Integrated pharmacological preconditioning in combination with adenosine, a mitochondrial K_{ATP} channel opener and a nitric oxide donor

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Background: Mitochondrial K_{ATP} channel activation is an essential component of ischemic preconditioning. These channels are selectively opened by diazoxide and may be up-regulated by adenosine and nitric oxide. Therefore, pharmacological preconditioning with diazoxide in combination with adenosine and a nitric oxide donor (triple-combination pharmacological preconditioning) may enhance cardioprotection.

Methods and Results: Isolated and perfused rat hearts underwent ischemic preconditioning with 3 cycles of 5 minutes of ischemia and 5 minutes of reperfusion before 5 minutes of oxygenated potassium cardioplegia and 35 minutes of ischemia. Pharmacological preconditioning was performed by adding adenosine, diazoxide, and a nitric oxide donor S-nitroso-N-acetyl-penicillamine each alone or in combinations for 25 minutes followed by 10 minutes washout before cardioplegic arrest. Only triple-combination pharmacological preconditioning conferred significant cardioprotection as documented by highly improved left ventricular function and limited creatine kinase release during reperfusion that was comparable to that afforded by ischemic preconditioning. Mitochondrial K_{ATP} channel activity assessed by flavoprotein oxidation was increased by diazoxide, but no further increase in flavoprotein oxidation was obtained by ischemic preconditioning and triple-combination pharmacological preconditioning. Significant activation of protein kinase C- ϵ was observed in only ischemic preconditioning and triple-combination pharmacological preconditioning. Pretreatment with the mitochondrial K_{ATP} channel inhibitor 5-hydroxydecanoate or the protein kinase C inhibitor chelerythrine abrogated activation of protein kinase C- ϵ and cardioprotection afforded by ischemic preconditioning and triple-combination pharmacological preconditioning.

Conclusions: Integrated pharmacological preconditioning is not simply mediated by enhanced mitochondrial K_{ATP} channel activation, but is presumably mediated through amplified protein kinase C signaling promoted by coordinated interaction of adenosine, mitochondrial K_{ATP} channel activation, and nitric oxide.

We have previously demonstrated that the efficacy of ischemic preconditioning (IPC) under potassium cardioplegia is dependent on the grade of IPC.¹ Low-grade IPC induced by a repeated brief period of ischemia does not enhance myocardial protection over potassium cardioplegia, although high-grade-IPC induced by a repeated longer period of ischemia enhances myocardial protection conferred by potassium cardioplegia. Although IPC is a powerful endogenous form of myocardial protection, application of high-grade

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ischemic stress as a preconditioning challenge is limited in the clinical setting of myocardial protection. This is because the graded phenomenon of IPC has not been demonstrated in human subjects, and clinical trials to identify optimal IPC protocols may not be allowed from an ethical point of view. Thus, pharmacological preconditioning (PPC) represents an ideal alternative to IPC, and greater effort should be exerted to invent pharmacological tools that mimic high-grade IPC.

A major issue in exploring the strategy for successful PPC was obtaining a reliable indicator for cardioprotective signaling. Although the exact signaling cascades generated by IPC have been poorly understood, accumulating evidence suggests that protein kinase C (PKC) activation is a key event in IPC-mediated signal transduction responsible for myocardial protection.^{2,3} Our previous study¹ demonstrated that low-grade IPC is associated with activation of only Ca^{2+} -dependent classical isoform of PKC, while high-grade IPC also activates Ca^{2+} -independent novel isoform of PKC associated with greater myocardial protection than low-grade IPC. Among the novel isoform of PKC, PKC- ϵ has been shown to be an essential molecular link between preconditioning and cardiomyocyte protection.^{4,5} Therefore, it is suggested that PKC- ϵ activity represents the magnitude of cardioprotective signaling provoked by IPC and PPC.

