Protective Effects of Verapamil and Adenosine Treatment on High Energy Phosphate Metabolism in Ischemic and Reperfused Myocardium

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SUMMARY

Isolated rat hearts were subjected to retrograde perfusion to investigate the protective effects of adenosine and verapamil on the myocardium. In group 1, the perfusate was standard Krebs-Henseleit buffer solution. The perfusate was changed to Krebs-Henseleit buffer containing verapamil (100 nM) in group 2, adenosine (100 μ M) in group 3 and both drugs in combination in group 4 for 30 min before ischemia and during 20 min of reperfusion. Group 2 displayed a recovery of creatine phosphate but not of ATP at the end of reperfusion. In group 4, the recovery of both ATP and creatine phosphate was significantly greater than in group 1. The coronary flow of group 4 was significantly higher than that of the other groups. Treatment with both verapamil and adenosine before and after global ischemia may protect the ischemic myocardium by improving high energy phosphate metabolism and coronary circulation. (Jpn Heart J 35: 455-465, 1994)

Key words: Verapamil Adenosine Myocardial ischemia
Reperfusion ATP Creatine phosphate ^{31}P -NMR spectroscopy

CORONARY thrombolytic therapy has been a recent development for treatment of myocardial infarction.¹⁾ However, postischemic injury may occur and recanalization of the coronary artery may not always be beneficial to myocardial cells. Various agents have been attempted to protect against ischemic and reperfusion injury. Experimental and clinical studies have indicated that treatment with verapamil protects the myocardium from ischemic and reperfusion injury.^{2,3)} This protection is accompanied by preservation of high

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energy phosphate stores and maintenance of the ATP-generating capacity of the mitochondria. However, verapamil provided insufficient ATP protection. Prolonged myocardial ischemia reduces both the tissue levels of ATP and phosphocreatine and the adenine nucleotide pool.⁴⁾ The loss of adenine nucleotide is thought to disturb ATP synthesis after reperfusion. Further, the development of ³¹P-NMR spectroscopy allowed continuous monitoring of myocardial metabolism during ischemia and reperfusion in identical and beating heart.⁵⁾ In this study, we investigated the protective effects of the calcium antagonist verapamil and adenosine separately and combined in a rat model of ischemic and reperfused myocardium.

MATERIALS AND METHODS

Perfusion method: Twenty-one male Wistar-Kyoto strain rats, from eight to nine weeks old, were used in these experiments. Heparin (800 U/Kg body weight) was injected into the tail vein and the rats were anesthetized individually with ether. The hearts were removed quickly. The ascending aorta was cannulated and the hearts were perfused retrogradely under a constant pressure of 100 cm H₂O with Krebs-Henseleit buffer solution. The perfusate was maintained at 37°C and was oxygenated continuously with a gas mixture consisting of 5% CO₂ and 95% O₂.

Nuclear magnetic resonance (NMR) method: The hearts were placed in test tubes and the tubes were inserted into an NMR apparatus. Creatine phosphate and adenosine triphosphate were measured by ³¹P-NMR spectroscopy. Spectra were obtained with a JEOL GX-270 spectrometer at 6.5 Tesla. The diameter of the probe was 15 mm. Spectra were collected from transients following 45-degree pulses delivered at 1-sec intervals for 2 min. A typical ³¹P-NMR spectrum from normal rat heart is shown in Figure 1. For each spectrum, the area of all peaks was integrated and the peaks of creatine phosphate and adenosine triphosphate (β -Phosphate of ATP) were expressed as percentiles of the total phosphate peaks. The chemical shift of the inorganic peak correlated with intracellular pH. Thus the intracellular pH was calculated from the chemical shift of the inorganic phosphate peak as described by Flaherty et al.⁶⁾

Hemodynamic measurements: A latex balloon was inserted in the left ventricle via the left atrial appendage. Left ventricular pressure was measured with a fluid-filled balloon connected to a pressure transducer. Left ventricular end-diastolic pressure was set at 5 mmHg by infusing bubble-free water into the balloon. The left ventricular developed pressure was calculated as the left ventricular peak systolic pressure minus the left ventricular end-diastolic pressure. Coronary flow was measured by vacuum aspiration of perfusate overflow.

