintenance of myocardial function during ischemic conditions (24, 25). Marone *et al.* (26) reported that TFP and W-7 inhibited lysosomal enzyme release from human polymorphonuclear leukocytes, and emphasized the possible role of calmodulin in the control of lysosomal enzyme release from these cells. However, the results of our study have demonstrated that in TFP treated hearts, leakage of lysosomal enzymes was significantly reduced, whereas stabilization of lysosomes by W-7 did not occur. This difference may be attributed to different actions of these drugs.

In conclusion, calcium-calmodulin dependent enzymes play an important role in the development of cellular damage of the myocardium during hypothermic ischemia. However, levels of lysosomal enzyme distribution may be unreliable predictors of functional recovery after hypothermic ischemia.

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