Differential Effect of Phosphodiesterase-3 Inhibitors on Sympathetic Hyperinnervation in Healed Rat Infarcts

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Background: The effect of phosphodiesterase-3 (PDE-3) inhibitors on arrhythmia remains controversial, so the purpose of this study was to determine their differential effects on sympathetic hyperinnervation and the involved mechanisms in a rat model of myocardial infarction.

Methods and Results: After ligating the coronary artery, male Wistar rats were randomized to cilostazol or milrinone, chemically unrelated inhibitors of PDE-3, or vehicle for 4 weeks. The postinfarction period was associated with increased myocardial norepinephrine levels and oxidant release, as measured by myocardial superoxide level and dihydrouethidine fluorescence staining. Infarcted rats in the milrinone- and cilostazol-treated groups had favorable ventricular remodeling with similar potency. Compared with milrinone, cilostazol significantly increased interstitial adenosine levels and reduced the production of myocardial cAMP and superoxide. Cilostazol significantly blunted sympathetic hyperinnervation, as assessed by immunofluorescent analysis of sympathetic innervation, and western blotting and real-time quantitative RT-PCR of nerve growth factor. Furthermore, the inhibitory effect of cilostazol on nerve growth factor was reversed by 8-cyclopentyl-1,3-dipropylxanthine, a selective A1 receptor antagonist, and enhanced by tempol administration. In spite of similar arrhythmic vulnerability during programmed stimulation in both the vehicle- and cilostazol-treated groups, cilostazol did not have proarrhythmic effects compared with milrinone.

Conclusions: Unlike milrinone, cilostazol has therapeutic neutrality in arrhythmias because of adenosine uptake inhibition, which antagonizes the PDE-3-induced increase of sympathetic reinnervation via mediation of an adenosine A1 receptor-mediated antioxidation. (Circ J 2014; 78: 366–376)

Key Words: Arrhythmia; Myocardial infarction; Phosphodiesterase-3 inhibitors; Sympathetic innervation; Remodeling

Cilostazol, a selective phosphodiesterase inhibitor, was approved by the US FDA in 1999 for the treatment of intermittent claudication. We have previously demonstrated that in patients with peripheral arterial disease it improves their ability to walk. The pharmacological effects of cilostazol apparently involve 2 different mechanisms of action, inhibiting both cyclic nucleotide PDE-3 and adenosine uptake. PDE-3 inhibition elevates the intracellular cAMP concentration, which has been implicated in the genesis of ischemia-induced dysrhythmias and lowering of the fibrillation threshold in the isolated rat heart. Previous studies have shown that PDE-3 inhibitors such as milrinone aggravate ventricular tachyarrhythmias; however, cilostazol is observed to be devoid of such adverse effects. A possible explanation for this different pharmacological profile might be the inhibition of adenosine uptake by cilostazol, but not milrinone, in the heart. In vitro, cilostazol inhibited adenosine uptake with a median effective concentration of 5 μmol/L, whereas milrinone, a selective PDE-3 inhibitor, was ineffective. However, this result has not been consistently observed across studies. Sanganalmath et al showed that cilostazol increased ventricular arrhythmias after myocardial infarction (MI). Thus, on the basis of the information available it is difficult to conclude whether cilostazol exerts arrhythmogenic or antiarrhythmogenic effects.

Increased sympathetic nerve density after myocardial injury is responsible for the occurrence of lethal arrhythmias and sudden cardiac death in humans. During the chronic stage of MI, a regional increase in sympathetic nerve density is commonly observed in the remote zone, and it is increased sympathetic...
nerve activity that plays an important role in the generation of ventricular arrhythmia and sudden cardiac death. Thus, nerve sprouting has been shown to be an important contributing factor in the occurrence of ventricular arrhythmias and sudden cardiac death in the healing or healed stage of MI in both animals and humans. Given the high coincidence of coronary artery disease and peripheral arterial occlusion disease, it is important to assess the long-term effect of cilostazol on arrhythmia. Based on the differential effect of cilostazol and milrinone, we can explore the role of cAMP and adenosine on nerve growth factor (NGF) expression. In the present study, we assessed whether chronic administration of PDE-3 inhibitors, (cilostazol and milrinone) results in hyperinnervation of the heart by MI by measuring the expression of NGF.

