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Effect of Ischemic Preconditioning on the Oxygen Free Radical Production in the Post-ischemic Reperfused Heart

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ABSTRACT

The protective effect of 'ischemic preconditioning (PC)' on ischemia-reperfusion injury of heart has been reported in various animal species, but without known mechanisms in detail. In an attempt to investigate the cardioprotective mechanism of PC, we examined the effects of PC on the myocardial oxidative injuries and the oxygen free radical production in the ischemia-reperfusion model of isolated Langendorff preparations of rat hearts. PC was performed with three episodes of 5 min ischemia and 5 min reperfusion before the induction of prolonged ischemia (30 min)-reperfusion(20 min).

PC prevented the depression of cardiac function (left ventricular pressure x heart rate) observed in the ischemic-reperfused heart, and reduced the release of lactate dehydrogenase during the reperfusion period. On electron microscopic pictures, myocardial ultrastructures were relatively well preserved in PC hearts as compared with non-PC ischemic-reperfused hearts. In PC hearts, lipid peroxidation of myocardial tissue as estimated from malondialdehyde production was markedly reduced. PC did not affect the activity of xanthine oxidase which is a major source of oxygen radicals in the ischemic rat hearts, but the myocardial content of hypoxanthine (a substrate for xanthine oxidase) was much lower in PC hearts. It is suggested from these results that PC brings about significant myocardial protection in ischemic-reperfused heart and this effect may be related to the suppression of oxygen free radical reactions.

Key Words: Heart, Ischemia, Reperfusion, Preconditioning, Oxygen free radical

INTRODUCTION

In the ischemic hearts, the late reperfusion does not reduce the extent of irreversible myocardial injury, and it rather aggravates the ischemia-induced cardiac malfunction and arrhythmia (Ganote *et al.*, 1975; Hearse, 1977). A notable recent progress in the development of protective intervention for the myocardial ischemia-reperfusion injury is the demonstration that repeated brief episodes of ischemia, which is not strong enough to result in irreversible injury, can render the heart more resistant to subsequent prolonged ischemic insult. This phenomenon was termed 'ischemic preconditioning (PC)' (Murry *et al.*, in 1986). The protection against ischemia by PC has been demonstrated

in various mammalian species including dog (Murry *et al.*, 1986), rabbit (Miura *et al.*, 1990), pig (Schott *et al.*, 1990), and rat (Tani and Neely, 1990I). Whether PC results in increased tolerance to ischemia in human is not certain, though Deutsch *et al.*, (1990) suggested in the clinical report that PC may help the heart tolerate ischemic challenge in human also.

In spite of many hypotheses for the cardioprotective effect of PC, the mechanism is still unclear. Several investigators have proposed that purine metabolites (Liu *et al.*, 1991), prostaglandines (Vegh *et al.*, 1990) or heat-shock proteins (Knowlton *et al.*, 1989) are involved in the protective effect, but failed to show the definitive evidence. To find the clue to elucidate the mechanism of PC, we simply guess that PC could eliminate some critical factors mediating ischemia-reperfusion injury. Since oxygen free radical is known to be a major mediator of ischemia-reperfusion injury (Hess and Marson, 1984; McCord, 1982), it is possible that PC might protect the heart from the ischemia-reperfusion injury by reducing the oxidative insult produced by oxygen free radicals. In the present study, we test the hypothesis that the suppression of oxygen radical reactions by PC might be responsible for the resistance to ischemia-reperfusion injury. For this purpose, we studied the effect of PC on the post-ischemic oxidative injury in the ischemia-reperfusion model of isolated rat hearts. Additionally, to examine whether the oxygen radical production is altered by PC we studied the effect of PC on hypoxanthine-xanthine oxidase system which is a major source of oxygen free radicals in rat hearts.

