Integrated pharmacological preconditioning and memory of cardioprotection: role of protein kinase C and phosphatidylinositol 3-kinase

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adenosine; apoptosis

MANY YEARS AGO, Murry et al. (28) demonstrated that repeated brief periods of ischemia significantly reduced infarct size after a prolonged period of coronary artery occlusion. This phenomenon has been termed ischemic preconditioning (IPC). Now, it is apparent that IPC is a powerful endogenous tool for myocardial protection against ischemia-reperfusion injury. However, clinical applications of IPC have been limited by ethical concerns. Thus pharmacological preconditioning (PPC) has emerged as an ideal alternative to IPC. PPC employs putative triggers of IPC, such as G protein-coupled receptor agonists, mitochandrial ATP-dependent K⁺ (Kₐtp) channel openers, and nitric oxide (NO) donors. However, the efficacy of early single-drug PPC is variable. Several investigators reported that single-agent PPC was as effective as IPC (10, 26, 29, 33, 39); however, other studies, including our own, showed only marginal cardioprotection by the single-PPC strategy and require a combined-PPC technique in which multiple agents are used to elicit optimal cardioprotection (7, 9, 43, 49). Such conflicting observations may be attributed to differences in species and experimental models but, more importantly, may be related to the strength and duration of cardioprotective signaling activity.

The unique feature of cardioprotection conferred by IPC is the cardioprotective memory, which lasts for up to 1 h after the discontinuation of the preconditioning stimulus (50). Although the exact mechanism whereby IPC acquires the memory of cardioprotection has not been fully elucidated, we previously demonstrated that the memory of cardioprotection that mimics IPC cannot be achieved by simply increasing the dose of drugs that have been employed as an IPC mimetic, i.e., a G protein-coupled receptor agonist, a mitochondrial Kₐtp channel opener, and an NO donor, but is successfully reproduced by combining these drugs (32). Because cardioprotective memory through combined PPC was thought to be mediated by coordinated interaction of cardioprotective signaling, we termed this technique the integrated PPC (43).

Cardioprotective signal transduction responsible for early IPC has been extensively investigated in the last decade. A growing body of evidence indicates that protein kinase C (PKC) and phosphatidylinositol 3 (PI3)-kinase play a crucial role in cardioprotective signal transduction mediated by IPC (3). Thus it is anticipated that PKC and PI3-kinase are also essential signaling elements in cardioprotection mediated by the integrated PPC. Accordingly, we have investigated the effect of single and combined PPC on cardioprotective memory by evaluating PKC and PI3-kinase activity during and after the PPC challenge and the relative contribution of these kinases to cardioprotection.

MATERIALS AND METHODS

Isolation and cultivation of neonatal rat cardiomyocytes. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publ. No. 85-23, Revised 1996) and approved by Kansai Medical University Institutional Committee of Animal Care and Use. Neonatal rat cardiomyocytes (CMCs) were isolated and cultured as described previously (42). Briefly, neonatal rats were killed by decapitation, and their hearts were rapidly removed and placed in ice-cold Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL, Rockville, MD) containing penicillin G (300 U/ml), streptomycin (300 μg/ml), and amphotericin B (0.75 μg/ml; GIBCO-BRL). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
ventricles were minced and incubated at 37°C for 10 min in Joklik’s modified Eagle’s medium (GIBCO-BRL) with 0.01% collagenase (Wako, Osaka, Japan). After incubation, minced pieces were stirred by pipette, and digested cell suspensions were transferred to DMEM with 10% FCS. Precipitate was removed and incubated in buffer with 0.01% collagenase at 37°C for 10 min. These procedures were repeated four to five times. The supernatants were centrifuged at 600 g and resuspended in DMEM with 10% FCS. Cells were plated for 30 min to reduce the number of contaminating non-CMCs. CMCs were pooled, counted, and incubated in DMEM with serum. CMCs (2 × 10^6 cells) were plated on a 60-mm culture dish and incubated for 48 h at 37°C under 5% CO2 in a tissue culture incubator.

