Infarct Size-limiting Effect of Calcium Preconditioning in Rabbit Hearts

Recent studies demonstrated that brief period of Ca^{2+} depletion and repletion (Ca^{2+} preconditioning, CPC) has strong protective effects against ischemia in a rat heart. CPC and classic preconditioning (IPC) were compared in relation with infarct size and protein kinase C (PKC) isozymes. Isolated Langendorff-perfused rabbit hearts were subjected to 45-min ischemia (Isc) followed by 120-min reperfusion (R) with or without IPC, induced by 5-min Isc and 10-min R. In the CPC hearts, 5-min Ca^{2+} depletion and 10-min repletion (CPC) were given before 45-min Isc, with or without concurrent PKC inhibition (calphostin C, 200 nmol/L). IPC enhanced recovery of LV function, while CPC did not. Infarct size was significantly reduced by both CPC and IPC (p<0.05 vs. ischemic control). Membrane PKC was significantly increased from 2.53 ± 0.07 (baseline, nmol/g tissue) to 3.11 ± 0.07, 3.34 ± 0.11, 3.15 ± 0.09, and 3.06 ± 0.08 by IPC, IPC and 45-min Isc, CPC and 45-min Isc, respectively (p<0.01). Immunoblots of membrane PKC were increased by IPC, IPC and 45-min Isc, and CPC. These effects were abolished by PKC inhibition. Thus, activation of PKC may have trigger role in the mechanism of cardioprotective effect by CPC.

Key Words: Ischemic Preconditioning Myocardial; Myocardial Infarction; Reperfusion Injury; Protein Kinase C; Rabbits; Heart

INTRODUCTION

A brief period of ischemia followed by reperfusion (ischemic preconditioning, IPC) can attenuate lethal injury to the heart as a result of a prolonged period of ischemia and/or reperfusion (1-3). IPC has been shown to limit infarct size in ischemic myocardium to a remarkable degree in a variety of laboratory animals (4-9). Recent evidences suggest that this powerful endogenous cellular adaptation is conserved in a human heart (10). Activation of adenosine A_{1} (11), e_{1} adrenergic (12), bradykinin B_{2} receptors (13), opening of K_{ATP} channels (14), preservation of myocardial high-energy phosphate (15), and attenuation of ischemia-induced acidosis (16) have been proposed as responsible mechanisms of IPC, but the exact mechanisms still remain inconclusive.

Ashraf et al. (17) have demonstrated that repetitive Ca^{2+} depletion and repletion for short duration (Ca^{2+} preconditioning, CPC) can protect the heart against ischemia/reperfusion injury via the activation of protein kinase C (PKC) in a rat heart. It has been well documented that the activation of PKC plays an important role in IPC-induced cardioprotection against the development of myocardial infarction and post-ischemic contractile dysfunction (3, 18, 19). However, the relationship between CPC and PKC and/or PKC isozymes in a rabbit heart remains unclear. In this respect, this study was performed to determine whether CPC is cardioprotective as IPC, and to assess whether PKCs are involved in Ca^{2+}-mediated preconditioning in rabbit hearts, if CPC is cardioprotective.

MATERIALS AND METHODS

Preparation of the heart

This study followed guidelines for the use of laboratory animals from the American Physiological Society. Male New Zealand White rabbits weighing 1.5 to 2.0 kg were used. They were fed ad libitum with balanced diet and water, and housed in an acclimated room throughout the experiments. The animal was stunned by a blow to the neck 30 min after intraperitoneal heparinization (300 IU/kg). The heart was rapidly isolated, transferred to a Langendorff perfusion apparatus (size 5, Hugo Sachs Elektronik, March-Hugstetten, Germany), and perfused with a constant flow rate (30 mL/min) of oxygenated Tyrode solution (containing in mmol/L: NaCl 140, KCl 4.4, CaCl_{2} 1.0, MgCl_{2} 1.0, HEPES buffer 3.0, and glucose 10.0) at 37°C. The pH of the perfusate was equilibrated a physiological range (7.3-7.4). A latex balloon connected to a pressure transducer was inserted into the left ventricular cavity through the mitral opening. Eight to 10 mmHg of the initial left ventricular end-diastolic pressure (LVEDP) was loaded, and the heart was constantly paced at 150 beats/min with an electrical stimulator (Harvard Apparatus, Edenbridge, U.K.). Hemodynamic parameters including left ventricular developed pressure (LVDP) and differentiation of the left ventricular developed pressure (LVDP) and differentiation of the left ventricular pressure were monitored throughout the experiment as previously described (5).
Experimental protocol