MATERIALS AND METHODS

Induction of ischemia-reperfusion injury and PC

Sprague-Dawley rats of either sex, weighing about 200 g, were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). Hearts were rapidly excised and perfused by the Langendorff technique with Krebs-Henseleit (K-H) solution. Aortic perfusion pressure was 80 cm H_2O and coronary flow was approximately 12 ml/min. Left ventricular pressure was monitored via a plastic catheter with a small balloon tip which was inserted into the left ventricle through the mitral valve. The balloon was swollen until the end diastolic pressure reached 5 mm Hg. Krebs-Henseleit solution contained (mM) NaCl 118, $NaHCO_3$ 27.2, KCl 4.8, $MgSO_4$ 1.2, KH_3PO_4 1, $CaCl_3$ 1.25, and glucose 10. The solution was gassed with a 95% O_2−5% CO_2 mixture to saturate oxygen and adjust its pH to 7.4. The solution and hearts were maintained at 37℃ during all procedures.

After an initial equilibration period (15 min), sustained global ischemia was induced by closing the 3-way valve in the aortic perfusion tube. When hearts were reperfused after 30 min ischemia, restoration of coronary flow was accomplished by opening the valve for 20 min. PC was performed with three episodes of 5 min ischemia, interrupted by 5 min periods of intermittent perfusion, before the sustained ischemic period.

Evaluation of cardiac function

The heart rate (HR), the left ventricular pressure (LVP) and the coronary flow rate were measured as indices for cardiac function. The balloon tip was connected to a pressure transducer of the physiograph 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 ($HR \times LVDP \times 10^{-3}$). The functional recovery was calculated from the percent value of the cardiac function index at the end of reperfusion (20 min) compared to the pre-ischemic value.

Measurement of cardiomyocyte injury

The activity of an intracellular enzyme, lactate dehydrogenase (LDH) released into coronary effluent, was measured as an index for cardiomyocyte injury. LDH activity was determined by the enzymatic method using UV-spectrophotometer (Bergmeyer and Bernt 1974). The coronary effluent was added into 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 (Hewlett-Packard, 8452A).

Measurement of the myocardial calcium content

The myocardial content of calcium was measured by using $^{45}Ca^{2+}$ according to the method of Tani and Neely (1990II). Hearts were perfused with K-H solution containing $^{45}Ca^{2+}$ (100 uCi/L) throughout all procedures and washed out with the K-H solution without $^{45}Ca^{2+}$ for 3 min prior to termination of the experiment. The hearts were frozen and pulverized in liquid nitrogen. The powdered tissue was homogenized with Polytron tissue disintegrator in 6% perchloric acid. The homogenate was centrifuged at 3,000 g for 10 min and the supernatant was neutralized with 5M K_2CO_3. The concentration of calcium was calculated by counting the radioactivity of the homogenate with liquid scintillation spectrometer (Packard, Tricarb 1600CA).

Preparation for electron-microscopic examination

The excised ventricular tissue was immediately cut into three small pieces (about 1 mm^3). The pieces were fixed for 2 h in cold mixture of 3% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). Fixed tissues were rinsed in 0.1 M cacodylate buffer at pH 7.4 and then post-fixed for 2 h in 1% osmium tetroxide buffered in 0.1 M cacodylate buffer at pH 7.4. After post-fixation, the tissues were dehydrated with a graded series of ethanol solution, treated with propylene oxide and embedded in Epon. Thin sections were cut with a MT-2B microtome, mounted on copper grids, and stained with uranyl acetate. Sections were examined with an electron microscope (JEM 100 CXII).

Measurement of lipid peroxidation

As an estimation of myocardial lipid peroxidation, the amount of malondialdehyde (MDA) in the coronary effluent was measured by thiobarbituric acid method (Yagi, 1982). An aliquot (0.6 ml) of 1:1 mixture of 0.67% thiobarbituric acid and glacial acetic acid was added into 2.4 ml coronary effluent sample. The reaction mixture was incubated in boiling water bath for 60 min and then cooled to room temperature. After cooling, the absorbance was measured at 532 nm with spectrophotometer. The amount of MDA was calculated using the molar extinction coefficient of 1.52×10^5/M/Cm (Placer *et al.*, 1966).