**Experimental protocols.** Experimental protocols are shown in Fig. 1. CMCs were cultured overnight in Krebs-Henseleit bicarbonate (KHB) buffer solution containing (in mM) 118 NaCl, 4.7 KCl, 1.7 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 25 NaHCO3, and 10 glucose, pH 7.4 with incubation in 5% CO2. CMCs were then treated with 50 mM adenosine (Sigma, Tokyo, Japan), 50 μM diazoxide (Sigma), and 50 μM S-nitroso-N-acetylpenicillamine (SNAP, Wako) alone or in combination for 15 min; then these drugs were washed out for 30 min before 2 h of incubation in GasPak (Becton Dickinson) in modified KHB buffer solution in which 10 mM glucose was replaced by 2-deoxy-D-glucose. In some experiments where the dose-response effect on PKC and PI3-kinase activities was examined, the dose of adenosine, diazoxide, and SNAP was increased fourfold. The PKC inhibitor chelerythrine (5 μM; Sigma) and the PI3-kinase inhibitor LY-294002 (10 μM; Calbiochem) were administered during the washout period and simulated ischemia. Control CMCs were treated with the vehicle (0.05% dimethylsulfoxide) for 45 min before hypoxia and for 2 h during simulated ischemia. CMCs were reoxy genated for 15 min in the isosmotic KHB or the hyp osmotic modified KHB buffer solution (140 mosM with reduced NaCl) to produce physical stress. Combined treatment with adenosine, diazoxide, and SNAP, each alone or in combination for 15 min; then these drugs were washed out for 30 min and centrifuged for 10 min at 200 g. The supernatant of the medium was quantitatively measured with a Cell Death Detection ELISA kit (Roche) as described previously (42). Briefly, CMCs (2 × 10^6) were centrifuged for 10 min at 200 g. The supernatant of the cell lysate or the supernatant and for 2 h during simulated ischemia. CMCs were then treated with 50 μM of protein, 50 μL of reaction buffer and 5 μL of the 4 mM DEVD-p-nitroanilide substrate (200 μM final concentration) were added, and the cells were incubated at 37°C for 1 h. p-Nitroanilide fluorescence was analyzed using a microtiter plate reader (Bio-Rad) at an emission wavelength of 405 nm.

**DNA fragmentation assay.** DNA fragmentation in CMCs was quantitatively measured with a Cell Death Detection ELISA kit (Roche) as described previously (42). Briefly, CMCs (2 × 10^6) were centrifuged for 10 min at 200 g. The supernatant of the cell lysate or the supernatant and for 2 h during simulated ischemia. CMCs were then treated with 50 μM of protein, 50 μL of reaction buffer and 5 μL of the 4 mM DEVD-p-nitroanilide substrate (200 μM final concentration) were added, and the cells were incubated at 37°C for 1 h. p-Nitroanilide fluorescence was analyzed using a microtiter plate reader (Bio-Rad) at an emission wavelength of 405 nm.

**Lactate dehydrogenase release.** CMC necrosis was determined by lactate dehydrogenase (LDH) release in the culture medium. LDH was measured using an LDH assay kit (Sigma).

**Statistical analysis.** Values are means ± SE. Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s post hoc test. The differences were considered significant at P < 0.05.

**RESULTS**

**Combined treatment with adenosine, diazoxide, and SNAP, but not each alone, provokes sustained activation of PKC-ε and PI3-kinase.** Because PKC-ε and PI3-kinase have consistently been demonstrated as cardioprotective kinases against acute ischemia-reperfusion injury (15, 20, 24), we measured these protein kinase activities as an index for cardioprotective signal transduction. PKC-ε was significantly activated by treatment with adenosine, diazoxide, and SNAP, each alone (Fig. 2A). Similarly, Akt phosphorylation, measured to evaluate PI3-kinase activation, was significantly increased by treatment with adenosine (Fig. 2B). Treatment with diazoxide and SNAP also tended to increase Akt phosphorylation. PKC-ε activity and Akt phosphorylation were additively increased by com-
bined treatment with adenosine, diazoxide, and SNAP. The activation of PKC-ε and the increase in Akt phosphorylation mediated by treatment with adenosine, diazoxide, and SNAP, each alone, dissipated 30 min after washout. In contrast, the combined addition of adenosine, diazoxide, and SNAP produced a sustained increase in PKC-ε activity and Akt phosphorylation after withdrawal of these drugs. Addition of chelerythrine or LY-294002 after withdrawal of adenosine, diazoxide, and SNAP, each alone, did not inhibit caspase-3 activity during reoxygenation (not shown). However, combined-PPC-mediated inhibition of caspase-3 activity was partially blocked by chelerythrine or LY-294002 alone but was completely abrogated by combined addition of chelerythrine and LY-294002.