Mitochondrial K_{ATP} (mito K_{ATP}) channels have been implicated in the trigger of IPC⁶ and, thus, mito K_{ATP} channel opener diazoxide has been employed as a tool of PPC.⁷ Diazoxide-induced mito K_{ATP} channel activation could be enhanced by adenosine and nitric oxide (NO), which are also known as the triggers of IPC.^{8,9} It has been shown that adenosine primes opening of mito K_{ATP} channels by diazoxide¹⁰ and NO enhances diazoxide-induced activation of mito K_{ATP} channels.¹⁰ Therefore, it is anticipated that the powerful myocardial protection conferred by IPC compared with any single PPC strategies may be accounted for by a highly activated state of mito K_{ATP} channels under the combined effect of these agents. If this assumption is true, PPC in combination with adenosine, diazoxide, and a NO donor (triple combination PPC; TPPC) could enhance the cardioprotection afforded by each drug alone by augmenting mito K_{ATP} channel activity. Alternatively, robust cardioprotection could be promoted by coordinated interaction of distinct cellular events induced by adenosine, mito K_{ATP} channel activation, and NO that amplifies PKC signaling responsible for cardioprotection. The present study was designed to address this issue.

Materials and Methods

Perfusion Technique

Male Sprague-Dawley rats weighing 250 to 300 g were used in the present study. All experiments were conducted in accordance with the "Guidelines for the Care and Use of Laboratory animals" (NIH

publication No. 86-23, revised 1985). The rat hearts were excised and perfused as described previously¹ except that CaCl_2 of Krebs-Henseleit bicarbonate (KHB) buffer solution was reduced to 1.8 mmol/L.

Isovolumic left ventricular (LV) function was measured as described previously.¹ The hearts with LV-developed pressure (LVDP) lower than 80 mm Hg or heart rate less than 240 bpm at the baseline were excluded from the study. LVDP, heart rate, and coronary flow were expressed as a percentage of the baseline values.

Experimental Protocol

The hearts were randomly assigned to 15 groups (Figure 1). Time-matched control hearts underwent 45 minutes of normal perfusion before 5 minutes of oxygenated potassium cardioplegia (PCP) followed by 35 minutes of global ischemia and 30 minutes of reperfusion. The composition of PCP was the same as that of KHB buffer solution except that the concentration of KCl was increased to 20 mmol/L with equimolar reduction of NaCl. IPC was introduced by 3 cycles of 5 minutes of ischemia and 5 minutes of reperfusion. The last reperfusion of IPC was extended to 10 minutes. PPC groups of hearts received 30 $\mu\text{mol/L}$ adenosine, 50 $\mu\text{mol/L}$ diazoxide, and 50 $\mu\text{mol/L}$ S-nitroso-N-acetyl-penicillamine (SNAP), with each drug either alone or in combinations of two or three drugs for 25 minutes followed by 10 minutes of drug-free perfusion before PCP. The mito K_{ATP} channel inhibitor 5-hydroxydecanoic acid (5-HD; 0.5 mmol/L) or the PKC inhibitor chelerythrine (5 $\mu\text{mol/L}$) was administered for 45 minutes before PCP with or without IPC or TPPC. Adenosine, diazoxide, and chelerythrine were obtained from Sigma Chemical Co. SNAP was purchased from Wako Pure Chemical Co, and 5-HD was obtained from Biomol Research Labs, Inc. Diazoxide, SNAP, and chelerythrine were dissolved in dimethylsulfoxide. The final concentration of dimethylsulfoxide was $<0.05\%$.

Coronary effluent was collected during 30 minutes of reperfusion, and creatine kinase (CK) activity was measured as described previously¹ to assess the severity of cardiomyocyte necrosis. At the end of experiments, a piece of the left ventricular tissue was excised to determine the wet/dry ratio.

Flavoprotein Fluorescence Measurement

The isolated rat heart was placed in a temperature-regulated heart chamber installed in a CAF-110 intracellular ion analyzer (Japan Spectroscopic Co) and was perfused as described above. The experimental system uses a dual-wavelength spectrofluorimeter for exciting and detecting flavoprotein autofluorescence. Fluorescence excitation was provided by a xenon lamp with a band-pass filter centered at 480 nm. The excitation light was diverted onto a circular region of the LV epicardium in diameter of 1 cm by a dichroic mirror. Fluorescence then passed through an emission filter centered at 530 nm before reaching a photomultiplier. Photomultiplier output was digitized and the fluorescence was displayed on a strip-chart recorder. At the end of each experiment dinitrophenol (DNP; 1 mmol/L) was added to elicit full oxidation of mitochondrial flavoprotein. Data were expressed as a percentage of the DNP-induced fluorescence.