Measurement of xanthine oxidase activity

Myocardial contents of NAD-dependent xanthine dehydrogenase (D-form), O_2-dependent xanthine oxidase (O-form) and intermediate D/O-form of xanthine oxidase were determined by the method of Kaminski and Jezewska (1979). Heart was quickly frozen and pulverized in liquid nitrogen. The powder was homogenized in 5 volume of the homogenation buffer (Tris/HCl 100 mM, EDTA 1 mM, DTT 10 mM, pH 8.1) with a Polytron tissue disintegrator. The homogenate was centrifuged for 20 min at 1,000 g and 4℃. The supernatant was re-centrifuged for 60 min at 30,000 g and 4℃. The resulting supernatant was precipitated by ammonium sulfate (1.6~2.4 M) addition and high speed centrifugation. The pellet was dissolved in 50 mM Tris/HCl buffer (pH 8.0) and used for the assay of xanthine oxidase activity. The standard reaction mixture contained 50 mM Tris/HCl (pH 8.0), 60 uM xanthine and sample (0.3~0.5 mg/ml), with or without 167.5 uM NAD. The enzyme activity was measured by monitoring the formation of uric acid at 290 nm and that of NADH at 340 nm with UV-spectrophotometer. The calculation of the activities of D-, O- and D/O-form was performed according to the method of Kaminski and Jezewska (1979).

Measurement of the contents of hypoxanthine and xanthine

Myocardial content of hypoxanthine and xanthine were measured by enzymatic method using xanthine oxidase (Jensen and Jorgensen, 1985). The molar absorption coefficient for the enzymatic transformation of xanthine to uric acid at 290 nm ($E=0.85\times10^4$/M/Cm) and that for the transformation of hypoxanthine to uric acid at 280 nm ($E=0.7\times10^4$/M/Cm) were used for the calculation.

RESULTS

The protective effect of PC against ischemia-reperfusion injury

Ventricular function could be maintained for more than 60 minutes when hearts were perfused with the oxygenated K-H buffer. After stopping coronary perfusion, hearts were completely arrested within 2 min and LVEDP increased significantly around 15 min of ischemic period. Just after reperfusion, hearts began to contract irregularly and LVEDP increased abruptly. The impairment of heart function

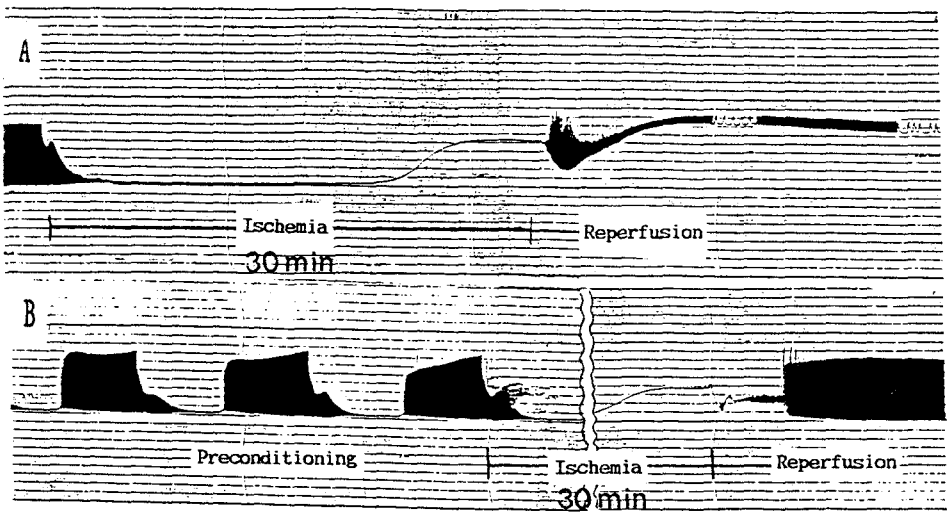


Fig. 1. The record of left ventricular pressure in isolated rat heart.
A: Ischemia-reperfusion of non-preconditioned heart.
B: Preconditioning and Ischemia-reperfusion of preconditioned heart.

