Korean J. of Pharmacology
Vol. 32, No. 1, pp. 31~37, 1996

Role of Adenosine and Protein Kinase C in the Anti-ischemic Process of Ischemic Preconditioning in Rat Heart

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ABSTRACT

The protective effect of 'ischemic preconditioning (IP)' on ischemia-reperfusion injury of heart has been reported in various animal species, but the mechanism is unclear. In an attempt to elucidate the mechanism of IP, we examined the effects of blockers against adenosine and protein kinase C in preconditioned heart of rat.

The hearts perfused with oxygen-saturated Krebs-Henseleit solution by Langendorff method were exposed to 30 min global ischemia followed by 20 min reperfusion. IP was performed with three episodes of 5 min ischemia and 5 min reperfusion just before ischemia-reperfusion.

IP prevented the depression of contractile function and the myocardial contracture in the ischemic-reperfused heart and reduced the release of lactate dehydrogenase during the reperfusion period. Polymyxin B, chelerythrine and colchicine, PKC inhibitors, attenuated almost completely the anti-ischemic effect of IP, while adenosine receptor antagonists did not.

These results indicate that PKC may be a crucial intracellular mediator in anti-ischemic action of IP in ischemic-reperfused rat heart, while adenosine may not be involved in the mechanism of IP.

Key Words: Reperfusion Injury, Ischemic Preconditioning, Protein Kinase

INTRODUCTION

Myocardial ischemia, caused by the occlusion of a coronary artery, initiates a series of cellular reactions which, if not checked, may lead to cell death and tissue necrosis. In the early stage of myocardial ischemia, tissue injury is reversible if adequate coronary flow is restored. However, as the duration of ischemia increases, the reperfusion may cause lethal ventricular arrhythmia and accelerate the progress of cell death (Ganote *et al.*, 1975; Hearse, 1977). Recent studies provided that repetitive episodes of brief regional ischemia do not produce a cumulative loss of myocardial function, but reduce infarct size and make recovery of regional myocardial function better during the reperfusion of ischemic heart, this phenomenon is called "ischemic preconditioning(IP)" (Murry *et al.*, 1986). This phenomenon has been demonstrated by many investigators in various mammarian species such as dog (Murry *et al.*, 1986), rabbit (Liu *et al.*, 1991), pig (Schott *et al.*, 1990), rat (Tani *et al.*, 1990), and human (Deutsch *et al.*, 1990).

Several investigators have proposed that

*This study was supported by a grant from Chosun University (1994) and a grant from Ministry of Science and Technology (1994).

adenosine, catecholamine, prostaglandine, nitric oxide, and oxygen free radicals may be acting as an endogenous mediators of IP (Parratt, 1994). However, but the precise mechanism of IP is not yet clearly understood.

Adenosine is an endogenous substance produced from the degradation of adenosine triphosphate (ATP); its concentration therefore rises during brief ischemia. Adenosine-mediated effects include vasodilatation and delaying of ischemia-induced contracture. Blockade of adenosine receptor abolishes the protective effect of IP and adenosine agonists can mimic IP effect (Liu *et al.*, 1991). These studies provide strong evidence that stimulation of A_1 adenosine receptor is the crucial role in anti-ischemic action of IP. Adenosine has various action in myocardium such as activation of Na^+-Ca^{2+} exchange, opening the ATP-dependent potassium channel, closing the voltage-dependent Ca^{2+} channel and inhibition of adenylate cyclase (Linden, 1991). However, cellular mechanism by which activation of this receptor provides resistance against ischemia to the myocardium is still unclear.

A hypothesis has recently been proposed that protein kinase C (PKC) may participate in the intracellular process of IP. Ytrehus *et al.* (1994) have found that the PKC inhibitor blocked anti-ischemic effect of IP in rabbits, and PKC activators could mimic IP. PKC is activated by various mediators and then is involved in many intracellular signal transduction pathways, but it is still unclear which mediators can participate in the activation of PKC in the IP. In the present study, we examine whether the adenosine may play a certain role as an extracellular mediator and the PKC may play a central role as an intracellular mediator in anti-ischemic process of IP in rat heart.