Figure 1. Experimental protocols. Control group ($n = 7$) underwent 5 minutes of normothermic oxygenated potassium cardioplegia (shaded box) followed by 35 minutes of global ischemia (filled box) and 30 minutes of reperfusion. Ischemic preconditioning (IPC) group ($n = 7$) received 3 cycles of 5 minutes of ischemia and 5 minutes of reperfusion. Single pharmacological preconditioning (SPPC) groups ($n = 7$) received adenosine (Ad) or diazoxide (Dz) or S-nitroso-N-acetyl-penicillamine (SNAP) for 25 minutes. Double combination pharmacological preconditioning (DPPC) groups ($n = 6$) received Ad + Dz or Ad + SNAP or Dz + SNAP for 25 minutes. Triple-combination pharmacological preconditioning (TPPC) group ($n = 7$) received Ad + Dz + SNAP for 25 minutes. All PPC groups underwent 10 minutes of drug-free perfusion before cardioplegic arrest. In 6 groups of hearts ($n = 6$ each) treated with 5-hydroxydecanoate (HD) or chelerythrine (Che), these drugs were administered for 45 minutes before cardioplegic arrest with or without IPC or TPPC.

Tissue Sample Preparation and PKC- ϵ -Selective Phosphorylation Activity Assay

In the experiments on PKC- ϵ -selective phosphorylation activity assay, LV myocardium of approximately 500 mg was excised just before cardioplegic arrest. The myocardial samples were immediately frozen in liquid nitrogen. The particulate and the cytosol fractions were isolated, and the phosphorylation activity of the ϵ -isoform of PKC was determined as described previously.¹¹ Briefly, 50 μ g of protein from either the particulate or the cytosol fraction were immunoprecipitated overnight with PKC- ϵ antibodies (Santa Cruz Biotechnology). The immunoprecipitates were then subjected to a phosphorylation assay using a PKC assay kit (Upstate Biotechnology).

Statistical Analysis

All numerical data are expressed as mean $\pm$ SEM. Statistical analysis was performed by 1-way ANOVA, followed by the Bonferroni post hoc test.

Results

IPC and TPPC Improve Postischemic Myocardial Performance

None of the baseline hemodynamic variables significantly differed among the experimental groups. LVDP was significantly ($P < .01$) depressed during IPC (Figure 2). Although adenosine, diazoxide, and their combinations had no significant ($P > .2$) effect on LVDP during preconditioning, SNAP and its combination with diazoxide significantly ($P < .05$) increased LVDP during preconditioning. However, TPPC did not significantly ($P > .2$) affect LVDP during preconditioning. Heart rate was significantly reduced by preconditioning with adenosine ($P < .01$) and its combination with diazoxide ($P < .01$) or SNAP ($P < .05$). Coronary flow was markedly ($P < .01$) increased by IPC. A similar increase in coronary flow was noted during PPC with adenosine and SNAP alone. Diazoxide also produced significant ($P < .05$) increase in coronary flow. PPC in combination

Figure 2. Left ventricular function after preconditioning and reperfusion. Isovolumic left ventricular (LV) function was measured just prior to cardioplegic arrest in the control and ischemic preconditioning (IPC) hearts. In pharmacological preconditioning (PPC), hearts with adenosine (Ad), diazoxide (Dz), and S-nitroso-N-acetylpenicillamine (SNAP), alone or in combination with two of them or all of them, LV function was measured at the end of PPC. LV function was also measured 30 minutes after reperfusion. LVDP, HR, and CF indicate LV-developed pressure, heart rate, and coronary flow, respectively. Each bar graph represents mean $\pm$ SEM of 6 or 7 experiments. * $P < .05$, ** $P < .01$ vs control.