**Combined treatment with adenosine, diazoxide, and SNAP, but not each alone, prevents DNA fragmentation during reoxygenation in a PKC- and PI3-kinase-dependent manner.** DNA fragmentation was not significantly increased during hypoxia but was increased during reoxygenation (Fig. 5). PPC with adenosine, diazoxide, and SNAP individually did not inhibit

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**Fig. 2. Effect of single and combined addition of PPC drugs on PKC-ε and phosphatidylinositol 3 (PI3)-kinase activities.**

**A:** PKC-ε activity assay. **B:** immunoblot analysis of phosphorylated Akt (phospho-Akt, p-Akt) and total Akt. PKC-ε activity and phospho-Akt were measured 15 min after addition of 50 μM adenosine, 50 μM diazoxide, 50 μM SNAP, or CP and 30 min after washout of adenosine (−Ad), diazoxide (−Dz), SNAP (−SNAP), or combined drugs (−CP). Chelerythrine and LY-294002 were administered alone or in combination during washout. Values are means ± SE of 5 experiments. †P < 0.05; ††P < 0.01 vs. control. *P < 0.05; **P < 0.01 vs. −CP.

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**Fig. 3. Effect of high dose of a single PPC drug on PKC-ε and PI3-kinase activities.**

**A:** PKC-ε activity assay. **B:** immunoblot analysis of phospho-Akt (p-Akt) and total Akt. PKC-ε activity and phospho-Akt were measured 15 min after addition of 200 μM adenosine, 200 μM diazoxide, or 200 μM SNAP and 30 min after washout of adenosine, diazoxide, or SNAP. †P < 0.05; ††P < 0.01 vs. control.
DNA fragmentation during reoxygenation. In contrast, the combined-PPC protocol significantly inhibited DNA fragmentation during reoxygenation. Chelerythrine and LY-294002 administered alone or in combination did not significantly increase DNA fragmentation during reoxygenation (not shown). However, combined-PPC-mediated inhibition of DNA fragmentation was partially blocked by chelerythrine or LY-294002 alone but was completely abrogated by combined addition of chelerythrine and LY-294002.

Combined treatment with adenosine, diazoxide, and SNAP, but not each alone, prevents LDH release during reoxygenation under the hyposmotic condition in a PKC- and PI3-kinase-dependent manner. No significant LDH release was observed during reoxygenation under the isosmotic condition (Fig. 6). Next, the hyposmotic buffer was employed to induce LDH release by imposing physical stress on the sarcolemmal membrane (4). Indeed, significant LDH release occurred during reoxygenation under the hyposmotic condition, which did not cause significant LDH release in normoxic CMCs. PPC with adenosine, diazoxide, and SNAP individually did not inhibit LDH release under the hyposmotic condition during reoxygenation. In contrast, combined PPC significantly inhibited LDH release under the hyposmotic condition during reoxygenation. Chelerythrine and LY-294002 administered alone or in combination did not significantly increase LDH release during reoxygenation under the hyposmotic condition (not shown). However, combined-PPC-mediated inhibition of LDH release was partially blocked by chelerythrine or LY-294002 but was completely abrogated by combined addition of chelerythrine and LY-294002.

DISCUSSION

We previously demonstrated that integrated PPC with three putative triggers of IPC, i.e., a G protein-coupled receptor agonist, a mitochondrial K_{ATP} channel opener, and an NO donor, at a dose that does not confer cardioprotection alone or in combination with two triggering agents, produced powerful cardioprotection comparable to that produced by IPC in the isolated rat heart model (32, 43). The present study confirmed the efficacy of this combined-PPC technique in the cultured CMC model and demonstrated that combined PPC with adenosine, diazoxide, and an NO donor confers the memory of cardioprotection by sustained activation of PKC-ε and PI3-kinase. The sustained increase in PKC-ε and PI3-kinase activity and cardioprotective memory could not be achieved by simply increasing the dose of each drug; however, it was successfully reproduced by combining these drugs. These results suggest that the sustained activation of PKC-ε and PI3-kinase and the memory of cardioprotection are mediated by combined PPC with adenosine, diazoxide, and SNAP.