MATERIALS AND METHODS

Heart perfusion and experimental protocol

Sprague-Dawley rats of either sex, weighing from 150 to 200 g, were killed by decapitation and hearts were rapidly removed and arrested with ice cold isotonic saline solution (4℃). Retrograde non-recirculating perfusion through the aorta was performed according to the technique of Langendorff. The pulmonary artery was cut in order to allow complete coronary drainage. The perfusion solution was Krebs-Henseleit bicarbonate buffer solution (K-H solution, NaCl 118 mM, $NaHCO_3$ 2.2 mM, KCl 4.8 mM, $MgSO_4$ 7H2O 1.2 mM, KH_2PO_4 1 mM, $CaCl_2$ 1.25 mM and glucose 11.1 mM) saturated with a 95% O_2—5% CO_2 gas mixture yielding a pH value of 7.4. The perfusion pressure was constantly maintained at 100 cm H_2O throughout the whole perfusion period. The heart was kept in a humidified chamber maintained at 37℃ during the perfusion. The isolated hearts were first perfused for 15 min with an perfusion solution. The hearts were subjected to global ischemia for 30 min. Global ischemia was induced by stopping the perfusion. During global ischemia, the hearts were immersed into hypoxic solution which was a glucose-free (substituted with equimolar mannitol) solution equilibrated with a 95% N_2—5% CO_2 gas mixture. After this period, the hearts were reperfused for 20 min. The reoxygenated solution was saturated with a 95% O_2—5% CO_2 gas mixture. IP was performed with three episodes of 5 min ischemia, interrupted by 5 min periods of intermittent perfusion, before the sustained ischemic period. All drugs were infused at a rate 0.5 ml/min from 5 min before the induction of IP to the period of grobal ischemia.

Evaluation of cardiac function

The heart rate (HR), the left ventricular pressure (LVP) were measured as indices for cardiac function. The balloon tip was connected to a pressure transducer of the physiogrph to monitor HR and LVP. The values of the left ventricular end-diastolic pressure (LVEDP) and the left ventricular systolic pressure (LVSP) were directly obtained from the monitored pressure. The left ventricular developed pressure (LVDP) could be calculated from the difference between LVSP and LVEDP. The ability of cardiac pumping action was estimated from the cardiac function index ($HRxLVDPx10^{-3}$). The functional recovery was calculated from the percent value of the cardiac function index at the end of reperfusion (20 min) compared to

the preischemic value.

Measurement of lactate dehydrogenase (LDH) activity

The activity of LDH released into coronary effluent, was measured as an index for cardiomyocyte injury. LDH activity was determined by an enzymatic method using UV-spectrophotometer (Bergmeyer and Bernt 1974). The coronary effluent was added to the reaction mixture containing 48 mM phosphate buffer (pH 7.4), 0.6 mM pyruvate and 0.18 mM NADH. The change of optical density was measured at 25℃ and 340 nm with UV-spectrophotometer (Gilford, 2600).

RESULTS

The protective effect of IP against ischemia-reperfusion injury

Ventricular function was maintained for more than 60 minutes without any functional impairment when hearts were perfused with the oxygenated K-H solution. When the supply of the oxygenated K-H solution was stopped, hearts were completely arrested within 2 min and LVEDP increased significantly about 15 min of ischemic period. After 30 min ischemia followed by reperfusion, hearts began to contract irregularly and LVEDP increased abruptly. The impairment of heart function was sustained even after 20 min of reperfusion. In preconditioned hearts, upon reperfusion LVEDP was markedly lowered and ventricular beating reappeared in regular rhythm. The ventricular function recovered to 75% of preischemic value after 20 min of ischemia (Table 1). The level of LDH in coronary effluent samples was significantly lower in preconditioned hearts than in non-preconditioned hearts (Table 1).

The effect of adenosine antagonists on IP

To determine whether adenosine is involved in IP, we used 8-(p-sulfophenyl)-theophylline (SPT) as a non-selective adenosine antagonist, and also used 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and xanthine amine congener (XAC) as A1 selective antagonists. All of the adenosine antagonists failed to abolish not only the recovery of myocardial function but also reduced LDH release in preconditioned hearts (Table 2).

Table 1. Effect of ischemic preconditioning on ischemia-reperfusion injury in rat heart

	NON		PRE	
	P	R	P	R
HR(beats/min)	322±44.7	221±11.7	319±34.8	296±37.5*
LVEDP(mm Hg)	5	54.8±10.9	5	13.5±9.60*
DP(mm Hg)	83.9±19.2	15.7±5.10	78.9±15.5	64.5±13.2*
DPxHRx10^{-3}(mm Hg/min)	26.8±0.2	3.48±1.21	25.2±5.54	18.9±3.93*
Recovery of function(%)		13.4±4.85		77.1±15.2*
LDH(U/g)		1.53±0.23		0.51±0.11*

NON: non-preconditioned hearts; PRE: preconditioned hearts; P: preischemia; R: reperfusion; HR: heart rate; LVEDP: left ventricular end-diastolic pressure; DP: developed pressure. Ventricular function was assessed by the product of DPxHRx10^{-3}. Recovery of function was calculated by division of the product at the end of 20 minutes of reperfusion by the product before induction of ischemia. LDH activities were measured in coronary effluents collected during the first 3 minutes of reperfusion. Each value is given as mean±SD of six hearts.