TABLE 1. Effect of ischemic preconditioning and pharmacological preconditioning on ischemic contracture, end-diastolic pressure, and peak systolic pressure after reperfusion

	TTC (minutes)	Peak contracture (mm Hg)	LVEDP R30 (mm Hg)	Peak systolic pressure R30 (mm Hg)
Control (n = 7)	21 ± 2	35 ± 2	34 ± 3	93 ± 4
IPC (n = 7)	15 ± 1*	50 ± 3*	18 ± 3*	103 ± 4
Ad (n = 7)	21 ± 1	36 ± 3	29 ± 3	93 ± 6
Dz (n = 7)	22 ± 1	36 ± 3	28 ± 3	91 ± 3
SNAP (n = 7)	18 ± 2	41 ± 3	27 ± 4	92 ± 6
Ad + Dz (n = 6)	21 ± 2	39 ± 3	26 ± 5	94 ± 4
Ad + SNAP (n = 6)	18 ± 2	41 ± 4	27 ± 2	95 ± 6
Dz + SNAP (n = 6)	19 ± 2	41 ± 4	24 ± 4	94 ± 6
Ad + Dz + SNAP (n = 7)	14 ± 1*	47 ± 3*	10 ± 3*	106 ± 5

TTC, Time to the onset of ischemic contracture; LVEDP R30, left ventricular end-diastolic pressure 30 minutes after reperfusion; IPC, ischemic preconditioning; Ad, adenosine; Dz, diazoxide; SNAP, S-nitroso-N-acetyl-penicillamine.

Data are expressed as mean ± SEM.

* $P < .01$ vs control.

with adenosine and SNAP further increased coronary flow, but the increase in coronary flow was not additive. No further increase in coronary flow was noted during TPPC.

Recovery of LVDP after reperfusion was significantly ($P < .01$) improved by IPC. Among the PPC groups, only TPPC conferred significantly ($P < .01$) better recovery of LVDP after reperfusion, which was comparable to that observed with IPC. Similarly, recovery of heart rate after reperfusion was significantly improved only by IPC ($P < .01$) and TPPC ($P < .01$). Coronary flow was also significantly improved only by IPC ($P < .05$) and TPPC ($P < .01$).

IPC and TPPC Accelerate Ischemic Contracture But Alleviate Postischemic Contracture

LVEDP was not changed during IPC and PPC with any treatment modalities. LVEDP rose during ischemia, a phenomenon known as “ischemic contracture.” Because we have previously shown that IPC accelerates ischemic contracture whereas potassium cardioplegia delays it,¹ we have asked whether PPC similarly enhances ischemic contracture without prior ischemia. Time to the onset of ischemic contracture defined as a 5 mm Hg increase in LVEDP from the baseline was significantly ($P < .05$) shortened by IPC (Table 1). Similar shortening of time to the onset of ischemic contracture was observed in only TPPC. Peak contracture was also significantly ($P < .05$) increased by IPC and TPPC. On the contrary, LVEDP 30 minutes after reperfusion was significantly lowered by only IPC ($P < .05$) and TPPC ($P < .01$), although PPC in combination with diazoxide and SNAP marginally ($P = .05$) lowered LVEDP.

IPC and TPPC Inhibit Postischemic Cardiomyocyte Necrosis

Although postischemic LV function was significantly improved by IPC and TPPC, functional recovery may not

always be associated with protection against cardiomyocyte necrosis. Therefore, we have measured CK release during reperfusion. CK release was significantly inhibited by only IPC ($P < .05$) and TPPC ($P < .01$) compared with control (Figure 3).

TPPC Does Not Enhance Diazoxide-Induced Flavoprotein Oxidation

Flavoprotein fluorescence has been measured as an index of mitoK_{ATP} channel activity.¹⁰ Therefore, we have measured flavoprotein fluorescence in the isolated and perfused rat heart to investigate correlation between mitoK_{ATP} channel activity and cardioprotection (Figure 4). Treatment with 0.5 mmol/L 5-HD, a selective mitoK_{ATP} channel blocker, modestly decreased flavoprotein fluorescence (<5% of DNP-induced flavoprotein oxidation) in the normally perfused heart, indicating that there was only a trivial mitoK_{ATP} channel activity under the baseline condition. Flavoprotein fluorescence decreased during each cycle of 5 minutes of ischemia and returned above the baseline during each reperfusion. Maximal flavoprotein oxidation was usually obtained between 10 and 15 minutes after the last reperfusion. Treatment with 5-HD before and during IPC abrogated the increase in flavoprotein oxidation induced by IPC, suggesting that IPC-induced flavoprotein oxidation was mediated by activation of mitoK_{ATP} channels. In contrast, DNP-induced flavoprotein oxidation was not affected by 5-HD (not shown). Diazoxide promptly increased flavoprotein fluorescence, which reached plateau within 5 minutes. The magnitude of flavoprotein oxidation was comparable to that induced by IPC. The increase in flavoprotein oxidation induced by diazoxide was abrogated by pretreatment with 5-HD. Adenosine treatment induced a transient decline of flavoprotein oxidation but did not increase flavoprotein oxidation thereafter nor did it enhance diazoxide-induced flavoprotein oxidation. Subsequent addition of SNAP had also