Methods

Animals

An expanded Methods section is available in the Supplementary file. All rats received humane care and the experiment was approved and conducted in accordance with local institutional guidelines of the China Medical University for the care and use of laboratory animals and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Protocol 1 (In Vivo Study)

Male Wistar rats (250–300 g) underwent ligation of the anterior descending artery as previously described, resulting in infarction of the left ventricular (LV) free wall. Rats were randomly assigned into 3 groups such that each group would have approximately the same number of survivors: (1) vehicle group (dimethyl sulfoxide); (2) milrinone (3.17 mg·kg⁻¹·day⁻¹, Sigma, St Louis, MO, USA); (3) cilostazol (5 mg·kg⁻¹·day⁻¹). To confirm the role of sympathetic hyperinnervation in arrhythmic vulnerability, we added an infarcted group treated with carvedilol ($\beta$-adrenoceptor blocking agent: 5 mg·kg⁻¹·day⁻¹, Sigma). The dosages of milrinone, cilostazol, and carvedilol were used in this study to exert biological effects. The 5 mg/kg dosage of cilostazol in rats in this study is equivalent to 100 mg in humans after correction of differences in body surface area. Cilostazol and milrinone were dissolved in dimethyl sulfoxide and carvedilol was dissolved in normal saline.

To avoid the confounding factor of limiting infarct size by cilostazol, drug administration began 24 h after infarction, a time when the drugs could produce maximum benefit. The study’s duration was designed to be 4 weeks because most of the myocardial remodeling process in the rat (70–80%) is complete within 3 weeks. Sham-operation served as a control to exclude the possibility of the drugs themselves directly affecting sympathetic reinnervation. In each treatment group, drugs were withdrawn approximately 24 h before the end of the experiments in order to eliminate their pharmacological actions.

Protocol 2 (In Vitro Study)

Cilostazol has inhibitory effects on both adenosine and PDE-3. To differentiate the role of adenosine and cAMP on NGF, we used 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective A1 receptor antagonist, affecting sympathetic reinnervation. In each treatment group, animals were dissolved in normal saline. Each heart was perfused with a noncirculating modified Tyrode’s solution containing (in mmol/L): NaCl 117.0, NaHCO3 23.0, KCl 4.6, NaH2PO4·0.8, MgCl2:1.0, CaCl2:2.0, and glucose 5.5, equilibrated at 37°C and oxygenated with a 95% O2–5% CO2 gas mixture. The perfusion medium was maintained at a constant temperature of 37°C with a constant flow at 4 ml/min as previously described. The dosages of milrinone, cilostazol, tempol, and DPCPX were used according to previous studies. Drugs were infused for 120 min. At the end of the study, all hearts (n=10 in each group) were used for performing western blot of NGF at the remote zone (>2 mm outside the infarct).

Hemodynamics and Infarct Size

At 28 days after operation, hemodynamic parameters were measured by Millar catheter. With respect to clinical importance, only rats with a large infarction (>30%) were selected for analysis.

Spontaneous and Induced Arrhythmias

To induce ventricular arrhythmias, pacing in a Langendorff apparatus was performed at a cycle length of 120 ms (S1) for 8 beats, followed by 1–3 extrastimuli (S2, S3, and S4) at shorter coupling intervals.

Real-Time RT-PCR of NGF

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described. For NGF, the primers were 5´-GGGTACCTGACACCAAATCT-3´ (sense) and 5´-GCGTCCAGAGAAAGCGAAG-3´ (antisense). For glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), the primers were 5´-CTTCAACCATGTGGAGCAAGG-3´ (sense) and 5´-GGCATGACACTGTTGCATGAG-3´ (antisense).

Western Blot Analysis of NGF

Rabbit polyclonal antibodies to NGF (Chemicon) were used as described previously.

Immunofluorescent Studies of Tyrosine Hydroxylase, Growth-Associated Factor 43, and Neurofilament

Tissue samples for assessing sympathetic innervation were incubated with antityrosine hydroxylase (1:200; Chemicon, Temecula, CA, USA), antigungrowth-associated protein 43 (43 a marker of nerve sprouting, 1:400; Chemicon), and antineurofilament antibodies (a marker of sympathetic nerves, 1:1,000; Chemicon).

In Situ Detection of Superoxide

For evaluating myocardial intracellular superoxide production using in situ dihydroethidium (DHE; Invitrogen Molecular Probes, Eugene, OR, USA) fluorescence, OCT-embedded tissues were incubated with DHE.