Experimental protocols are shown in Fig. 1. After commencing retrograde aortic perfusion, hearts were allowed to stabilize for 50 min, and baseline hemodynamics were measured. Coronary flow was measured by timed collection of coronary venous effluent as previously described (5). The hearts were randomly assigned to the following experimental groups: 1) in the non-preconditioned ischemic control (n=7), hearts were subjected to 45-min global ischemia followed by 120-min reperfusion; 2) in the ischemic preconditioning (IPC) group (n=8), hearts were preconditioned with 5-min global ischemia followed by 10-min reperfusion, and then subjected to 45-min global ischemia and 120-min reperfusion; 3) in the Ca2+-preconditioning (CPC) group (n=10), hearts were preconditioned with 5-min Ca2+-free perfusion followed by 10-min perfusion with Tyrode solution (containing 1.0 mmol/L CaCl2), and then subjected to 45-min global ischemia and 120-min reperfusion; 4) in the CPC+PKC inhibitor-treated (CPC+PKCi) group (n=8), the experimental method was as similar as in the CPC group, except calphostin C (200 nmol/L) was given for 10 min during CPC. B. baseline. TTC: triphenyltetrazolium.

Fig. 1. Schematic illustration of the experimental protocol. Hearts were subjected to 5-min global ischemia and 10-min reperfusion in the ischemic preconditioned (IPC) group and to 5-min Ca2+-depletion and 10-min repletion in the Ca2+-preconditioned (CPC) group, before 45-min ischemia. In the CPC+PKCi (PKC inhibitor) group, calphostin C (CalC) was given for 10 min and washed out for 5 min during CPC. B. baseline. TTC: triphenyltetrazolium.

PKC assay and Western blot analysis of PKC isozymes

The left ventricle including interventricular septum was homogenized in 3 vols of buffer solution (containing in mmol/L: Tris-HCl 20.0, sucrose 250.0, iodoacetic acid 1.0, phenylmethylsulfonyl fluoride 1.0, EDTA 1.0, EGTA 1.0, and β-mercaptoethanol 10.0; pH 7.4 at 4 °C) using Ultra-Turrax homogenizer (10,000 rpm, 3 times for 30 sec each). Crude homogenate was centrifuged (360 g at 4 °C for 10 min). The supernatant was centrifuged at 100,000 g at 4 °C for 1 hr and the resulting supernatant was used for the measurement of cytosol PKC activity. Pellet was resuspended in 2 vols of the same buffer containing 0.3 vol% of Triton X-100 and gently stirred at 4 °C for 1 hr, centrifuged at 100,000 g for 1 hr, and the resulting supernatant was used for measurement of membrane PKC activity. PKC activity was determined by using a commercial kit (PKC Assay System, RP177A, Amersham) as previously described (22, 23), and the protein concentration in each fraction was determined by using DC Protein Assay (Bio-Rad).

Cytosol and membrane PKC samples (each sample containing 50 μg of protein) were processed by SDS-PAGE (7.5% separating gel) at 25 mA constant current for 5 hrs. Bio-Rad Kaleidoscope prestained standards were used to determine molecular masses. Proteins were transferred from gels to PVDF membrane (Hoefer Scientific). The PVDF membrane was incubated in blocking buffer (pH 7.6) containing 5% skim milk, 25 mmol Tris-HCl, 100 mmol NaCl and 0.1% Tween 20 for 1 hr at room temperature, and then incubated for 1 hr at room temperature with primary monoclonal antibodies (α, β, γ, ε, and θ). Transduction Laboratory) at indicated concentrations (1:1,000). After intensive washing with 5% skim milk-free blocking buffer, blots were incubated for 1 hr in buffer con-
taining goat anti-mouse peroxidase conjugate (1:1,000, Bio-
Rad). Finally, immunoreactive bands were visualized by en-
hanced chemiluminescence method (Amersham).