Table 1. Effect of ischemic preconditioning on cardiac function in post-ischemic reperfused heart of rat

	Non-preconditioned		Preconditioned	
	Pre-ischemia	Reperfusion	Pre-ischemia	Reperfusion
HR (beats/min)	302±14.8	228±25.6	308±8.37	284±15.2*
LVEDP (mm Hg)	5	77.1±9.05	5	23.5±12.1*
LVDP (mm Hg)	58.4±13.0	10.6±8.70	58.5±9.45	57.9±20.9*
LVDP×HR×10^{-3}(mm Hg/min)	17.4±4.04	2.58±2.46	18.0±2.74	16.6±5.86*
Recovery of function (%)		14.1±10.4		91.0±25.7*
Coronary flow(ml/min)	12.4±3.50	5.08±1.51	11.7±4.21	9.76±4.01*

HR, heart rate; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure.
Ventricular function was assessed by the product of LVDP×HR×10^{-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.
Values are given as mean±SD of six hearts.
*$p < 0.01$ versus values of non-preconditioned hearts.

Table 2. Effect of ischemic preconditioning on the LDH release in post-ischemic reperfused heart

	Release of LDH (unit/min/g wet wt.)	
	0~5 min	5~10 min
Non- preconditioned	3.98±2.26	4.26±2.06
Preconditioned	1.40±0.57*	0.98±0.26*

Values are given as mean±SD of six hearts.
*$p < 0.01$ versus values of non-preconditioned hearts.

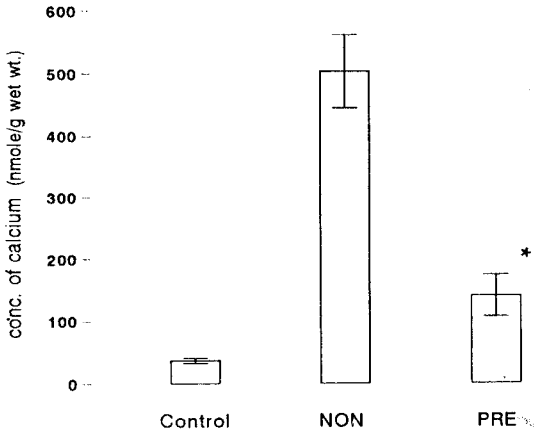


Fig. 2. Myocardial calcium level of post-ischemic reperfused myocardium. Isolated rat hearts were perfused with $^{45}Ca^{2+}$-containing K-H soluton(100uCi/l) under the conditions described in Methods. Control: continuous perfusion with normal K-H solution for 60 min. NON: 20 min reperfusion after 30 min global ischemia of non-preconditioned hearts. PRE: 20 min reperfusion after 30 min global ischemia of preconditioned hearts. Each bar represents mean ±SEM of five experiments. *: $p<0.01$ versus non-preconditioned group.

with severe contracture was sustained even after 20 min of the reperfusion. In preconditioned hearts, upon reperfusion LVEDP was markedly lowered and ventricular beating was reappeared in regular rhythm. The cardiac function recovered almost completely at 20 min of the reperfusion (Fig. 1, Table 1). Additionally, the coronary flow rate after reperfusion was also higher in preconditioned hearts than in non-preconditioned hearts (Table 1).

LDH released into coronary effluent was significantly reduced in preconditioned hearts. LDH release in non-preconditioned heart was continuously higher throughout the reperfusion period (Table 2).