Figure 3. Creatine kinase release during reperfusion. Coronary effluent was collected during 30 minutes of reperfusion and measured for creatine kinase release. Ischemic preconditioning (IPC) and pharmacological preconditioning with adenosine (Ad), diazoxide (Dz), and S-nitroso-N-acetyl-penicillamine (SNAP), alone or in combination with two of them or all of them, were performed according to the protocol as shown in Figure 1. Each bar graph represents mean $\pm$ SEM of 6 or 7 experiments. * $P < .01$ vs control.

no appreciable effect on diazoxide-induced flavoprotein oxidation. Quantitative analysis showed that maximal flavoprotein oxidation induced by IPC and TPPC was not significantly different ($P > .2$) from that induced by diazoxide alone. Flavoprotein oxidation induced by IPC, diazoxide, and TPPC was significantly inhibited by pretreatment with 5-HD.

IPC and TPPC Activate PKC- ϵ and Inhibition of PKC- ϵ Activity by 5-HD or by a PKC Inhibitor Abrogates Cardioprotection

Because activation of PKC- ϵ is an essential molecular link between IPC and myocardial protection,^{4,5} we have measured activity of this novel PKC isoform in preconditioned hearts. PKC- ϵ activity in the particulate fraction was significantly ($P < .01$) increased by only IPC and TPPC (Figure 5). IPC- and TPPC-induced activation of PKC- ϵ in the particulate fraction was abrogated by pretreatment with 5-HD or a PKC inhibitor chelerythrine. PKC- ϵ activity in the cytosol fraction was not affected by IPC or PPC with any combinations, but was significantly decreased from the baseline level by treatment with chelerythrine alone ($P < .01$) or in combination with IPC or TPPC ($P < .05$).

Pretreatment with 5-HD or chelerythrine had no significant ($P > .1$) effect on baseline hemodynamic variables nor did it affect IPC-induced and TPPC-induced changes in LVDP, heart rate, and coronary flow (not shown). Pretreatment with 5-HD or chelerythrine had also no effect on IPC-induced and TPPC-induced acceleration of ischemic contracture (Figure 6). However, pretreatment with 5-HD or

chelerythrine inhibited improvement of functional recovery (Figure 6) and reduction of CK release (Figure 7) induced by IPC and TPPC, although 5-HD or chelerythrine by itself had no significant effect ($P > .2$) on functional recovery and CK release during reperfusion compared to control.

Discussion

We have examined a cardioprotective effect of adenosine, a mitoK_{ATP} channel opener diazoxide, and an NO donor, SNAP, and compared their efficacy with that conferred by IPC. We have employed these drugs with a preconditioning rather than a pretreatment modality because the former modality provides better therapeutic potential than the latter. Preconditioning is known to carry a memory of cardioprotection that lasts 1 to 2 hours after discontinuation of the preconditioning stimulus and is restored 12 to 24 hours later and lasts 3 to 4 days. Using the experimental model, which fulfills the criterion of early preconditioning, we were able to show that TPPC with adenosine, diazoxide, and SNAP conferred powerful myocardial protection that was comparable or even superior to IPC, although each drug alone or in combination with two of them exerted no significant cardioprotection.