Statistics

Data were presented as mean ±SEM; they were analyzed
by paired t-test within the same experimental group and by
one-way ANOVA for repeated measures within different ex-
perimental groups. When significant differences were ob-
erved, the mean values were evaluated with Tukey’s post-hoc
test. A p value less than 0.05 was considered significant.

RESULTS

Functional parameters

The baseline values of LVDP, +dP/dt, LVEDP and coro-
nary flow were not different between experimental groups.
Ischemia caused a rapid drop in LVDP, +dP/dt and coronary
flow; these parameters stayed at zero point during ischemia
for 45-min. On reperfusion, IPC enhanced LVDP and +dP/
dt recovery, but CPC did not. LVDP and dP/dt recovery on
reperfusion were greater in the IPC group (>90% of pre-
ischemic value) than in the ischemic control group (<70% of
preischemic value), however, these parameters in the CPC and
CPC+PKCi groups were less than 50% of preischemic values
(Fig. 2). Coronary flow recovery in the IPC group was greater
than in the ischemic control group. It reached near to pre-
ischemic value and maintained approximately at >90% of
preischemic value in the IPC group, but difference between
the IPC and ischemic control groups (80-90% of preischemic
value) was not evident. In contrast, CPC and CPC+PKCi
depressed the coronary flow recovery. Coronary flow recovery
was smaller in the CPC group (<60% of preischemic value)
than that in the ischemic control group (Fig. 2), but the dif-
ference was not evident between the CPC and CPC+PKCi
groups. In all experimental groups, LVEDP was increased by
35 min after ischemia and further increased immediately after
the onset of reperfusion. The increased levels of LVEDP last-
ed throughout reperfusion both in the ischemic control and
IPC group, however, the increase was less prominent in the
IPC group (p<0.05). This result means that IPC attenuated
myocardial contracture during reperfusion. In the IPC group,
LVEDP reached a plateau (>100 mmHg, p<0.01 vs ischemic
control group) in the earlier period of reperfusion and pro-
gressively decreased afterwards (approximately 50 mmHg
at the end of reperfusion).

---

Fig. 2. Changes of the recovery rate of left ventricular developed pressure (LVDP), contractility (+dP/dt), left ventricular end-diastolic pres-
sure (LVEDP), and coronary flow during ischemia and reperfusion. *p<0.05, **p<0.01, ischemic control vs. IPC; *p<0.05, CPC+PKCi vs.
CPC; *p<0.05, ischemic control vs. CPC+PKCi.
Infarct size (%) of left ventricle

Infarct size was reduced by IPC and CPC but reduction of infarct size is abolished by PKC inhibition with calphostin C (CPC+PKCi). *p<0.05, vs. ischemic control.

Infarct size and LDH leakage

Infarct size (Fig. 3, 4) was expressed as a percentage of the left ventricle including the interventricular septum. We did not assume risk area since the entire myocardium might be at risk in global ischemia. Compared with the ischemic control (45.2±2.4%), the infarct size was reduced by IPC (21.0±1.3%, p<0.05) and CPC (25.5±2.2%, p<0.05); however, infarct size was not reduced or limited by PKC treatment (CPC+PKCi, 37.9±2.5%).

LDH leakage at the baseline in the coronary effluent of the ischemic control, IPC, CPC, and CPC+PKCi groups were 6.00±0.62, 5.29±0.50, 5.50±0.54, 5.81±0.60 unit/mL, respectively. It was significantly increased in the ischemic control and CPC+PKCi groups on reperfusion (p<0.05 vs CPC group, 5-20 min), however, no significant difference was found between the IPC and CPC groups (Fig. 5).

PKC activity and Western blot analysis of PKC isozymes

Total PKC activity in the baseline, after 45-min ischemia, IPC, IPC and 45-min ischemia, CPC, CPC and 45-min ischemia, CPC+PKCi, and CPC+PKCi and 45-min ischemia were 8.57±0.06, 8.31±0.04, 8.75±0.12, 8.81±0.10,
were analyzed by We-

during IPC, in part, result-

and by CPC, while cytosol fraction between groups is not signifi-

cantly different. Base, baseline; PKCi, PKC inhibition with cal-

are increased by IPC, IPC and 45-min ischemia (IPC+45-min Isc),

PKC were evidently increased by IPC, IPC and 45-min ischemia,

PKC inhibition did not increase the activity. These results

ticate that PKC was activated and translocated from the cytosol

to the cell membrane by IPC or CPC.