Interstitial Adenosine Measurement In Vivo

To compare interstitial adenosine levels after administering PDE-3 inhibitors, 12 supplementary infarcted rats (n=4 in each group) were allocated to the vehicle, milrinone, and cilostazol group. At 4 weeks after inducing MI, cardiac microdialysis was performed as previously described. Microdialysis probes (13×0.2 mm ID; PAN-1200; Asahi Chemical, Tokyo, Japan) with a molecular mass cutoff of 50 kDa were implanted in the LV myocardium remote from the LV scar in the MI group and in a nearly similar area of myocardium in the sham group. The inflow dialysis probe was perfused with Ringer solution at a constant temperature of 37°C with a constant flow of 4 ml/min as previously described. The dosages of milrinone, cilostazol, tempol, and DPCPX were used according to previous studies. Drugs were infused for 120 min. At the end of the study, all hearts (n=10 in each group) were used for performing western blot of NGF at the remote zone (>2 mm outside the infarct).
Differences among the infarcted groups in terms of mortality were not found during the study. Neither milrinone nor cilostazol had an effect on cardiac gross morphology in the sham-operated rats (data not shown). Given that only rats with a large infarction >30% were selected, the infarcted area of the LV was very thin and totally replaced by fully differentiated scar tissue 4 weeks after infarction. The weight of the LV inclusive of the septum remained essentially constant among the infarcted groups during the 4 weeks (**Table S1**). Compared with the vehicle-treated infarcted rats, the maximal rate of LV dP/dt max and −dP/dt min was significantly increased and the right ventricular weight/body weight ratio, and lung weight/body weight ratio were significantly lower in the milrinone- or cilostazol-treated infarcted rats, consistent with favorable LV remodeling. LV end-systolic pressure and infarct size did not differ among the infarcted groups.

**Myocardial cAMP, NE, Superoxide, and Interstitial Adenosine Levels**

Infarction was associated with a significant increase in cAMP content (413±78 vs. 254±62 pmol/g LV in sham, \( P<0.01 \); Figure 1A). Treatment with cilostazol resulted in a significant increase in the cAMP levels and cAMP further increased in milrinone-treated infarcted rats.

Discussion of the results and implications for future research are provided in the final section of the manuscript.

**Figure 1.** Myocardial (A) cAMP, (B) norepinephrine, (C) superoxide and (D) interstitial adenosine levels from the remote zone of the LV myocardium after MI in rats. *P<0.01, compared with sham; †P<0.05, compared with vehicle and cilostazol; ‡P<0.05 compared with vehicle. LV, left ventricle; MI, myocardial infarction.

Rate of 3 μl/min. Dialysate sample collection was started 60 min after probe implantation. A sample period lasted 4 min. Samples were immediately frozen, and stored at −70°C until analysis. We measured dialysate adenosine levels using high-performance liquid chromatography.

**Laboratory Measurements of Myocardial cAMP, Norepinephrine, and Superoxide Levels**

LV myocardial tissue samples from the remote zone were pulverized to measure cAMP content using a colorimetric cAMP immunoassay kit (R&D Systems).

Myocardial norepinephrine (NE) levels were measured using a commercial ELISA kit (Noradrenalin ELISA, IBL Immuno-Biological Laboratories Co, Hamburg, Germany).

Superoxide production by the myocardium in the remote zone was measured using lucigenin (5 μmol/L bis-N-methyl-acridinium nitrate; Sigma) enhanced chemiluminescence as previously described. The specific chemiluminescence signal was calculated after subtraction of background activity and expressed as counts per minute per milligram weight.

**Statistical Analysis**

Results are presented as mean±SD. Statistical analysis was performed using the SPSS statistical package (SPSS, version 12.0, Chicago, IL, USA). Differences among groups of rats were tested by an ANOVA. Subsequently analysis for significant differences between 2 groups was performed with a multiple comparison test (Scheffe’s method). Electrophysiological data (scoring of programmed electrical stimulation-induced arrhythmias) were compared by a Kruskal-Wallis test followed by a Mann-Whitney test. Significance was assumed at \( P<0.05 \).

**Results**

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sham and further increased in that from milrinone-treated infarcted rats (Figure 1C). The chronic administration of cilostazol to MI animals lead to a significant decrease in superoxide levels compared with vehicle.

Cilostazol-treated infarcted rats had significantly increased adenosine levels in the interstitial dialysate compared with the sham and further increased in that from milrinone-treated infarcted rats (Figure 1B). When compared with milrinone-treated infarcted rats, cilostazol-treated infarcted rats had significantly lower LV NE levels.