*$p<0.01$ versus values of non-preconditioned group.

Table 2. Effects of adenosine receptor antagonists on anti-ischemic action of ischemic preconditioning in rat heart

	PRE		PRE+SPT		PRE+DPCPX		PRE+XAC	
	P	R	P	R	P	R	P	R
HR(beats/min)	319±34.8	296±37.5	295±31.4	285±29.4	310±32.0	293±28.2	288±26.5	276±32.6
LVEDP(mm Hg)	3	13.5±9.60	5	14.5±8.12	5	15.6±6.31	5	14.0±7.52
DP(mm Hg)	78.9±15.5	64.5±13.2	75.6±13.0	61.2±15.3	72.5±12.2	62.3±11.4	77.4±14.3	60.0±14.9
DPxHRx10^{-3}(mm Hg/min)	25.2±5.52	18.9±3.83	22.5±8.10	17.5±6.41	22.3±6.83	18.2±4.20	21.4±5.41	16.8±5.70
Recovery of function(%)		77.1±15.2		75.3±15.1		78.2±14.2		74.9±14.9
LDH(U/g)		0.51±0.11		0.58±0.06		0.50±0.13		0.53±0.05

PRE: preconditioned hearts; P: preischemia; R: reperfusion; HR: heart rate; LVEDP: left ventricular end-diastolic pressure; DP: developed pressure. Ventricular function and LDH were measured as same as in Table 1. Final concentrations of SPT, DPCPX and XAC were 1.0×10^{-6} M, 1.5×10^{-8} M and 5.0×10^{-7} M, respectively. Each value is given as mean±SD of six hearts.

Table 3. Effects of protein kinase C inhibitors on anti-ischemic action of ischemic preconditioning in rat heart

	PRE		PRE+Polymixin B		PRE+chelerythrine		PRE+colchicine	
	P	R	P	R	P	R	P	R
HR(beats/min)	319±34.8	296±37.5	275±26.1	245±88.7	280±29.8	282±44.1	284±19.3	291±63.7
LVEDP(mm Hg)	5	13.5±9.60	5	37.1±7.26**	5	29.5±12.3*	5	21.9±21.5**
DP(mm Hg)	78.9±15.5	64.5±13.2	59.3±14.6	27.7±21.0*	75.7±12.8	31.6±3.61*	77.5±5.83	27.1±17.0*
DPxHRx10^{-3}(mm Hg/min)	25.2±5.54	18.9±3.83	16.2±2.23	5.69±3.28**	21.2±6.02	9.82±1.04*	21.7±3.06	8.11±5.03*
Recovery of function(%)		77.1±15.2		27.4±24.0*		43.8±15.1*		38.2±23.7*
LDH(U/g)		0.51±0.11		1.67±0.33**		1.23±0.32**		1.42±0.21**

PRE: preconditioned hearts; P: preischemia; R: reperfusion; HR: heart rate; LVEDP: left ventricular end-diastolic pressure; DP: developed pressure. Ventricular function and LDH were measured as same as in Table 1. Final concentrations of polymixin B, chelerythrine and colchicine were 1.0×10^{-5} M, 1.0×10^{-6} M and 5.0×10^{-6} M, respectively. Each value is given as mean±SD of six hearts.

*$p<0.05$; **$p<0.01$ versus values of PRE.

The effect of PKC inhibitor on IP

To investigate whether PKC plays a role in IP, polymyxin B and chelerythrine, specific antagonists of PKC, and colchicine, an inhibitor of PKC translocation, were used to determine whether IP could be blocked. The increase of myocardial function recovery and reduction of LDH release by IP were significantly abolished when the PKC inhibitor, polymyxin B or chelerythrine, was administered during the 5 min prior to precondition period. The treatment of colchicine showed also the same block effect on IP as the inhibitors of PKC (Table 3).

DISCUSSION

There is evidence in support of adenosine being the most important endogenous mediator of protection in preconditioned heart of some animals (Wyatt *et al.*, 1989; Lasely *et al.*, 1990; Teresa *et al.*, 1993), but adenosine dose not appear to play the same role in the rat. Although Linden (1991) have induced protection from ischemia-reperfusion injury in the rat by giving adenosine and an analogue of adenosine, Cave *et al.* (1993) has been unable to reproduce the effect of IP in the rat with adenosine. In our study, both non-selective adenosine antagonist and A1 selective antagonist failed to block the protective effect of IP (Table 2). These results suggested that although adenosine may be involved in IP in the rat, it is not the most important mediator. Despite this apparent difference in the endogenous mediators of protection between species, IP has been clearly shown to be protective in all the species (Parrett, 1994).