Five PKC isozymes (α, β, δ, ε, and γ) were analyzed by West-

ern blot with corresponding monoclonal antibodies against
each isozymes. Distribution and intensity of PKCε isozymes

except PKCε were not different between experimental groups
(data not shown). In comparison with the baseline, the mem-
brane fraction of PKCε increased after IPC, IPC and 45-min ischemia, and CPC (Fig. 7).

**DISCUSSION**

As already established, the myocardial cell uses Ca^{2+} as the
essential final step in excitation-contraction coupling, the
process by which depolarization of the cell surface membrane ini-
tiates the interactions between the contractile proteins that
lead to tension development and shortening in the wall of the
heart. Ca^{2+} also mediates stimulus-secretion coupling in a vari-
ety of non-motile cells. As indicated, Ca^{2+} ions, by carrying
signals generated at the cell surface to a variety of intracellu-
lar organelles and proteins, can be viewed as the most impor-
tant of the intracellular messengers (24). It is generally believed
that a high Ca^{2+} influx during ischemia/reperfusion (Ca^{2+} over-
load) causes pathological changes in the ischemia/reperfusion
injury; however, in a rat heart, Ashraf et al. (17) have demon-
strated that repetitive Ca^{2+} depletion and repletion for a short
duration (Ca^{2+} preconditioning, CPC) had induced a signifi-
cant functional recovery and remarkable preservation of cell
structure against ischemia/reperfusion injury. Our results are
partly consistent with Ashraf et al. (17). However, functional
recovery by CPC was not seen, although infarct size was sig-
ificantly reduced by IPC and CPC; IVDP and dP/dt recovered
only 25–30% of the baseline values, compared with those
in the IPC group (80–90% of baseline values) or even in the
ischemic control group (60–70% of baseline values). The rea-
son of depressed functional recovery by CPC in this study is
unclear. Differences in species and experimental methods could
be causing factors, but they are not satisfactory explanations.
Since the early increase of LVEDP on reperfusion after 45-min ischemia in the CPC group was evident, it was doubtful whether
the CPC regimen in this study would cause Ca^{2+} paradox
injury, in which necrosis of the cell is a common feature, or not.
However, functional parameters after Ca^{2+} depletion and reple-
tion in the CPC group were not significantly different from the
baseline values, and LDH leakage was not different between
the IPC and CPC groups after 45-min ischemia. Moreover,
infarct size was reduced or limited by CPC as seen in the IPC
group. From these results, it could be concluded that 5-min Ca^{2+}
depletion and 10-min Ca^{2+} repletion did not cause Ca^{2+} para-
dox injury. Reduced response or sensitivity of contractile pro-
teins to normal Ca^{2+} concentration or reduced energy produc-
tion on reperfusion due to myocardial stunning by CPC could
be another possible factor, although intracellular Ca^{2+} and ener-
gy metabolism were not considered. They can cause a retarded
functional recovery with diastolic abnormalities.

The physiological function of protein kinases is not yet fully
understood, but PKC is activated upon external stimulation of
the myocyte by various ligands including hormones, neuro-
transmitters, and growth factors. Many lines of evidence indi-
cate that PKC is involved in IPC of the heart. In this study,
membrane PKC activity was increased by IPC and CPC, while
PKC inhibition did not increase the activity. These results
indicate that PKC was activated by IPC and CPC, and translo-
cated from the cytosol to the cell membrane. It has been sug-
gested that the elevation of [Ca^{2+}] during IPC, in part, result-
ed from Ca^{2+} entry via voltage-dependent Ca^{2+} channel (8, 25)
triggers activation of PKC. However, cytosol PKC was not
significantly decreased in both IPC and CPC in this study as
in a rat heart (19), in contrast to other studies (8, 26). The
reason for this is unclear, however, there is a possibility that
a small change in the cytosol PKC by CPC or IPC did not
affect the total amount of cytosol PKC, since the amount of
cytosol PKC is much greater than that of membrane PKC.