Figure 1. A typical ^{31}P -NMR spectrum from normal rat heart. This spectrum demonstrates the resonance from hexamethylphosphoric triamide, inorganic phosphate (Pi), creatine phosphate (CP) and adenosine triphosphate (ATP).

Experimental protocol: After a 10- to 20- min equilibration period, the hearts were divided into four groups. In group 1 ($n=6$), the perfusate was standard Krebs-Henseleit buffer solution. The perfusate of the other three groups was changed to Krebs-Henseleit buffer containing verapamil (100 nM) in group 2 ($n=5$), adenosine (100 μM) in group 3 ($n=5$) and both drugs in group 4 ($n=5$). Preliminary studies at our institute showed that most hearts stopped beating after perfusion with Krebs-Henseleit buffer containing 2 μM verapamil, but hearts perfused with 100 nM verapamil continued to beat. It was also found that 1 μM of adenosine was not effective for improving the recovery of ATP after reperfusion. Previous research revealed that 100 μM adenosine stimulates glycolysis in the myocardium.⁷⁾ Based on these findings, 100 nM verapamil and 100 μM adenosine were chosen as the drug concentrations used in this study. After a 30-min equilibration period, total interruption of aortic inflow was accomplished by cross-clamping the perfusion line for 20 min. Another 20 min of reperfusion followed the release of the clamp.

Statistics: Results are expressed as mean $\pm$ SEM. The Student's t-test for unpaired observation and analysis of variance was used if appropriate, taking $p < 0.05$ as the limit of significance.

RESULTS

Hemodynamic data: Table I shows the pressure rate product (heart rate $\times$ developed pressure, PRP) in all four groups during the infusion period, global ischemia and the reperfusion period. The sequential changes of developed pressure, beating rate and PRP are shown in Figure 2. After 30 min of drug administration (verapamil, adenosine and both compounds), the PRP decreased significantly. All of the hearts stopped beating after about 3 min of ischemia. The hearts resumed beating soon after reperfusion. The PRP of the group 3 hearts was significantly lower than that of group 1 throughout the reperfusion period.

Coronary flow: As shown in Figure 3, verapamil ($126.3 \pm 7.0\%$) and adenosine ($133.3 \pm 4.3\%$) significantly increased coronary flow during the pre-ischemic period. On the other hand, there were no significant differences between groups 1 and 2 during the reperfusion period. Group 4 maintained higher coronary flow values throughout the reperfusion period. In group 3, decreased coronary flow occurred during reperfusion.

Intracellular pH: Figure 4 shows the changes in intracellular pH throughout the experimental period. Twenty minutes of global ischemia induced severe myocardial acidosis. During the first few minutes of ischemia, group 4 maintained a higher pH level than the other groups. After reperfusion, intracellular pH recovered rapidly to pre-ischemic levels.

High energy phosphate measurement: Figure 5 illustrates serial 120-second spectra during normal perfusion, ischemia and reperfusion in 4 groups. As shown in Figure 6, the creatine phosphate content of group 4 was higher than that of group 1 after 2.5 min of ischemia ($17.7 \pm 0.5\%$ vs. $12.7 \pm 1.0\%$). Creatine phosphate recovered very rapidly after reperfusion. The creatine phosphate concentrations of groups 2 and 4 exhibited small overshoots above the preischemic baseline values after reperfusion. The creatine phosphate content of group 3 did not differ from that of group 1 throughout ischemia and reperfusion. In group 4, the cardiac creatine phosphate content remained higher than group 1 during

Table I. Pressure Rate Products ($\times 1000$ mmHg/min, Mean $\pm$ SEM)