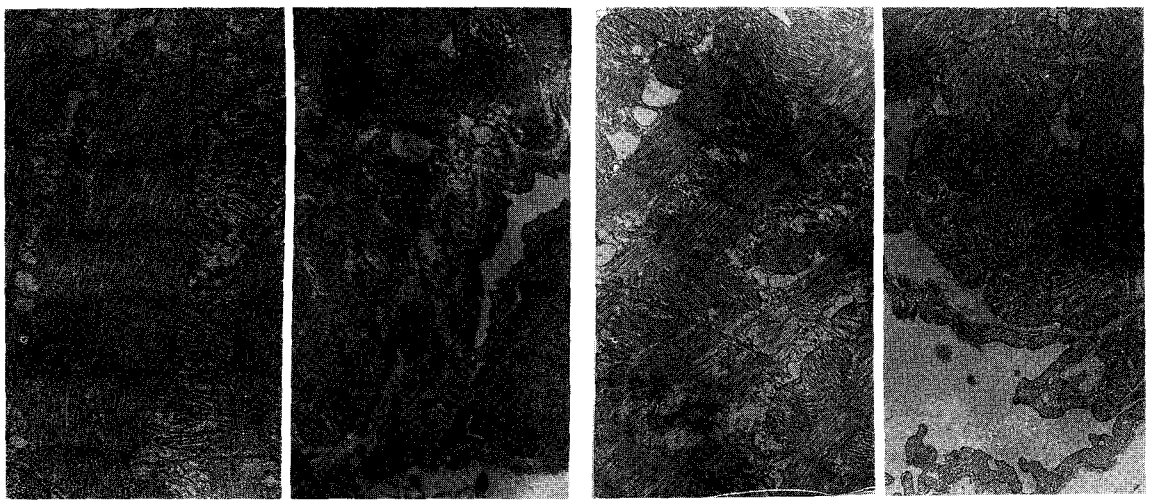
The myocardial content of calcium was 36 nmole/g wet wt in normal hearts and was increased more than 10 times by ischemia-reperfusion procedure. In the preconditioned hearts, however, the increase in the calcium content (139 nmole/g wet wt) was much lower than that in non-preconditioned hearts (Fig. 2).

The damage of the cardiomyocyte was analyzed with electron microscopic findings. The preconditioning procedure itself caused no cellular damage. Microscopic examination of post-ischemic reperfused heart showed the typical feature of reperfusion injury (Fig. 3-c). Heterochromatines were increased in nucleus and many dense granules, which are thought to be calcium-phosphate precipitates, were shown in swollen mitochondria. The direction of myofibrils and the distance between Z-bands were irregular. The separation of intercalated disc was often found, representing severe contracture of heart. In the preconditioned heart, however, it was found that many mitochondria of myocytes got together near the cellular and nuclear membrane, and the number of transport vesicles increased in the endothelial cells (Fig. 3-b). Any dense granules in mitochondrial matrix or any abnormalities of myofibrils and intercalated discs could not be found (Fig. 3-d).

The effect of PC on oxygen radical reaction

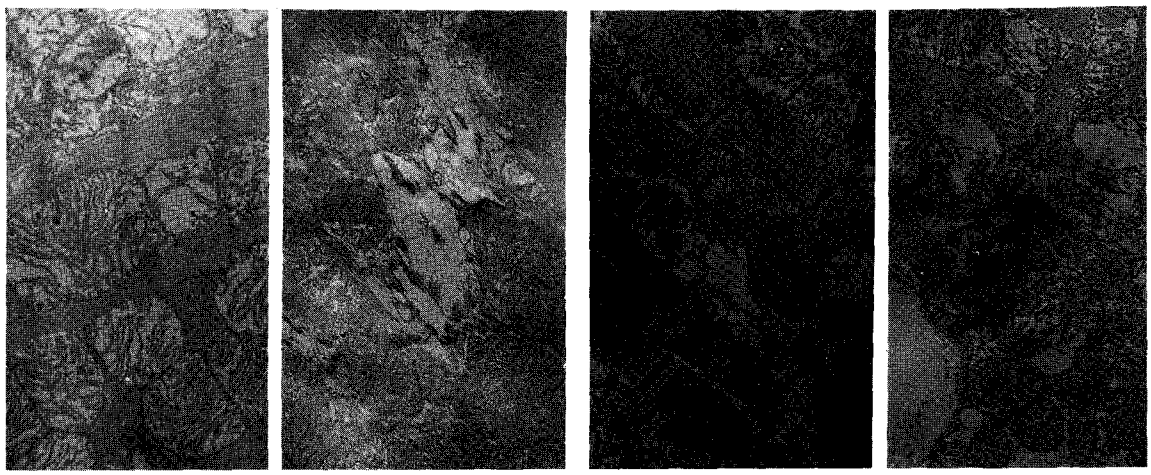
The amount of MDA in the coronary effluent collected during early 10 min of reperfusion was 6.1 and 13.2 nmol/g wet weight after 30 min and 60 min ischemia. PC significantly reduced the MDA production in the both ischemic conditions. Especially, the MDA was much decreased to 0.53 nmol/g wet wt in the 30 min ischemic condition (Fig. 4).

