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Allitridum mimics effect of ischemic preconditioning by activation of protein kinase C

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KEY WORDS allitridum; myocardial ischemia; reperfusion injury; polymyxin B; protein kinase C

ABSTRACT

AIM: To investigate whether allitridum has the effect of pharmacological preconditioning and whether protein kinase C (PKC) plays a role in myocardial protection. **METHODS**: Thirty-four isolated rabbit hearts which subjected to 30 min of regional myocardial ischemia and 2 h reperfusion, were randomly divided into 5 groups: control group, ischemic preconditioning (PC) group, allitridum (A) group, polymyxin B (Poly B) group, allitridum + polymyxin B (A + PolyB) group. Infarct size was determined by triphenyltetrazolium staining. **RESULTS**: Pharmacological preconditioning in hearts with a 5-min allitridum infusion 10 min before the prolonged regional ischemia resulted in significantly smaller infarcts (7 % ± 6 % of risk area) than in control hearts (25 % ± 7 %, $P < 0.05$). There is no significant difference in infarct size between (A + Poly B) group and control hearts (23 % ± 5 % *vs* 25 % ± 7 %, $P > 0.05$). **CONCLUSION**: These data indicate that allitridium can precondition rabbit ischemic myocardium and this protection can be effectively blocked by administration of Poly B, an inhibitor of PKC, implying that PKC has an important role in preconditioning.

INTRODUCTION

Brief periods of myocardial ischemia and reperfusion prior to a longer episode of myocardial ischemia and reperfusion appear to activate a cascade of events that render it resistant to ischemic injury. This unique myocardial adaptive phenomenon has been termed as ischemic preconditioning[1]. It also appears to be a protective mechanism in human cardiomyocytes[2]. Protein kinase C (PKC), a major signal transduction mechanism, has been shown to play an important role in protecting the ischemic-preconditioned heart[3]. Allitridum is an extract of garlic, which in China has been eaten popularly for about 3000 years and written in classic works that garlic could promote circulation. There are some investigations to prove that allitridum has a good effect on treating patients with cardiovascular deseases[4]. Furthermore, a previous unpublished study from our laboratory has shown that allitridum can cause a fall in coronary flow and finally protect the ischemic hearts, suggesting that allitridum might have the effects of ischemic preconditioning. The aim of the present study was to determine (1) whether allitridum mimic the effect of ischemic preconditioning; (2) whether this protection can be mediated by PKC.

MATERIALS AND METHODS

Surgical preparation Experimental healthy rabbits of either sex, weighing 1.8 - 2.6 kg, were supplied by China-Japan Friendship Hospital Animal Center (Grade Ⅱ, Certificate № 01-2100). Sodium pentobarbital was injected into an ear vein until the rabbit was flaccid. A tracheotomy was performed, and a large bore polythylene cannula was inserted into the trachea and connected to positive pressure respirator. The rabbits were ventilated with 100 % oxygen. The respiratory rate and inspiratory/expiratory phase ratio was adjusted to maintain a normal arterial pH and P_{CO_2}. The hearts was rapidly excised and mounted on a Langendorff apparatus by the aortic root. Isolated rabbit hearts were instrumented as previously described[5]. The hearts was perfused with Krebs-Henseleit bicarbonate buffer consisting of (mmol/L) NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2,

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KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 24.8, and glucose 10. A 95 % O_2/5 % CO_2 gas mixture was bubbled through the perfusate. The left atrial appendage was retracted, and the superficial branches of the left coronary artery were identified. A 3 - 0 silk suture on a small curved needle was passed through the myocardium beneath the proximal segment of the large arterial branch coursing down the middle of the anterior surface of the left ventricle (if there are higher branches than the middle point, all branches will be tied). A saline-filled latex balloon connected by a polyethlene catheter to a pressure transducer was inserted into the left ventricle and inflated to produce an end-diastolic pressure of 5 mmHg. Atrial pacing was performed at 180 beats per minute if the spontaneous rate was slower. The heart was allowed to stabilize for 10 min before the experiment was begun.

