Cardioprotective Effect Afforded by Transient Exposure to Phosphodiesterase III Inhibitors

The Role of Protein Kinase A and p38 Mitogen-Activated Protein Kinase

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Background—Phosphodiesterase III inhibitors (PDE-III-Is) improve the hemodynamic status of heart failure via inotropic/vasodilatory effects attributable to the increase in intracellular cAMP level. Direct cardioprotection by PDE-III-Is and its underlying mechanisms, however, have not been identified. We tested the infarct size–limiting effect of PDE-III-Is and the roles of cAMP, protein kinase (PK) A, PKC, and mitogen-activated protein kinase (MAPK) families in open-chest dogs.

Methods and Results—Milrinone, olprinone (PDE-III-Is), or dibutyryl-cAMP (db-cAMP) was injected intravenously 30 minutes before 90-minute ischemia, followed by 6 hours of reperfusion. Olprinone was also examined with an intracoronary cotreatment with a PKA inhibitor (H89), a PKC inhibitor (GF109203X), an extracellular signal–regulated kinase kinase (MEK) inhibitor (PD98059), or a p38 MAPK inhibitor (SB203580) throughout the preischemic period. Either PDE-III-Is or db-cAMP caused substantial hemodynamic changes, which returned to control levels in 30 minutes. Collateral flow and percent risk area were identical for all groups. Both PDE-III-Is and db-cAMP increased myocardial p38 MAPK activity during the preischemic period, which was blocked by H89, but not by GF109203X. Both PDE-III-Is and db-cAMP reduced infarct size (19.1 ± 4.1%, 17.5 ± 3.3%, and 20.3 ± 4.8%, respectively, versus 36.1 ± 6.2% control, P < 0.05 each). Furthermore, the effect of olprinone was blunted by either H89 (35.5 ± 6.4%) or SB203580 (32.6 ± 5.9%), but not by GF109203X or PD98059. H89, GF109203X, PD98059, or SB203580 alone did not influence infarct size.

Conclusions—Pretreatment with PDE-III-Is has cardioprotective effects via cAMP-, PKA-, and p38 MAPK–dependent but PKC-independent mechanisms in canine hearts. (Circulation. 2001;104:705-710.)

Key Words: phosphodiesterase ■ infarction ■ kinases

Phosphodiesterase type III inhibitors (PDE-III-Is) used for the treatment of severe heart failure have 2 major cardiovascular effects,1 ie, vasodilatory and inotropic effects, via the elevation of intracellular cAMP levels in both vascular smooth muscle cells2 and cardiomyocytes.3 The former effect reduces vascular resistance, and the latter increases myocardial contractility to improve hemodynamic status,4 with less cardiac oxygen demand than catecholamines.5 It has been shown,6 however, that long-time exposure to either inotropic or cardiotonic agents fails to provide any better outcomes. Conversely, Adamopoulos et al7 reported that intermittent short-time exposure to dobutamine, a potent β-adrenoceptor agonist, for 3 weeks causes better functional recovery and exercise tolerance for several months in patients with chronic heart failure. Furthermore, Lochner et al8 showed that transient β-adrenergic stimulation before sustained ischemia mimics the cardioprotection of ischemic preconditioning.9 Thus, we hypothesized that there is a novel cardioprotective mechanism afforded by transient but not persistent use of inotropic/cardiotonic agents that overcome or mask the demerits of long-term β-adrenergic stimulation, such as β-adrenoceptor downregulation10 or proarrhythmic effects.6

Treatment with PDE III-Is or catecholamines increases intracellular calcium levels ([Ca2+]i) in cardiomyocytes.1 We have reported that a transient increase in myocardial [Ca2+]i mimics the infarct size limitation by ischemic preconditioning via protein kinase C (PKC) activation.11 Therefore, transient pretreatment with PDE-III-Is may reduce infarct size,
Experimental Protocols