After 30 min ischemia, parts of D-and D/O-form of xanthine oxidase were converted into



3-a Control

3-b PRE only



3-c ISC-REP

3-d PRE-ISC-REP

Fig. 3. Electron micrograph of rat myocardium.
PRE: Preconditioning, ISC: Ischemia, REP: Reperfusion

O-form which can produce oxygen radicals. In both non-preconditioned and preconditioned hearts, there was no difference between the ratios of each form of enzymes (Table 3). However, the hypoxanthine content of preconditioned heart was significantly lower than that of non-preconditioned heart. As hypoxanthine is a substrate for xanthine oxidase, the result gives a suggestion that the oxygen radical production by xanthine oxidase system may be lower in preconditioned heart (Fig. 5).

DISCUSSION

It was generally considered in the earlier concept that the repeated, brief episodes of ischemia could be cumulative to cause irreversible damage (Geft *et al.*, 1982). Recently, howev-

Table 3. The activity of xanthine oxidase in non-preconditioned and preconditioned ischemic myocardial tissues

	Xanthine oxidase (unit/mg prot.)			
	D-form	O-form	D/O-form	Total
Pre-ischemic	1.74±0.45	0.023±0.019	0.39±0.086	2.15±0.26
Non-preconditioned	1.29±0.37	0.79±0.16	0.025±0.015	2.09±0.43
Preconditioned	1.21±0.32	0.75±0.22	0.031±0.014	2.10±0.16

Pre-ischemic heart was continuously perfused for 60 min with oxygenated K-H solution. The XOD activities of ischemic hearts were assayed from isolated rat hearts after 30 min global ischemia. Values are given as mean± SD of six hearts.

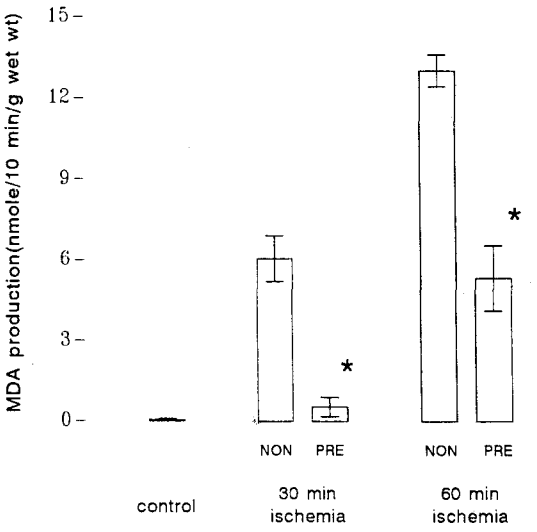


Fig. 4. Lipid peroxidation in post-ischemic reperfused rat hearts. Isolated rat hearts were reperfused after 30 min and 60 min global ischemia. Lipid peroxidation of myocardial tissue was estimated from malondialdehyde(MDA) released into the coronary effluent collected during the first 10 min reperfusion, MDA was measured with thiobarbituric acid as described in Methods. Each bar represents mean±SEM. *: $p<0.01$ versus non-preconditioned hearts.