Experimental protocol In all hearts, regional ischemia was produced by pulling the snare for 30 min, then the snare was released, allowing reperfusion for 2 h. Myocardial ischemia was confirmed by a decrease in left ventricular systolic pressure (LVSP) and a fall in coronary flow. The animals were divided into 5 groups: 1) the control group had only 30 min of ischemia and 120 min of reperfusion; 2) the ischemic preconditioning (PC) group received 5 min of global ischemia during cessation of aortic perfusion, followed by 10 min of reperfusion before 30-min regional ischemia; 3) the allitridum (A) group received the allitridum (60 μmol/L) for 5 min in lieu of ischemia, followed by a 10 min drug free interval before the 30-min ischemic period; 4) The polymyxin B (Poly B) group received Poly B (50 μmol/L), 20 min before and continuing to the end of ischemia; 5) the allitridum + polymyxin B (A + Poly B) group, allitridum and poly B were infused together as outlined in group 3 and 4.

Chemicals Allitridum (Diallyl Trisulfate, $C_6H_{10}S_3 = 178.33$, Shanghai Harvest Pharmaceutical Co Ltd) was obtained from Dept of Pharmacy, China-Japan Friendship Hospital. Triphenylcetrazolium chloride (TTC), Evan's blue, and pentobarbital were obtained from Beijing Chemical Reagent Company. Poly B was purchased from Sigma Chemical Co. Allitridum and Poly B were dissolved in Krebs-Henseleit buffer directly.

Infarct size meassurement At the end of the study, the coronary branch was reoccluded and 0.25 % Evan's blue was infused into the perfusate to demarcate the perfused myocardium and thus mark the nonblue perfusion field of the occluded artery (the risk area). The heart was frozen for 3 h, and then cut into 2-mm transverse slices. The heart slice were incubated for 20 min at 37 ℃ in a 1 % solution of TTC in sodium phosphate buffer, PH 7.4. Viable tissue stained by TTC was deep red, necrotic tissue was unstained and appeared tanned. After staining, the slices were immersed in 10 % formalin to enhance the contrast between stained and unstained tissue. The areas of infarct and risk zone for each slice were meassured by Tracor Northern picture analysor, and volumes were obtained by multiplying the area by the thickness of the section. Infarct size was normalized by expressing as a percentage of the area at risk.

Statistics All values are given as $\bar{x} \pm s$. The significance of differenes in hemodynamics after an intervention in any group was determined by ANOVA with replication and Newman-Keuls test. Differences in risk zone or infarct volumes or infarct-to-risk zone ratios between control and experimental groups were evaluated by unpaired t-test.

RESULTS

Data were obtained from 34 rabbits. All hearts used in the study were perfused with buffer within 1 min after excision and the study was continued only if LVSP was in excess of 80 mmHg after 10 min of equilibration.

Basal values At baseline, the values of heart rate, LVSP, maximum positive and negative values of its first derivative (dp/dt), and coronary flow (CF) were compared in Tab 1. There were no significant difference among any of the groups.

Changes in LVSP during 10 - 30 min As in Tab 2, at 16 min, 1 min after infusion of 50 μmol/L allitridum or global ischemia, LVSP in the PC group and A group were significantly reduced; both poly B and A + Poly B lowered the LVSP, but did not significantly change it ($P > 0.05$). At 20 min, 5 min after infusion of 50 μmol/L allitridum or global ischemia, allitridum reduced LVSP and LVSP in PC group fell to zero, while Poly B and A + Poly B mildly reduced their LVSP ($P > 0.05$). At 30 min, in a drug-free interval just before the 30-min regional ischemia, LVSP returned to predrug level in PC group, and in A group partially recovered to (77 ± 14) mmHg, while in poly B and Poly B + A groups, LVSP continued to be mildly depressed.

Infarct size data Body weights, heart weights, and volumes of risk zone were similar in the 5 groups (Tab 3). Infarct sizes were also indicated in Tab 3. Average infarct size in control rabbits was 25 % ± 7 % of

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Tab 1. Baseline hemodynamic parameters and coronary flow. $\bar{x} \pm s$.