The memory of cardioprotection conferred by preconditioning and the underlying mechanisms have been a subject of extensive research for many years. It has been suggested that the mitochondrial K_{ATP} channel and PKC create a positive-feedback loop during the memory phase of IPC (44). Recent studies (21, 38) raised the hypothesis that reactive oxygen species (ROS) generated through activation of mitochondrial K_{ATP} channels play a pivotal role in the memory of cardioprotection. Juhaszova and associates (21) suggested that characteristic memory of PPC is mediated by moderate, reversible, and sustained mitochondrial swelling, which causes increased...
generation of ROS and, consequently, redox activation of PKC-ε. The persistence of mitochondrial swelling appears to be promoted by feedback amplification of mitochondrial K$_{ATP}$ channel activity by PKC-ε.

Although adenosine alone can activate mitochondrial K$_{ATP}$ channels and the endothelial isoform of NO synthase through the activation of PKC and PI3-kinase, respectively (35, 48), the present study suggests that coordinated interaction of distinct and overlapping downstream signaling generated by diacylglycerol and SNAP is necessary to amplify PKC and PI3-kinase activities. Adenosine stimulates adenosine A$_1$, A$_2$, and A$_3$ receptors, which are coupled with Gi/o proteins (31). Gi/o protein activation results in membrane translocation and phosphorylation of PKC in a Ca$^{2+}$-dependent (in the case of the classical PKC isoforms) and Ca$^{2+}$-independent (in the case of the novel PKC isoforms) manner by generating the lipid second messenger diacylglycerol (12). The heterotrimeric G proteins can also activate PI3-kinase via the transactivation of receptor tyrosine kinases through the generation of ROS in certain cell types (27, 45). Although several Gi/o-coupled receptor agonists can generate ROS through the activation of mitochondrial K$_{ATP}$ channels in the heart (10), ROS generation by Gi/o-coupled receptor stimulation may be transient, and the redox signaling is attenuated over time. Persistent generation of ROS can be obtained by diazoxide administration through the opening of mitochondrial K$_{ATP}$ channels (25). This mechanism is also essential for activation of PKC by diazoxide (22). Indeed, Sato and associates (35) demonstrated that the primary effect of PKC activation by adenosine on mitochondrial K$_{ATP}$ channel activation was to prime and enhance the channel activation by diazoxide but not to open the channels. On the other hand, NO-induced activation of PKC-ε occurs through ROS-dependent tyrosine nitration of PKC-ε (6) or through cGMP-dependent activation of mitochondrial K$_{ATP}$ channels (47). Moreover, cardioprotective signal transduction induced by NO proceeds via the formation of the PKC-ε-Src kinase signaling module (46), each element of which may interdependently upregulate the other’s kinase activity (19). Src kinase then leads to PI3-kinase activation through protein-protein interaction (1). Thus it seems likely that ROS and NO signaling produced by adenosine is insufficient for sustained activation of PKC-ε and PI3-kinase after the withdrawal of adenosine, and additional ROS and NO are necessary to provoke positive feedback and feedforward amplification of PKC-ε and PI3-kinase activities. However, it has been shown that acetylcholine and adenosine provoke sustained activation of PI3-kinase after withdrawal of these drugs in the isolated rabbit heart (23). In that model, acetylcholine and adenosine, each alone, appear to be capable of producing the memory of cardioprotection. Therefore, in certain experimental models, but not in others, G protein-coupled receptor agonists alone can produce ROS and NO or alternative molecules in an amount sufficient to integrate the positive amplification loop for sustained activation of PKC-ε and PI3-kinase.

We investigated the relative position of PKC and PI3-kinase in the integrated PPC-mediated cardioprotective signal transduction by employing the PKC inhibitor chelerythrine and the PI3-kinase inhibitor LY-294002. Administration of chelerythrine and LY-294002, each alone, during the washout period partially inhibited PKC-ε and PI3-kinase activities mediated by integrated PPC, whereas combined addition of chelerythrine and LY-294002 completely inhibited activation of PKC-ε and PI3-kinase. These results suggest that the cross-talk between PKC-ε and PI3-kinase exists in a parallel position, and the activities of each of these kinases are regulated, at least in part, by the other’s kinase, as is the case for the PKC-ε-Src kinase signaling module (46). It has been demonstrated that PI3-kinase exists upstream of PKC in cardioprotection mediated by IPC in the isolated and perfused rat heart model (40). Such a disparate observation may arise from the different experimental models and the preconditioning protocol. However, caution must be taken to interpret our results, because chelerythrine and LY-294002 are not completely specific for PKC and PI3-kinase, respectively.