Protocol 1: Effects of PDEIII-Is or cAMP on Infarct Size

We used 34 dogs in this protocol. After hemodynamic stabilization, we administered the PDEIII-Is milrinone (Yamanouchi, 30 μg/kg) or olprinone (Eisai, 20 μg/kg), dibutyryl cAMP (db-cAMP, Sigma, 5 mg/kg), or saline intravenously (Mil group, n=8; Olp group, n=8; db-cAMP group, n=9; and control group, n=9, respectively) and observed hemodynamic changes for 30 minutes. The agents were dissolved in saline. Then, the bypass tube was occluded for 90 minutes, followed by 6 hours of reperfusion. The doses of these agents, determined according to our preliminary experiments (data not shown), provide significant hemodynamic effects and return to the control level in 30 minutes.

Protocol 2: Effects of Inhibition of Calcium, PKA, PKC, and p38 MAPK on the Infarct Size–Limiting Effect of the PDEIII-Is

In another 40 dogs, procedures identical to those in protocol 1 were performed with olprinone, with an intracoronary infusion into the LAD of the calcium chelator EGTA (Sigma; 0.57 mg/kg · min⁻¹ · 34 mg/mL at an infusion rate of 0.0167 mL · kg⁻¹ · min⁻¹), the selective PKA inhibitor H89 (Alexis; 1.35 μg · kg⁻¹ · min⁻¹; 80 μg/mL at the same rate), the selective PKC inhibitor GF109203X (GFX, Sigma; 0.3 μg · kg⁻¹ · min⁻¹; 18 μg/mL at the same rate), the selective MEK inhibitor PD98059 (PD, Calbiochem; 13.5 μg · kg⁻¹ · min⁻¹; 0.8 mg/mL at the same rate), or the selective p38 MAPK inhibitor SB203580 (SB, Calbiochem; 1.18 μg · kg⁻¹ · min⁻¹; 70 μg/mL at the same rate), between 5 minutes before intravenous injection and the onset of bypass occlusion (Olp+EGTA group, n=10; Olp+H89 group, n=7; Olp+GFX group, n=7; Olp+PD group, n=7; and Olp+SB group, n=9, respectively). The drugs were dissolved in saline with polyethylene glycol and ethanol (vehicle; final dose of <1%). The dose of EGTA was confirmed in our previous report to block the calcium-induced preconditioning effect without any systemic hemodynamic changes. The doses of other drugs were determined according to previous reports. We were also evaluated to inhibit the individual kinases specifically in this system in the present study. In 46 other dogs, we administered EGTA, H89, GF109203X, PD98059, SB203580, or vehicle alone identically for 35 minutes before bypass occlusion to check their effects on infarct size (EGTA group, n=6; H89 group, n=8; GFX group, n=7; PD group, n=7; SB group, n=9; and vehicle group, n=9, respectively). In protocols 1 and 2, we measured myocardial collateral blood flow at 80 minutes of ischemia by injecting nonradioactive microspheres into the left atrium and comparing the content of accumulated spheres in the ischemic myocardium with those in the arterial blood, as described previously. Infract size was evaluated as described previously. After 6 hours of reperfusion, the LAD was reoccluded, and Evans blue dye was injected intravenously to determine the risk/nonischemic area. The heart was then removed and sliced 6 to 7 mm in width. The nonischemic area was identified.
by blue stain, and the ischemic region was incubated for triphenyl-tetrazolium chloride staining to determine the infarct area.