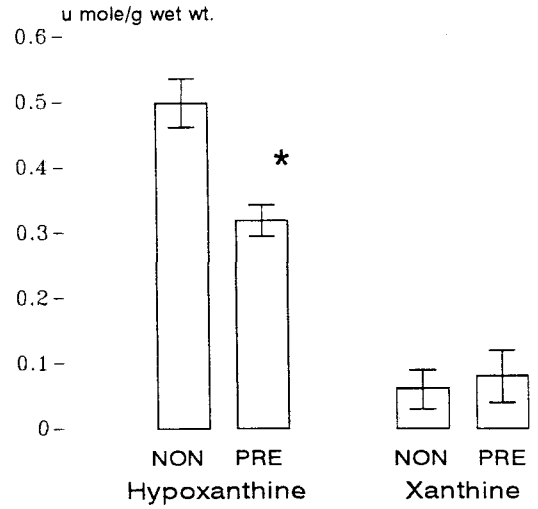


Fig. 5. Contents of hypoxanthine and xanthine in non-preconditioned and preconditioned rat hearts. Hypoxanthine and xanthine were assayed from isolated rat hearts after 30 min grobal ischemia. Each bar represents mean ±SEM of six experiments. NON: non-preconditione, PRE: preconditioned. *: $p<0.01$ versus non-preconditioned group.

er, the concept has changed and it is accepted that the short-term ischemic challenges do not cause damage to the heart, rather render it more resistant to the prolonged ischemia (Murry *et al.*, 1986). In this experiment, there was no evidence of tissue injury by three episodes of 5 min ischemia either (Fig. 2). Prolonged ischemia followed by reperfusion resulted in severe contracture, increased LDH release, decreased coronary flow and elevated calcium influx (Fig. 2, Table 1, 2). These findings were consistent with electronmicroscopic findings, such as destruction of myofibrils and Z-bands, injured membrane struc-

tures, deformation of endothelium, and calcium granules in mitochondrial matrix (Fig. 3). The severity of these parameters indicating the ischemia-reperfusion injury was markedly reduced in preconditioned hearts, suggesting that PC protects the heart from the injury at the cellular level.

The possible mechanisms for the cardio-protective effects of preconditioning have been proposed, but not yet clearly resolved. One potential explanation is an increase in collateral coronary blood flow. However, since the protective effect of PC is remarkable even in a globally ischemic heart in which no collateral circulation exists, the increase in collateral circulation during ischemia is unlikely to be responsible. Another hypothesis about the changes in energy metabolism by PC has been proposed (Murry *et al.*, 1990; Reimer *et al.*, 1986). They reported that, even though some amount of ATP is lost during PC per se, ATP depletion occurs in a slower rate during the subsequent ischemic periods in the preconditioned hearts, and that glycogenolysis and anaerobic glycolysis occur much more slowly with the resultant reduction of accumulation of toxic catabolites in the tissue. Alternatively, there are interesting hypotheses that endogenous substances produced during brief episode of ischemia may be responsible for the cardioprotective effect of PC. The possible substances include the stress proteins (Knowlton *et al.*, 1989), adenosine (Liu *et al.*, 1991) and prostaglandines (Vegh *et al.*, 1990). In the present study, we propose that PC may eliminate oxygen radical-mediated reactions which play an important role in causing the ischemia-reperfusion injury.

Oxygen radicals, superoxide anion ($O_2^- \cdot$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH \cdot$) and singlet oxygen ($^1O_2$) can easily oxidize lipids, resulting in cell membrane damage. In our experiments, a significant amount of lipid peroxides were detected in coronary effluent of post-ischemic/reperfused heart and PC lowered the amount of lipid peroxides (Fig. 4). It suggests that PC may protect the heart against reperfusion injury by reduction of oxidative insult.