In addition, in this study, immunoblots of the membrane
PKCε were evidently increased by IPC, IPC and 45-min isch-
emia and by CPC, while concurrent PKC inhibition abol-
ished the increase of membrane PKCε. These results strongly
suggest that the activation of membrane PKCε is closely
related with the infarct size-limiting effects of IPC and CPC.
There are some evidences that specific PKC isozymes were
responsible for the mechanism of IPC or CPC. Ping et al. (27)
described that IPC caused selective translocation of PKCε and

Fig. 7. Western blot analyses of PKCε. Membrane (Memb) PKCε is increased by IPC, IPC and 45-min ischemia (IPC+45-min Isc), and by CPC, while cytosol fraction between groups is not signifi-
cantly different. Base, baseline; PKCi, PKC inhibition with cal-
photin C.
PKC, without demonstrable changes in total myocardial PKC activity in the conscious rabbit heart, and our previous study (20) demonstrated that PKC is a trigger role for cardioprotective effect of IPC in the isolated rabbit heart. In contrast, Miyawaki et al. (19) demonstrated that immunolocalization of PKC\_\alpha and PKC\_\delta increased in the cell membrane of rat hearts. However, there is a controversy over the different PKC-coupled receptor systems, which might be involved in IPC. PKC activator did not reduce infarct size in canine hearts (22), and in porcine hearts, even the infarct size reduced by PKC inhibitor (28). These inconsistent results might have been derived from the following possibilities: 1) activation of PKC was not sufficient to phosphorylate certain key proteins; 2) expression of specific PKC isozymes was not significant to participate in the protection; or 3) mechanical factors of IPC or CPC protocol itself caused an aggravation of myocardial stunning. These experimental data provide that the activation of PKC may not be an universally applicable mechanism for cardioprotection by both IPC and CPC.

**ACKNOWLEDGMENT**

This study was supported by the research grant from Sungkyunkwan University School of Medicine (No. 2002-0145-000), 2002-2003.