	Group 1 (Control) (n = 6)	Group 2 (VER) (n = 5)	Group 3 (ADO) (n = 5)	Group 4 (ADO & VER) (n = 5)
Baseline	15.3 $\pm$ 2.0	12.6 $\pm$ 0.7	14.2 $\pm$ 0.9	14.7 $\pm$ 1.6
30 min after drug infusion	15.7 $\pm$ 2.1	7.1 $\pm$ 0.7*	9.5 $\pm$ 1.1*	6.6 $\pm$ 1.1*
Ischemia 2.5 min	2.7 $\pm$ 2.1	2.5 $\pm$ 0.5	2.4 $\pm$ 0.5	2.3 $\pm$ 0.2
Reperfusion 5.0 min	4.6 $\pm$ 1.1	4.4 $\pm$ 1.0	0.2 $\pm$ 0.0*	4.2 $\pm$ 1.0
Reperfusion 20.0 min	8.5 $\pm$ 2.6	3.3 $\pm$ 0.9	0.9 $\pm$ 0.3*	3.4 $\pm$ 0.8

ADO = adenosine; VER = verapamil. * = Difference vs. group 1, $p < 0.05$.

Figure 2. Changes in developed pressure (DP), beating rate (BR) and pressure rate product during the infusion period, global ischemia and the reperfusion period.

both the early ischemic period and the reperfusion period.

The ATP contents of the tissue were determined from the integrated β -phosphate values of the ATP peak. As shown in Table II, the ATP content of group 2 was higher than group 1 after either 5 min ($16.0 \pm 0.9\%$ vs. $12.2 \pm 0.7\%$) or 10 min ($14.9 \pm 1.0\%$ vs. $12.0 \pm 0.7\%$) of ischemia. The ATP contents were also significantly higher in group 4 than group 1 controls ($14.7 \pm 0.8\%$ for group 4 vs. $11.5 \pm 1.0\%$ for group 1 after 20 min of ischemia).

Figure 3. Changes in the coronary flow of perfused hearts treated with verapamil: group 2 (Δ), adenosine: group 3 ($\square$) and adenosine + verapamil: group 4 ($\times$). Group 1 ($\circ$) served as a control. The data are expressed as % value of baseline. The data at time 0 represent values after 30 min of drug administration. *=Significantly different from group 1.

Figure 4. Changes in the intracellular pH of perfused hearts treated with verapamil: group 2 (Δ), adenosine: Group 3 ($\square$) and adenosine + verapamil: group 4 ($\times$). Group 1 ($\circ$) served as a control. *=Significantly different from group 1.

Figure 5. Serial ^{31}P -NMR spectra during the infusion period, global ischemia and the reperfusion period in 4 groups.

Figure 6. Changes in the creatine phosphate (CP) contents of perfused hearts treated with verapamil: group 2 (Δ), adenosine: group 3 ($\square$) and adenosine + verapamil: group 4 (X). Group 1 ($\circ$) served as a control. * = Significantly different from group 1.

Table II. Myocardial Tissue Levels of ATP (% Value of Total Phosphate, Mean $\pm$ SEM)

	Group 1 (Control) (n = 6)	Group 2 (VER) (n = 5)	Group 3 (ADO) (n = 5)	Group 4 (ADO & VER) (n = 5)
30 min after drug infusion	18.4 $\pm$ 0.9	17.9 $\pm$ 0.9	19.2 $\pm$ 1.3	18.0 $\pm$ 1.1
Ischemia 5.0 min	12.2 $\pm$ 0.7	16.0 $\pm$ 0.9*	13.3 $\pm$ 0.5	14.7 $\pm$ 1.4
Ischemia 10.0 min	12.0 $\pm$ 0.7	14.9 $\pm$ 1.0*	11.0 $\pm$ 0.3	11.7 $\pm$ 0.8
Ischemia 20.0 min	8.9 $\pm$ 1.3	11.0 $\pm$ 0.5	9.6 $\pm$ 1.3	10.2 $\pm$ 2.5
Reperfusion 5.0 min	10.4 $\pm$ 1.0	12.5 $\pm$ 1.2	10.6 $\pm$ 0.9	16.6 $\pm$ 1.3*
Reperfusion 10.0 min	10.3 $\pm$ 0.9	8.4 $\pm$ 1.7	10.4 $\pm$ 1.2	12.9 $\pm$ 0.7*
Reperfusion 20.0 min	11.5 $\pm$ 1.0	13.7 $\pm$ 0.8	11.0 $\pm$ 1.0	14.7 $\pm$ 0.8*

ADO = adenosine; VER = verapamil. * = Difference vs. group 1, $p < 0.05$.