There is now some evidence for the role of PKC in IP. Ytrehus *et al.* (1994) demonstrated the involvement of PKC in IP in an isolated perfused rabbit heart model by treating with a phorbol myristate acetate (PMA). Recently other investigators also demonstrated that administering PKC blockers abolished the protective effect of IP in an in vivo rabbit or rat model (Black *et al.*, 1993; Speechly *et al.*, 1994). We also found that polymyxin B and chelerythrine abolished IP's effect on functional recovery and reduced the LDH release (Table 3). These results suggest that PKC may play a central role in IP heart of rat.

PKC has been recognized as a critical component of intracellular signal transduction pathways for a decade. It is known to have a vital role in cellular regulation, tumor promotion, and probably oncogenesis (Hug *et al.*, 1993). In the quiescent myocyte, most of the PKC located in the cytosol. However, some stimuli initiate translocation of the cytosol PKC pool into the membranes and activated membrane bound PKC (Azzi *et al.*, 1992). We found that disrupting microtubles with colchicine abolished the protective effect of IP (Table 3). Thus, this result provide evidence that translocation of PKC into the membrane of the cardiocyte is a key event in the IP. Recently, acetylcholine and norepinephrine have been also shown to induce IP-like effect (Fleming *et al.*, 1992). Both acetylcholine and norepinephrine activate phopholipase C via a G protein and then causes the breakdown of phosphatidylinositol 4,5-diphosphate and phosphatidylcholine to produce diacylglycerol and inositol-1,4,5-triphosphate; increased levels of diacylglycerol cause translocation and activation of cytosolic PKC (Loke-Winter *et al.*, 1991). Since adenosine does not contribute to the protective effect of IP, it is considerable that acetylcholine or norepinephrine may be involved in the IP via PKC pathway in the rat heart. Therefore, it is necessary to study whether acetylcholine or norepinephrine may play a certain role in ischemic preconditioned heart of rat.

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=국문초록=

허혈전처치의 허혈심장 보호과정에서 Adenosine 및 Protein Kinase C의 역할

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허혈전처치(IP)의 허혈-재관류손상에 대한 심근 보호작용의 기전을 규명하기 위한 일환으로 adenosine에 의한 PKC자극이 허혈전처치의 주요 기전으로 작용할 가능성을 조사하였다.

흰쥐 적출심장의 Langendorff 관류 표본에서 실험적인 허혈(30분)-재관류(20분)손상을 유도하였고, 허혈전처치는 허혈-재관류 손상 유도 전에 5분 허혈-5분 재관류를 3회 반복하여 시행하였다. 심근 손상의 지표로 심수축기능, 세포질효소 유출을 측정하였다. Adenosine이 허혈전처치의 심보호 효과에 관여하는지를 관찰하기 위하여 adenosine수용체 억제제인 8-(p-sulfophenyl)-theophylline(SPT), Xanthine amine congener(XAC) 및 8-cyclopentyl-1,3-dipropylxanthine (DPCPX)을 허혈전처치 유도 전에 투여하였다. 또한 PKC가 허혈전처치의 세포내 매개인자로 관여 할 가능성을 관찰하기 위하여 PKC활성 억제제인 polymyxin B 및 chelerythrine과 PKC translocation 억제제인 colchicine을 허혈전처치 유도 전에 투여하였다.

연구성적은 다음과 같다.

1) 허혈전처치는 허혈재관류 심장의 심기능의 저하를 현저히 회복시켜 심기능 회복률은 75%에 달하였다.

2) 허혈-재관류 심장에서 lactate dehydrogenase유출증가는 허혈전처치에 의해 현저히 저하되었다.

3) Adenosine 비선택적 차단제인 SPT와 A1 선택적 차단제인 DPCPX 및 XAC의 투여가 허혈전처치에 의한 심기능회복 및 LDH 유출 감소에 영향을 미치지 않았다.

4) PKC활성 억제제인 polymyxin B 와 chelerythrine을 처치시 허혈전처치 심장의 심기능 회복률이 현저히 감소되었으며 LHD 유출 역시 대조군 심장의 수준으로 증가하였다.

5) PKC translocation을 방해하는 colchicine도 허혈전처치의 심보호 효과를 억제시켰다.

이상의 결과들로부터 adenosine은 흰쥐 심장에서 허혈전처치의 심보호효과에 중요한 세포외 매개물질로 작용할 가능성이 희박하며, PKC는 흰쥐 심장에서 허혈전처치시 세포내 매개 인자로 관여하여 허혈전처치에 의한 심보호효과에 중요한 역할을 할 수 있으리라 사료된다.