**REFERENCES**

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124-36.
2. Sumeray MS, Yellon DM. Ischaemic preconditioning reduces infarct size following global ischaemia in the murine myocardium. Basic Res Cardiol 1998; 93: 384-90.
3. Yabe K-I, Tanonaka K, Koshimizu M, Katsuno T, Takeo S. A role of PKC in the improvement of energy metabolism in preconditioned heart. Basic Res Cardiol 2000; 95: 215-27.
4. Cave AC, Hearse DJ. Ischemic preconditioning and contractile function: Studies with normothermic and hypothermic global ischemia. J Mol Cell Cardiol 1992; 24: 1113-23.
5. Kim H, Kim DJ, Chung HS, Shim SJ, Yoo UH, Rah BJ, Kim HD. Evidence of protein kinase C translocation by ischemic preconditioning in global ischemia model. J Korean Med Sci 1998; 13: 473-82.
6. Kitakaze M, Hori M, Takashima S, Sato H, Kamada T. Augmentation of adenosine production during ischemia as a possible mechanism of myocardial protection in ischemic preconditioning. Circulation 1991; 84 (Suppl I): I-306.
7. Li Y, Kloner RA. The cardioprotective effects of ischemic ‘preconditioning’ are not mediated by adenosine receptors in rat hearts. Circulation 1993; 87: 1642-6.
8. Nade K, Kitakaze M, Sato H, Minamino T, Komamura K, Shinozaki Y, Morii H, Hori M. Role of intracellular Ca\(^{2+}\) in activation of protein kinase C during ischemic preconditioning. Circulation 1997; 96: 1257-65.
9. Schott RJ, Rohmann S, Braun ER, Schaper W. Ischemic preconditioning reduces infarct size in swine myocardium. Circ Res 1990; 66: 1133-42.
10. Matsubara T, Minatoguchi S, Matsuo H, Hayakawa K, Segawa T, Matsuno Y, Watanabe S, Arai M, Uno Y, Kawasako M, Noda T, Takeamura G, Nishigaki K, Fujiwara H. Three minute, but not one minute, ischemia and nicorandil have a preconditioning effect in patients with coronary artery disease. J Am Coll Cardiol 2000; 35: 345-51.
11. Liu GS, Thornton JD, Van Winkle DM, Stanley AWH, Olsson RA, Downey JM. Protection against infarction afforded by preconditioning is mediated by AI-adenosine receptors in rabbit heart. Circulation 1991; 84: 350-6.
12. Mitchell MB, Meng X, Ao L, Brown JM, Harken AH, Banerjee A. Preconditioning of isolated rat heart is mediated by protein kinase C. Circ Res 1995; 76: 73-81.
13. Goto M, Liu Y, Yang X, Ardell JL, Cohen MV, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. Circ Res 1995; 77: 611-21.
14. Schulz JEJ, Hsu AK, Gross GJ. Morphine mimics the cardioprotective effect of ischemic preconditioning via a glibenclamide-sensitive mechanism in the rat heart. Circ Res 1996; 78: 1100-4.
15. Vuorinen K, Ylitalo K, Peuhkurinen K, Raatikainen P, Ala-Rami A, Hassinen IE. Mechanism of ischemic preconditioning in rat myocardium: roles of adenosine, cellular energy state, and mitochondrial F\_F\_ATPase. Circulation 1995; 91: 2810-8.
16. De Albuquerque CP, Gerstenblith G, Weiss RG. Importance of metabolic inhibition and cellular pH in mediating preconditioning contractile and metabolic effects in rat heart. Circ Res 1994; 74: 139-50.
17. Ashraf M, Salekman I, Ahmad M. Ca\(^{2+}\) preconditioning elicits a unique protection against the Ca\(^{2+}\) paradox injury in rat heart. Role of adenosine. Circ Res 1994; 74: 360-7.
18. Armstrong SC, Hoover DB, Delacey MH, Ganote CE. Translocation of PKC, protein phosphatase inhibition and preconditioning of rabbit cardiomyocytes. J Mol Cell Cardiol 1996; 28: 1479-92.
19. Miyawaki H, Zhou X, Ashraf M. Calcium preconditioning elicits strong protection against ischemic injury via protein kinase C signaling pathway. Circ Res 1996; 79: 137-46.
20. Kim H, Kim HC, Chung ST, Kim TH, Kim DJ, Rah BJ, Kim HD. Cardioprotective effect of the ischemic preconditioning: its relation to activation of protein kinase C. Korean Circ J 1999; 29: 602-12.
21. Belkin MB, Brown RD, Wright JG, LaMorte WW, Hobson RW. A new quantitative spectrophotometric assay of ischemia-reperfusion injury in skeletal muscle. Am J Surg 1988; 156: 83-6.
22. Przyklenk K, Sussman MA, Simkhovich BZ, Klomer RA. Does ischemic preconditioning trigger translocation of protein kinase C in the canine model? Circulation 1995; 92: 1546-57.
23. Simkhovich BZ, Przyklenk K, Hale SL, Patterson M, Kloner RA. Direct evidence that ischemic preconditioning does not cause protein kinase C translocation in rabbit heart. Cardiovasc Res 1996; 32: 1064-70.

24. Katz A. Physiology of the Heart, 2nd ed, New York: Raven Press; 1992. pp. 178-9.

25. Miyawaki H, Ashraf M. Ca\textsuperscript{2+} as a mediator of ischemic preconditioning. Circ Res 1997; 80: 790-9.

26. Ping P, Zhang J, Zheng Y-T, Li RCX, Dawn B, Tang X-L, Takano H, Balafanova Z, Bolli R. Demonstration of selective protein kinase C-dependent activation of Src and Lck tyrosine kinases during ischemic preconditioning in conscious rabbits. Circ Res 1999; 85: 542-50.

27. Ping P, Zhang J, Qu Y, Tang X-L, Manchikalapudi S, Cao X, Bolli R. Ischemic preconditioning induces selective translocation of protein kinase C isoforms ε and η in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. Circ Res 1997; 81: 404-14.

28. Vogt A, Barancik M, Weihrauch D, Arras M, Podzuweit T, Schaper W. Protein kinase C inhibitors reduce infarct size in pig hearts in vivo. Circulation 1994; 90 (Suppl I): I-647.