Group	n	HR/ beats·min^{-1}	LVSP/ mmHg	± dp/dt/ mmHg·s^{-1}	CF/ mL·min^{-1}·g^{-1}
Control	6	182 ± 17	103 ± 20	6000 ± 510	7.2 ± 0.6
PC	6	187 ± 16	98 ± 14	5483 ± 1008	7.4 ± 0.2
A	8	191 ± 16	98 ± 9	6150 ± 1140	6.9 ± 0.7
Poly B	6	184 ± 14	100 ± 11	5874 ± 1013	7.1 ± 0.6
A + Poly B	8	189 ± 15	99 ± 13	5645 ± 1004	7.1 ± 0.8

Tab 2. Changes in LVSP during 10 – 30 min. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* control.

Group	n	Time course/min			
		10	16	20	30
Control	6	103 ± 20	103 ± 20	103 ± 20	103 ± 20
PC	6	98 ± 14	52 ± 11^b	0	98 ± 15
A	8	98 ± 9	70 ± 1^b	32 ± 12^b	77 ± 14
Poly B	6	100 ± 11	89 ± 12^a	84 ± 13^a	65 ± 11
A + Poly B	8	99 ± 13	85 ± 14^a	80 ± 12^a	51 ± 1

Tab 3. Infarct size data. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* control. $^dP > 0.05$ *vs* PC group.

Group	n	Body Weight/ kg	Heart Weight/ g	Infarct/ cm^3	Risk Zone/ cm^3	I/R/ %
Control	6	2.23 ± 0.21	8.70 ± 1.46	0.135 ± 0.054	0.557 ± 0.025	25 ± 7
PC	6	2.20 ± 0.15	7.70 ± 0.40	0.034 ± 0.008	0.64 ± 0.07	5.3 ± 1.4^b
A	8	2.21 ± 0.20	8.21 ± 0.52	0.045 ± 0.046	0.63 ± 0.11	7 ± 6^{bd}
Poly B	6	2.24 ± 0.20	8.54 ± 0.52	0.114 ± 0.020	0.48 ± 0.08	24 ± 5^a
A + Poly B	8	2.21 ± 0.18	8.05 ± 0.44	0.118 ± 0.037	0.53 ± 0.15	23 ± 5^a

that in the risk zone. As expected, ischemic preconditioning significantly reduced infarction to 5.3 % ± 1.4 % of the myocardium at risk ($P < 0.05$). Allitridum greatly reduced infarction ($P < 0.05$), and compared to PC, there was no significant difference ($P > 0.05$). Poly B, which itself had no direct effect on infract size, aborted the protective effect of allitridum 23 % ± 5 % *vs* control ($P > 0.05$).

DISCUSSION

The present study has shown that allitridum can precondition the heart against infarction and this protective effect may be dependent on reduction of LVSP during allitridium infusion. Furthermore, the protection derived from allitridum stimulation could be blocked with a PKC inhibitor.

Effect of changes in LVSP and differences in risk zone on infarct size As noted in Tab 3, risk zones were comparable in all groups. Poly B, a specific inhibitor of the enzyme activity of PKC, caused a mild fall during LVSP for the duration of the infusion, and the decrease was comparable in rabbits receiving this drug alone or with allitridum. Allitridum caused a singnificant fall in LVSP. According to Tsuchidu[5], these changes would be expected to have little effect on infarct size. If anything, a mild decrease in contractility should diminish infarction. In fact, the present study showed that Poly B had no specific effect on infarct size, while allitridum could reduce myocardium infarct size. Difference between allitridum and Poly B was the extent of recovery in LVSP during drug-free interval.