DISCUSSION

In this study, we observed different protective effects of adenosine and verapamil alone and combined in isolated perfused rat hearts subjected to global ischemia. The effects of adenosine were assessed because diffusible adenine nucleotides such as adenosine will be washed out at the onset of reperfusion.⁸⁾ There are three major pathways for the synthesis of ATP. The salvage pathway recovers deaminated breakdown products such as adenosine and inosine. The second pathway is the *de novo* synthesis of adenine nucleotide. The third pathway is also active: adenosine kinase. Mauser et al⁹⁾ reported that exogenous adenosine restored ATP levels after 3 hours of reperfusion. In addition, direct effects of adenosine have been reported. Adenosine inhibits platelet aggregation¹⁰⁾ and attenuates neutrophil activities, including intravascular accumulation,¹¹⁻¹³⁾ superoxide generation,^{14,15)} and neutrophil-mediated endothelial cell damage.¹⁶⁾ Furthermore, the stimulation of adenosine A₁ receptors leads to opening of myocardial K_{ATP} channels, as demonstrated in rat ventricular myocytes.¹⁷⁾ Thus, endogenously released adenosine, particularly during the initial brief period of coronary artery occlusion, may play a crucial role in the pathogenesis of myocardial stunning and may be an important endogenous mechanism that provides a cardioprotective effect during repetitive, brief episodes of myocardial ischemia.¹⁸⁾ However, in this study, exogenous adenosine was not effective for the recovery of ATP, possibly because the 20-min reperfusion period may have been too short to activate the salvage pathway.

The mechanisms of action of verapamil are complex and involve the antagonism of transmembrane calcium fluxes, vasodilation of coronary and peripheral blood vessels and reductions in cardiac contractility and automaticity.^{19,20)} Verapamil may have a direct effect on calcium fluxes within intracellular compartments, which would have resulted in a protective action on reversibly injured myocytes.²⁾ Nayler et al²¹⁾ and Mori et al²²⁾ reported that verapamil spared myo-

cardial ATP against ischemic and reperfusion injuries and suggested that the verapamil-induced protection was due to a direct effect of the drug and not to a decrease in heart work. In this study, verapamil reduced energy consumption during the pre-ischemic period, but not during the reperfusion period. With respect to myocardial metabolism, verapamil was beneficial for the recovery of creatine phosphate. Therefore, the protection of ATP by verapamil was observed in the early ischemic period but not in the reperfusion period. The mechanism of the protective effects of verapamil seems to include a reduction in energy consumption. Reduction of energy consumption alone may not be beneficial for the myocardium because the decrease induced by adenosine could not spare myocardial ATP during reperfusion.

To our knowledge, this is the first report concerning the protective effects of combined therapy with verapamil and adenosine. The mechanism of the protective effects of combined therapy is not clearly understood at present, but both verapamil and adenosine might cause a rapid cardiac arrest when they are used in combination. As a result, oxygen consumption in myocardial ischemia could be reduced very quickly by combination therapy, and the heart could be protected. Our experiment showed that treatment with both verapamil and adenosine provided sufficient recovery of coronary flow and myocardial levels of creatine phosphate and ATP. We could not evaluate the potential of the mechanical protective effect of this therapy because both verapamil and adenosine have primarily negative inotropic and chronotropic effects on the heart and because we did not use pacing methods.

For the evaluation of ventricular contractility, the beating rate should be maintained constant by pacing, because increasing the frequency *per se* was reported to exert a positive inotropic effect.²³⁾ Further, in order to use the drug as an agent in practical therapy, its chronotropic effects cannot be neglected. From the perspective of myocardial metabolism, coronary flow was considered to be more important for protection of the myocardium. The combination of verapamil and adenosine may protect against cell swelling and inhibit the no-reflow phenomenon, or it may increase coronary flow due to a direct effect on the coronary vascular bed. We could not determine whether the increase in coronary flow was the primary mechanism for myocardial protection. It is certain that verapamil and adenosine interacted synergistically to maintain increased coronary flow and to preserve high energy phosphates during reperfusion. This phenomenon appeared in the early stage of reperfusion. Further studies such as controlled coronary flow experiments are needed to clarify the mechanism of this protective effect.

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