**Protocol 3: Specificity of the Individual Kinase Inhibitors and Effect of PDEIII-Is on the Kinase Activity in the In Vivo Canine Model**

We evaluated the specificity of the individual kinase inhibitors used in this study on inhibiting PKA, PKC, or MAPKs (ERK, JNK, and p38 MAPK) in the in vivo canine system. Thirty other dogs received intravenous bolus injection of either db-cAMP (5 mg/kg), phorbol 12-myristate 13-acetate (PMA; 50 μg/kg), or anisomycin (10 μg/kg), with intracoronary infusion of saline, H89, PD98059, or SB203580 into the LAD (see Figure 1 for details) (n=3 each). After 20 minutes of each injection, we quickly placed samples of myocardial tissue supplied by the LAD into liquid nitrogen and stored them at −80°C. In this protocol, the kinase activities were assayed as follows: for PKC, each myocardial sample was homogenized, separated into membrane and cytosolic fractions by centrifugation, immunoprecipitated, and subjected to Western blotting with specific antibodies against PKC-α, -β, and -γ (Amersham) as described previously.19 For PKA, JNK, and ERK, each myocardial sample was homogenized, immunoprecipitated, and subjected to Western blotting as described previously20,21 with specific antibodies against phospho-CREB (Upstate Biotechnology) for PKA, specific antibodies against phospho-Jun (Upstate Biotechnology) for JNK, and specific antibody against phospho ERK-1/2 (Upstate Biotechnology), respectively. Other dogs, after hemodynamic stabilization, received intravenous bolus injection of either saline, db-cAMP, milrinone, or olprinone (control, db-cAMP, Mil, or the Olp groups, respectively; n=4 each), along with an intracoronary infusion of H89 or GF109203X into the LAD from 5 minutes before the intravenous injection and for 25 minutes (Mil+H89, Olp+H89, Mil+GFX, or Olp+GFX group, respectively; n=4 each; see Figure 1 for details). Thereafter, we quickly sampled myocardial tissue into liquid nitrogen and stored it at −80°C. One gram of each myocardial tissue sample in protocol 3 was homogenized, immunoprecipitated, and subjected to in vitro p38 MAPK activity assay as reported previously.22 Then, homogenates in extraction buffer were incubated with rabbit antiserum raised against the COOH-terminal peptide sequence of p38 MAPK (Upstate Biotechnology) or recombinant protein G-Sepharose 4B (Zymed Laboratories) was added, they were incubated and washed with extraction buffer and PAN buffer. For kinase assay, the immunoprecipitates were suspended in assay buffer containing a recombinant amino-terminal fragment of ATF-2 (20 to 50 ng) (Upstate Biotechnology) as a substrate and [γ-32P]ATP. After the reaction was terminated, the mixture was boiled, separated by electrophoresis on an SDS gel, and subjected to autoradiography. The kinase activity was quantified with a PhosphorImager (Molecular Dynamics).

All details of the above protocols are given in Figure 1.

**Criteria for Exclusion**

To ensure that all of the animals included in the data analysis of infarct size were healthy and exposed to similar extents of ischemia, the previously described standards18 were used.

**Statistical Analysis**

Each value was expressed as mean±SEM. Statistical analyses were performed by use of ANOVA with Fisher’s post hoc test to determine significance at the P<0.05 level for group pairs that exhibited statistically significant differences.

**Results**

**Mortality and Exclusions**

One hundred twenty dogs were randomly divided into 15 groups for assessment of infarct size and ventricular fibrillation. Eight and 13 dogs were matched to the exclusion criteria of ventricular fibrillation during sustained ischemia and during reperfusion, respectively. Eleven were also excluded because of myocardial collateral blood flow. Therefore, 88 dogs were used for data analysis.

**Changes in Hemodynamic Parameters, Risk Area, and Collateral Blood Flow**

The current changes of mean ABP and HR were compared in the 15 groups in protocols 1 and 2 before the onset of...
coronary occlusion (Figure 2). PDEIII-Is and db-cAMP caused an ≈15% decrease in mean ABP (2A) and ≈15% increase in HR (2B), which was not significantly influenced by the intracoronary administration of EGTA, H89, GF109203X, PD98059, or SB203580 and returned to the control level in 30 minutes. The mean ABP or HR during ischemia and reperfusion and either risk area or collateral blood flow were comparable in all of the 15 groups in protocols 1 and 2 (data not shown).