Effect of PKC on ischemic preconditioning Previous experiments showed ischemic preditioning in the rabbit heart could be blocked if PKC was inhibited with an inhibitor with infarct size used as the end point in an *in vitro* model[6]. Yang concluded that mechanism of preconditioning involved two phase: a trigger phase char-

acterized by a phosphorylation-independent process and a mediation phase occurring during the prolonged ischemic period and requiring phosphorylation by PKC[7]. Consequently, goto conclude that ischemia or drugs could trigger the myocardial protection only if they induced enough PKC activation, and this phenomenon applied to rule of "all or not"[8].

PKC has been recognized to play a central role as a critical component of intracellular signalling in ischemic precondtioning. According to the follwing mechanism: an endogenous ligand binds to its receptor on the myocyte surface, which then activates phospholipase C via a G protein; activated phopholipase C then causes the break down of phosphotidylinsitol 4, 5-diphosphate and phosphatidylcholine to produce diacylglycerol and inositol-1, 4,5-triphosphate; increased levels of diacylglycerol then activated PKC phophorylation and activation of cytosolic PKC, and this activated PKC phophorylates a secondary effector, which was able to induce the protective effects of preconditioning[9]. The present study shows that allitridum can mimic the effect of ischemic preconditioning by activation of PKC. Is there a relationship between allitridum and those receptors?

Though there is no report showing that allitridum can activate those receptors, some investigations shows some interests to us: Adel and his colleague found that adenosine could be extracted in garlic[10]; Das group found that garlic could activate NOS on platelet and concluded that there was a close relationship between garlic and NO[11]. Does those mean that allitridum, one of its extract, may activate one of those receptors, especially adenosine receptor or NO receptor? It needs further investigation.

Calcium and allitridum preconditioning Under physiological conditions, a small amount of Ca^{2+} entering through the L-type Ca^{2+} channels in the sarcolemmal membrance induces a release of Ca^{2+} from the sarcoplasmic reticulum stores and thus results in cardiac contraction[12]. Ischemia causes increased Ca^{2+} fluxes into cells because the depolarization and transmitter release could open the voltage-sensitive Ca^{2+} channels and the receptor-operated Ca^{2+} channels. Ischemia-reperfusion injury are known to be association with the occurrence of intracellular Ca^{2+} overload[13]. Chen observed that allitridum had the similar effect as calcium channal antagonist verapami on vascular smooth muscle and indicated that allitridum may antagonize the calcium channel[14]. Ma *et al* found that allitridum could reduce the Ca^{2+} fluxes via either voltage-sensitive Ca^{2+} channels blockade or receptor-operated Ca^{2+} channels blockade[15]. From those finding, we think the inhibitory effects of allitridum on $[Ca^{2+}]_i$ elevation are beneficial to its protection of post-ischemia myocardium and may be useful to understand the mechanism of allitridum preconditioning.

In summary, allitridum may bind to one or some known receptors (such as adenosine, NO, etc) to induce the PKC activation as much as possible, which triggers the myocardial protection, or block Ca^{2+}-influx to protect the ischemic hearts.

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大蒜素通过激活蛋白激酶 C 模拟心肌缺血预适应

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关键词　大蒜素；心肌缺血；再灌注损伤；多粘菌素 B；蛋白激酶 C

目的：研究大蒜素是否具有药物性预处理的作用，并探讨蛋白激酶 C 在大蒜素预处理中的重要作用. **方法**：34 只离体家兔心脏挂上 Langendorff 装置，以 95 % O_2/5 % CO_2 混合的 Krebs-Henseleit 液灌流，均接受 30 min 局部缺血和 2 h 复灌. **结果**：在 30 min 局部缺血前 10 min 予 5 min 药物性预处理的大蒜素组较之对照组明显缩小心肌梗塞范围(7 % ± 6 % *vs* 25 % ± 7 %，$P<0.05$)，大蒜素 + 多粘菌素 B 组的心肌梗塞范围与对照组相比无显著性差异(23 % ± 5 % *vs* 25 % ± 7 %，$P>0.05$). **结论**：大蒜素具有模拟缺血预处理样保护作用，其作用可被抑制剂多粘菌素 B 所阻断，表明大蒜素通过激活蛋白激酶 C 发挥药物性预处理作用.

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