Acute cardioprotective efficacy of single PPC with adenosine, diazoxide, or NO donors remains a controversial issue. There are a number of studies demonstrating that PPC with each drug alone confers significant myocardial protection,^{7,12,13} but at the same time a considerable number of studies have failed to substantiate cardioprotective effects of

Figure 4. Flavoprotein oxidation during ischemic preconditioning and pharmacological preconditioning. Flavoprotein fluorescence was measured as described in Materials and Methods. The upper panel shows representative traces of flavoprotein fluorescence during ischemic preconditioning (IPC) or pharmacological preconditioning with diazoxide (Dz) alone or in combination with adenosine (Ad) and S-nitroso-N-acetylpenicillamine (SNAP) in the presence or absence of 5-hydroxydecanoate (HD). Flavoprotein oxidation was expressed as a percentage of the dinitrophenol (DNP)-induced fluorescence. Filled boxes represent ischemia and a scale bar indicates 5 minutes. The lower panel shows quantitative analysis of maximum flavoprotein oxidation. Each bar graph represents mean $\pm$ SEM of 6 experiments. ** $P < .01$ vs IPC, †† $P < .01$ vs Dz, and # $P < .01$ vs Ad + Dz + SNAP.

Figure 5. Protein kinase C (PKC)-ε-selective phosphorylation activity assay. PKC-ε-selective phosphorylation activity after ischemic preconditioning (IPC) or pharmacological preconditioning with adenosine (Ad), diazoxide (Dz), and S-nitroso-N-acetyl-penicillamine (SNAP), in the presence or absence of 5-hydroxydecanoate (HD) or chelerythrine (Che), was measured in the particulate and the cytosol fractions as described in Materials and Methods. Each bar graph represents mean $\pm$ SEM of 6 experiments. * $P < .05$, ** $P < .01$ vs control, † $P < .05$, †† $P < .01$ vs IPC, and # $P < .05$ vs Ad + Dz + SNAP.

a single drug treatment.¹⁴⁻¹⁶ To the best of our knowledge, no studies using a single PPC strategy have reported such a powerful cardioprotection as documented in our study employing TPPC. It is not surprising to find that only TPPC mimics IPC, because IPC is mediated by complex signaling cascades arising from orchestration of multiple triggers. It is possible that in certain experimental models single PPC can exert some cardioprotection when other essential co-triggers are already activated. In support of this notion is the fact that in a sheep model of regional ischemia and reperfusion a bolus injection of adenosine before ischemia provided only marginal protection against stunning and infarction, while

IPC supplemented with adenosine markedly increased cardioprotection.¹⁷ Our recent study¹⁸ has also demonstrated that adenosine preconditioning had no early benefit in functional protection after global ischemia, although administration of adenosine during IPC was capable of significantly enhancing IPC-induced protection. These observations suggest that adenosine enhancement of IPC-induced cardioprotection is elicited by robust activation of G protein-coupled adenosine receptors in concert with other yet unidentified triggers produced by preconditioning ischemia and reperfusion. In this context, it is speculated that mitoK_{ATP} channel activation and NO are the likely co-triggers of successful

Figure 6. Effect of 5-hydroxydecanoate and chelerythrine on ischemic contracture and the recovery of LV function during reperfusion. *TTC* indicates time to the onset of ischemic contracture. *LVDP R30*, *HR R30*, *CF R30*, and *LVEDP R30* indicate LV-developed pressure, heart rate, coronary flow, and LV end-diastolic pressure measured at 30 minutes after reperfusion, respectively. Ischemic preconditioning (*IPC*) or pharmacological preconditioning with adenosine (*Ad*), diazoxide (*Dz*), and S-nitroso-N-acetyl-penicillamine (*SNAP*), in the presence or absence of 5-hydroxydecanoate (*HD*) or chelerythrine (*Che*), was performed according to the protocol as shown in Figure 1. Each bar graph represents mean $\pm$ SEM of 6 or 7 experiments. **P* < .05, ***P* < .01 vs control, †*P* < .05, ††*P* < .01 vs IPC, and #*P* < .05, ##*P* < .01 vs Ad + Dz + SNAP.