Infarct Size
Figure 3 shows infarct size in the 15 groups of protocols 1 and 2. Premischemic single treatment with either olprinone, milrinone, or db-cAMP significantly attenuated infarct size (19.1±4.1%, 17.5±3.3%, and 20.3±4.8%, respectively, P<0.05 each versus control). This cardioprotective effect of olprinone was blunted by cotreatment with either H89 or SB203580 (35.5±6.4% and 32.6±5.9%, respectively), but not by EGTA, GF109203X, or PD98059 (15.8±2.9%, 19.1±3.9%, and 21.0±4.1%, respectively). Treatment with EGTA, H89, GF109203X, PD98059, or SB203580 alone did not affect infarct size (37.3±5.3%, 41.0±6.6%, 43.3±7.4%, 38.7±4.5%, and 38.6±6.1%, respectively).

Specificity of the Individual Kinase Inhibitors in This Model and Effect of PDEIII-Is on the Kinase Activity
Figure 4 shows the results for the measurement of p38 MAPK activity, with representative cases (4A) and mean values (4B). Milrinone, olprinone, and db-cAMP equally activated p38 MAPK (2.6- to 2.9-fold), which was completely blunted by H89 and unaffected by GFX.

Figure 5 shows the measurement of PKA (5A), PKC (5B), ERK (5C), or JNK (5D) activity, with the representative cases (top of each panel) and the mean values (bottom of each panel) for each group (mean±SEM). See text for details. Olprinone increased PKA activity in myocardium after 20 minutes of treatment (4.6-fold), which was completely blocked by H89 but was unaffected by SB203580. PMA significantly increased the PKC translocation and the ERK activity (2.8-fold), but olprinone did not activate either of them.

Figure 4. p38 MAPK activity estimated by amount of phosphorylated substrate of activated p38 MAPK. Immunoblotting of representative specimens (A) and changes in mean value (B) (mean±SEM). Each bar indicates mean of n=4 each. Abbreviations as in Figure 1. *P<0.05, **P<0.005 vs control.

Figure 5. Measurement of PKA (A), PKC (B), ERK (C), and JNK (D) activity, with representative cases (top panels) and mean values (bottom panels) for each group (mean±SEM). See text for details. Each bar indicates mean of n=3 each. Abbreviations as in Figure 1.
Neither H89 nor SB203580 affected the translocation of PKC by PMA. PD98059 blunted the activation of ERK, indicating that the dose of PD98059 in this study can inhibit ERK in this system. Anisomycin activated JNK (3.8-fold), whereas olprinone did not. In addition, the dose of SB203580 in this study did not affect JNK by anisomycin.

**Discussion**

We have shown that the transient pretreatment with PDEIII-Is can limit infarct size independently of systemic hemodynamic changes and collateral blood flow. Furthermore, either the augmentation of PKA activity or subsequent p38 MAPK activation mediates this effect. This observation may explain the preconditioning effect by transient β-adrenergic stimulation.8

**Specificity of the Individual Kinase Inhibitors in the In Vivo Canine System**

First, olprinone activated both PKA and p38 MAPK, but not PKC, ERK, or JNK in this system. Of course, in the present canine model, we confirmed that PMA activated both PKC and ERK and anisomycin activated JNK.

Second, SB203580 in this protocol blocked only p38 MAPK activation, not PKA, PKC, or JNK activation, indicating that the dose of SB203580 in this system is specific to p38 MAPK. Although we did not check the direct effect of SB on ERK, both the inability of olprinone to activate ERK and the observation that PD98059, which blocked ERK activation in this system, failed to blunt the infarct size limitation by olprinone confirmed that the effect of SB203580 to blunt the cardioprotective effect by olprinone is afforded by blocking p38 MAPK.

Furthermore, H89, which blocked cardioprotection by olprinone, did block PKA, whereas it did not block PKC, indicating that (1) PKC is not related to the olprinone-induced cardioprotection and (2) the dose of H89 in this study is selective for PKA and does not affect PKC in this system.