Oxidative tissue injury mediated by reactive oxygen radicals could be prevented by suppression of the radical generation. We tried to test the possibility that PC inhibits the generation of oxygen radicals from xanthine oxidase system which is a major source of oxygen radicals in rat hearts (Chambers *et al.*, 1985; Lim and Kim, 1988). Xanthine oxidase is an oxidoreductase which catalyzes the oxidation of hypoxanthine and xanthine to uric acid, and it exists biologically in three different forms, D-, O-and D/O-form (Parks and Granger, 1986). The O-form which uses molecular oxygen as an electron acceptor is a candidate for the oxygen radical source in vivo. In the ischemic condition, the activity of O-form increases as a result of Ca^{2+}-dependent proteolytic conversion of D-and D/O-form to O-form (Chambers *et al.*, 1985; Hearse *et al.*, 1986; Park *et al.*, 1988), and its substrates, hypoxanthine and xanthine, are accumulated as a consequence of ATP degradation during ischemia (Jennings *et al.*, 1981). In our present results, PC did not produce any changes in either the O-form activity or the D to O conversion of xanthine oxidase. However, the myocardial hypoxanthine content after 30 min ischemia was significantly lower in preconditioned hearts than in non-preconditioned hearts (Fig. 5). It is suggested from this result that the lowered hypoxanthine content may inhibit the oxygen radical production and be responsible for the decrease in oxidative stress in the preconditioned hearts.

Increased calcium influx aggravates the myocardial injury to irreversible necrosis in ischemic-reperfused hearts (Nayler, 1981). About the mechanism of calcium increase, it has been suggested that extracellular calcium is likely to be influxed through Na^+-Ca^{2+} exchange in response to increased intracellular Na^+ that accumulated during ischemia (Murphy *et al.*, 1988; Tani and Neely, 1989). Therefore, to explain how the calcium influx was markedly suppressed by PC, it is necessary to study the effect of PC on ionic movements.

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허혈전처치(ischemic preconditioning)의 재관류심근손상 보호작용과 그 기전을 규명하기 위한 연구의 일환으로 허혈전처치가 심근세포의 산소라디칼 생성능력에 미치는 영향을 검토하였다.

흰쥐 적출심장의 Langendorff 관류표본에서 실험적인 허혈(30분)-재관류(20분)손상을 유도하였고, 허혈전처치는 재관류손상 유도전에 5분 허혈-5분 재관류를 3회 반복하여 시행하였다. 허혈심근 재관류손상의 지표로 심수축기능, 세포질효소 유출, 칼슘 유입 및 미세형태학적 변화를, 그리고 심근세포의 산소라디칼 생성기전으로 xanthine oxidase system의 변동을 허혈전처치와 비전처치 재관류 심장들에서 비교검토하였다.

연구성적은 다음과 같다.

1. 허혈전처치는 허혈-재관류 심장의 관상혈류량, 심박수, 좌심실압 등 심기능의 저하를 현저히 회복시켰다(회복률; 91%)

2. 허혈-재관류 심장에서 lactate dehydrogenase 유출증가는 허혈전처치에 의해 현저히 저하되었다.

3. 허혈-재관류 심근세포의 전자현미경상 미세구조는 허혈전처치시 비교적 잘 보존되었으며, 특히 myofibril 구조의 보존이 매우 뚜렷하였다.

4. 허혈-재관류시 산화성 조직손상 척도의 하나인 malondialdehyde 생성이 허혈전처치에 의하여 현저히 감소되었다.

5. 허혈전처치 심장에서 xanthine oxidase(D, O 및 D/O형)활성은 변화되지 않았으나 그 기질인 hypoxanthine의 조직함량은 현저히 감소되었다.

이상의 결과들로 부터 허혈전처치는 세포수준에서 허혈심근의 재관류손상을 방지하며, 허혈전처치에 따른 산소라디칼 생성 감소가 재관류손상 방지에 일부 기여할 수 있으리라 사료된다.

색인단어: 심장, 허혈, 재관류, 허혈전처치, 산소라디칼