Figure 7. Effect of 5-hydroxydecanoate and chelerythrine on creatine kinase release during reperfusion. Ischemic preconditioning (IPC) or pharmacological preconditioning with adenosine (Ad), diazoxide (Dz), and S-nitroso-N-acetyl-penicillamine (SNAP), in the presence or absence of 5-hydroxydecanoate (HD) or chelerythrine (Che), was performed according to the protocol as shown in Figure 1. Each bar graph represents mean $\pm$ SEM of 6 or 7 experiments. * $P < .01$ vs control, † $P < .05$ vs IPC, and # $P < .05$, ## $P < .01$ vs Ad + Dz + SNAP.

preconditioning with adenosine. Diazoxide was shown to generate reactive oxygen species,^{6,7} which have been implicated in the trigger of IPC.¹⁹ NO has also been implicated in the trigger of IPC.⁹ A recent study²⁰ has shown that stimulation of G protein-coupled receptors requires concomitant presence of reactive oxygen species and NO to form a signaling complex, which is potentially involved in enhanced PKC- ϵ activity by IPC.^{11,21} Although it has been shown that NO alone induces activation of PKC- ϵ ,²² our study demonstrating significant activation of PKC- ϵ by only TPPC suggests that this treatment modality is necessary to promote robust cardioprotective signaling.

Although mitoK_{ATP} channel activation appears to be an essential trigger in both IPC and TPPC because inhibition of mitoK_{ATP} channels with 5-HD abolished cardioprotection conferred by IPC and TPPC, IPC- and TPPC-induced cardioprotection was not associated with enhanced mitoK_{ATP} channel activity over diazoxide alone. Thus, synergistic cardioprotection induced by TPPC is thought to require signaling events not only mediated by mitoK_{ATP} channel activation but also those generated by adenosine and an NO donor. Furthermore, 5-HD or chelerythrine abrogated IPC-induced and TPPC-induced activation of PKC- ϵ and cardioprotection. Therefore, it is hypothesized that integrated PPC is achieved by coordinated interaction of three distinct signaling events mediated by adenosine, mitoK_{ATP} channel

activation, and NO, which amplifies PKC signaling responsible for cardioprotection.

The role of mitoK_{ATP} channels in acquisition of tolerance to ischemia afforded by preconditioning is a considerable matter of debate. The notion that mitoK_{ATP} channels act as a trigger of preconditioning comes from a study demonstrating that diazoxide confers myocardial protection and simultaneous treatment with 5-HD abolishes protection, while treatment with 5-HD after diazoxide treatment can not block protection.⁶ In contrast, the argument for the distal effector role of mitoK_{ATP} channels in preconditioning is based on the fact that 5-HD blocked cardioprotection when administered during ischemia and even reperfusion.²³ Putative actions of mitoK_{ATP} channel activation on energy conservation and protection against mitochondrial Ca²⁺ overload^{24,25} are consistent with an effector role of mitoK_{ATP} channels in preconditioning. Because the present study confirms only the former hypothesis that mitoK_{ATP} channel activation acts as a trigger of cardioprotection conferred by TPPC, an effector role of mitoK_{ATP} channels remains to be investigated.

MitoK_{ATP} channel activity during IPC and TPPC was evaluated by measuring the native autofluorescence of mitochondrial flavoproteins to monitor the redox state of the mitochondria.¹⁰ The rationale of measuring flavoprotein fluorescence is based on the hypothesis that opening of any

potassium-selective ion channels in the inner mitochondrial membrane would tend to dissipate the membrane potential established by the proton pump. Such dissipation accelerates electron transfer by the respiratory chain and leads to net oxidation of the mitochondrial matrix. However, there is an argument against the occurrence of significant uncoupling of mitochondrial electron transport by activation of mitoK_{ATP} channels.²⁴ We have observed nearly 50% increase in flavoprotein oxidation during IPC and TPPC. Increased flavoprotein oxidation during these treatments may not be attributed solely to uncoupling, because addition of the mitochondrial uncoupler DNP abolished contraction and provoked contracture within a few minutes, associated with a comparable increase in flavoprotein oxidation to that observed with IPC, diazoxide, and TPPC. In addition, the increase in flavoprotein oxidation induced by IPC, diazoxide, and TPPC but not DNP was inhibited by 5-HD. These findings suggest that mitoK_{ATP} channel activation may also affect flavoprotein oxidation in a manner distinct from uncoupling of mitochondrial respiration.