Taken together, we can conclude that the pharmacological interventions in this study work properly to activate or block the individual kinases selectively in this canine model.

**Direct Link Between PKA and p38 MAPK**

The present results suggest that p38 MAPK activation plays a crucial role in mediating cardioprotection afforded by short-time exposure to PDEIII-Is, after a transient increase in intracellular cAMP level and PKA activity. PDEIII-Is cause a prompt increase in cAMP in cardiomyocytes, which is maximized within 10 minutes,4 followed by a rapid activation of PKA. Furthermore, PKA activation is reported to cause sufficient augmentation of p38 MAPK activity through a protein tyrosine phosphatase, completely independently of PKC activation.23-24 Therefore, PDEIII-Is can rapidly activate p38 MAPK. We understand, however, that this interpretation of the results is perfectly confirmed when we observe that H89 has no effect on p38 MAPK activity when added directly to the in vitro p38 MAPK activity assay.

**Timing for the Activation of p38 MAPK to Provide Cardioprotection**

The next issue is the time window in which activation of p38 MAPK can mediate cardioprotection. The transient activation of p38 MAPK in the preischemic phase may cause cardioprotection, because we showed here that the concomitant administration of either H89 or SB203580 alone before ischemia abolishes either the enhancement of p38 MAPK activity in the preischemic period or the infarct size–limiting effect. This may be further supported by a recent report.25

Conversely, we do not have direct data on whether the transient use of PDEIII-Is during ischemia/reperfusion is cardioprotective. Nakano et al15 reported that the activation of p38 MAPK is beneficial, because ischemic preconditioning elicits the activation of p38 MAPK during ischemia and protects the myocardium, which does not occur in control ischemia. There are contradictory reports, however, to elucidate the role of p38 MAPK activation during ischemia; the activation of p38 MAPK is unfavorable,25-27 because sustained ischemia and reperfusion activate p38 MAPK in either the ischemic or reperfused phase, which enhances myocardial apoptosis or myocardial death, which is prevented by p38 MAPK inhibition during ischemia or in the early phase of reperfusion. Furthermore, persistent p38 MAPK activation has been established by recent reports28 to be deleterious to the heart, because it can induce myocardial hypertrophy, apoptosis, and fibrosis, leading to cardiac maladaptation or myocardial dysfunction. Thus, we should further consider the role of p38 MAPK activation during ischemia or in early reperfusion in the cardioprotection in this study.

**Further Downstream Effectors of p38 MAPK**

There are some candidates for the downstream effectors of p38 MAPK, which may also be the final effectors of cardioprotection. Because persistent p38 MAPK activation fails to protect myocardium, identification and stimulation of the downstream effectors that are tolerable for persistent stimulation may be essential for beneficial clinical use.

The translocation of HSP27 occurs after ischemic preconditioning through the transient activation of p38 MAPK25 and protects myocardium from ischemic injuries. The previous study reported that the translocated HSP27 accumulates in the Z bands of myofibril29 and prevents conformational changes or fragmentation in myofibril and cytoskeleton.30,31 This may also explain the cardioprotection against contraction failure, because a recent report showed that the structural components of myofibrils, especially in Z bands, are disorganized in failing human heart.32

In addition, it has also been reported that both the opening of ATP-sensitive potassium channels on mitochondria33 and nitric oxide34 are activated or induced by p38 MAPK and also mediate the cardioprotection. Further investigations concerning this issue may reveal the complete cellular mechanisms of cardioprotection afforded by β-adrenergic preconditioning or the mechanism of cardioprotection against ischemia/reperfusion injury.

Finally, taking these arguments together, there may be at least 2 possible ways to extend our present observations to the clinical situation. The direct short-time activation of p38 MAPK instead of transient administration of PDEIII-Is may be useful, because the transient, but not the persistent, activation of p38 MAPK is cardioprotective. Another way may include gene transfer or direct activation of HSP27 to elicit maximal cardioprotection.
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