We also investigated the relative contribution of PKC and PI3-kinase to the integrated PPC-mediated cardioprotection against apoptosis and necrosis. The apoptotic cascade was not activated during hypoxia in CMCs, as evaluated by caspase-3 activity and DNA fragmentation, but was activated during reoxygenation. This finding agrees with the previous observation that mitochondrial permeability transition pores, the regulatory channels for mitochondria-mediated death pathways (16), remain closed during ischemia, even though they are primed for activation but open on reperfusion (17). However, reoxygenation did not induce necrosis in CMCs under the isomotic condition, as evaluated by LDH release, but reoxygenation indeed induced necrosis under the hypoxic condition, which did not cause significant LDH release in normoxic CMCs. This finding suggests that fragility of the sarcolemma underlies the mechanism of necrosis in CMCs during reoxygenation. It has long been known that sarcolemmal fragility is a characteristic feature of reperfusion injury in the heart (14). Treatment with chelerythrine and LY-294002, each alone, before simulated ischemia was partially effective in blocking the inhibitory effect of integrated PPC on caspase-3 activation, DNA fragmentation, and LDH release during reoxygenation, whereas coadministration of chelerythrine and LY-294002 completely abrogated the combined-PPC-mediated cardioprotection against apoptosis and necrosis. These results suggest that the distal effectors activated by PKC-ε and PI3-kinase are different but ultimately converge on protection of mitochondria, because apoptosis and necrosis occur primarily as a consequence of mitochondrial damage.

Although the discrimination of apoptosis and necrosis is critical in the pathophysiology of ischemia and reperfusion injury, the present study did not provide a definite answer for this issue. It should be noted that DNA fragmentation is not specific for apoptosis but is also induced by necrosis associated with random DNA fragmentation at a late stage by release of lysosomal DNases (36). Our study demonstrating that caspase-3 activation and DNA fragmentation occurred without significant LDH release during reoxygenation under the isomotic condition suggests that necrosis made no major contribution to DNA fragmentation in this experimental model. Nevertheless, more specific methods for the detection of apoptosis and the use of small interfering RNA targeting caspase-3 mRNA or a dominant-negative caspase-3 overexpression would be necessary to unequivocally address the role of caspase-3 in CMC injury during reoxygenation. In addition, because activation of the mitochondria-mediated cell death pathway upstream from caspase-3 is determined by the quantitative ratio of antiapoptotic to proapoptotic Bcl-2 family proteins, the role of PI3-kinase activation in this context remains to be elucidated.
proteins in mitochondria (34), it would be intriguing to elucidate the relation between mitochondrial association of Bcl-2 family proteins and cardioprotection of PPC.

Among the distal effectors of PKC-ε, mitochondrial K\textsubscript{ATP} channels (30), sarcolemmal K\textsubscript{ATP} channels (2), and mitochondrial voltage-dependent anion channels (5) are the targets of PKC-ε responsible for cardioprotection. PI3-kinase is also known to activate signal transduction that converges on mitochondrial protection. It has been demonstrated that PI3-kinase confers cardioprotection by phosphorylating Akt, the substrate of PI3-kinase, which then modulates the phosphorylation status of several downstream kinases, such as glycogen synthase kinase-3β and the target of rapamycin (18). A growing body of evidence suggests that inactivation of glycogen synthase kinase-3β by Akt-dependent phosphorylation plays a cardioprotective role by inhibiting mitochondrial permeability transition (21). Akt also phosphorylates apoptosis-regulatory molecules such as Bad, procaspase-9, and cAMP-responsive element-binding protein in certain cell types (8, 11, 13). Recently, Uchiyama and associates (41) demonstrated that dominant-negative Akt abolished an antiapoptotic effect mediated by hypoxic preconditioning, and this detrimental effect was associated with the abrogation of phosphorylation of Bad in conjunction with the release of cytochrome c and the activation of caspase-3 in adult rat ventricular myocytes.

In conclusion, integrated PPC in combination with adenine, diazoxide, and SNAP, but not individually, confers cardioprotective memory against CMC apoptosis and necrosis during reoxygenation. This memory of cardioprotective signal transduction is likely to be mediated by coordinated interaction of distinct cellular events produced by the G protein-coupled receptor agonist, the mitochondrial K\textsubscript{ATP} channel opener, and the NO donor, promoting interdependent amplification of PKC-ε and PI3-kinase signaling.

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REFERENCES