A limitation of our study is that flavoprotein fluorescence changes are representative of a restricted region of the left ventricle. The excitation light hits the circular region of inferoposterior LV wall 1 cm in diameter and penetrates through the epicardium less than 0.1 mm deep into the myocardium. The fluorescence change in the middle myocardium and the subendocardium may be different from that in the subepicardium. It should also be considered that the fluorescence signal is originated from both cardiomyocytes and nonmyocytes, although we are interested mainly in mitoK_{ATP} channel activity in cardiomyocytes. Because of such a potential limitation in evaluating mitoK_{ATP} channel activity in the intact heart, caution must be exerted to address a complex role of mitoK_{ATP} channel activation in cardioprotection conferred by IPC and TPPC.

Integrated PPC mimics IPC not only with respect to the efficacy of cardioprotection but also with respect to some other physiological responses, such as an increase in coronary flow. More interestingly, ischemic contracture was accelerated by IPC and TPPC. Although accelerated ischemic contracture by IPC has also been observed by other investigators using animal models with global ischemia,^{26,27} this phenomenon has not been reported in animal models with regional ischemia. Increased contracture may also occur in the regionally ischemic myocardium undergoing IPC, but elevation of LVEDP is probably compensated by enhanced relaxation of the nonischemic myocardium. It is known that ischemic contracture usually occurs as a result of ATP depletion at the cessation of anaerobic glycolysis. However, because IPC preserves ATP during index ischemia,²⁸ accelerated ischemic contracture may not simply be explained by cellular ATP depletion. In addition, because TPPC accelerated ischemic contracture without prior sub-

mission to ischemia, ischemic insult is not an essential prerequisite for IPC-induced acceleration of ischemic contracture. This observation in turn suggests that common signaling pathways are involved in enhanced ischemic contracture induced by IPC and TPPC. The fact that the enhanced ischemic contracture was not affected by treatment with 5-HD or chelerythrine, which effectively inhibited cardioprotection conferred by IPC and TPPC, indicates that the mechanism of accelerated ischemic contracture is not mediated by mitoK_{ATP} channel or PKC activation.

Doses of pharmacological agents are of prime importance in determining the efficacy of PPC. We employed conventional doses of adenosine, diazoxide, and SNAP. However, adenosine is known to exert cardioprotection via 2 distinct mechanisms. A micromolar order of adenosine generated during a brief period of ischemia acts as a trigger of IPC rather than a mediator of cardioprotection, but a millimolar order of adenosine administered upon ischemia may be directly cardioprotective via a cardioplegic property. Such a high dose of adenosine may not be applicable in the clinical setting of myocardial protection because of profound systemic hypotension. The dose of adenosine used in the present study was 30 μ mol/L, which has been shown to increase cardiac interstitial adenosine concentration at least several times higher than that produced by IPC.²⁹ Equivalent doses of adenosine have been employed in clinical trials of adenosine myocardial protection without a considerable adverse hemodynamic effect.³⁰ We found that this dose of adenosine produced acceptable decrease of heart rate, and combination with diazoxide and SNAP even mitigated an adenosine-induced negative chronotropic effect. Duration of preconditioning is also crucial, because preconditioning stimulus usually imposes stress on the heart. It has been shown that prolonged treatment with diazoxide before ischemia discounts the efficacy of preconditioning with diazoxide.³¹ NO donors also act as a promoter and an inhibitor of cardiomyocyte cell death, depending on the dose and duration of treatment.³² In the present study, 25 minutes of PPC was chosen to match the time frame with the IPC protocol. Our preliminary study demonstrated that 15 minutes of TPPC was long enough to confer myocardial protection, which was equipotent to the present PPC protocol, although 5 minutes of TPPC provided no significant protection (H.O. and colleagues, unpublished observation).

In conclusion, integrated PPC in combination with adenosine, diazoxide, and an NO donor confers synergistic cardioprotection that is comparable to that induced by IPC. This pronounced cardioprotection is not simply mediated by enhanced mitoK_{ATP} channel activation but is presumably mediated through coordinated interaction of distinct signals derived from G protein-coupled receptors, mitoK_{ATP} chan-

nels, and NO, which amplifies PKC signaling responsible for cardioprotection.

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