Superoxide production was significantly increased in remote LV tissue from vehicle-treated infarcted rats compared with

Figure 2. Detection of superoxide in rat myocardium by DHE staining. Compared with sham, the DHE fluorescence intensity in the myocardium of the vehicle-treated infarcted group was significantly increased. (A) Sham (n=10); (B) infarction treated with vehicle (n=12); (C) infarction treated with milrinone (n=14); (D) infarction treated with cilostazol (n=13). Bar=50 μm. (Bottom) DHE staining (%) at the remote zone. Each column with bar represents the mean±SD. *P<0.01, compared with sham; †P<0.05, compared with vehicle and cilostazol. Arb.U, arbitrary units.
milrinone group (Figure 1D). There were similar adenosine levels in the sham, vehicle and milrinone groups.

**DHE Staining of Myocardium**

DHE reacts with superoxide radicals to form ethidium bromide, which in turn intercalates with DNA to provide nuclear fluorescence as a marker of superoxide radical generation. As shown in Figure 2, postinfarction remodeling was associated with increased intensity of DHE staining in the vehicle-treated rats compared with sham. When compared with milrinone-treated rats, cilostazol-treated rats had a significantly reduced intensity of the fluorescent signal.

**Figure 3.** Immunofluorescent (fluorescein isothiocyanate) staining for tyrosine hydroxylase from the remote regions (original magnification ×400). Tyrosine hydroxylase-positive nerve fibers are located between myofibrils and oriented longitudinal to the myofibrils. (A) Sham (n=10); (B) infarction treated with vehicle (n=12); (C) infarction treated with milrinone (n=14); (D) infarction treated with cilostazol (n=13). Bar, 50 μm. (Bottom) Nerve density area fraction (%) at the remote zone. Each column with bar represents the mean±SD. *P<0.01, compared with sham; †P<0.05, compared with vehicle and cilostazol.
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significantly lower nerve density than milrinone-treated infarcted rats ($P<0.05$). Similar to the tyrosine hydroxylase results, densities of growth-associated protein 43-positive nerve fibers (data not shown) were significantly attenuated in the cilostazol-treated infarcted rats compared with the milrinone-treated infarcted rats. These

**Immunofluorescent Analysis**

Tyrosine hydroxylase-immunostained nerve fibers appeared to be oriented in the longitudinal axis of adjacent myofibers (Figure 3). Tyrosine hydroxylase-positive nerve density was significantly increased in the vehicle-treated infarcted rats than in the sham group. Cilostazol-treated infarcted rats showed significantly lower nerve density than milrinone-treated infarcted rats ($P<0.05$). Similar to the tyrosine hydroxylase results, densities of growth-associated protein 43-positive nerve fibers (Figure 4) and neurofilament-positive (data not shown) nerves were significantly attenuated in the cilostazol-treated infarcted rats compared with the milrinone-treated infarcted rats. These
Figure 5. (Upper) Western blot analysis of nerve growth factor (NGF; MW: 13 kDa) in LV homogenates. When compared with milrinone-treated infarcted rats, vehicle- and cilostazol-treated infarcted rats had significantly lower NGF levels by quantitative analysis. Relative abundance was obtained by normalizing the density of NGF protein against that of β-actin. Results are mean±SD of 3 independent experiments. *P<0.01, compared with sham; †P<0.05, compared with vehicle and cilostazol. (Lower) Western blot analysis of NGF to confirm the effect of adenosine A1 receptor and antioxidant on NGF level in LV homogenates in a rat isolated heart model (n=10 each group). A significantly increased NGF level is noted in the group treated with a combination of cilostazol (Cil) and DPCPX compared with cilostazol alone. After adding tempol (Tem), either milrinone or cilostazol shows markedly decreased levels of NGF compared with each alone. Densitometric quantification of NGF was expressed as the ratio of the density of β-actin. Each point is an average of 3 separate experiments. *P<0.01, compared with vehicle and cilostazol; †P<0.001, compared with milrinone alone; ‡P<0.001 compared with cilostazol alone.
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decreased compared with those in milrinone-treated infarcted rats.

Electrophysiological Stimulation
To further elucidate the physiological effect of attenuated sympathetic hyperinnervation, ventricular pacing was performed. The arrhythmia score in sham rats was 0 (Figure 7). Ventricular tachyarrhythmias consisting of ventricular tachycardia and ventricular fibrillation were inducible by programmed stimulation in vehicle-treated infarcted rats. Milrinone treatment significantly increased the inducibility of ventricular tachyarrhythmias compared with the cilostazol-treated infarcted rats (P<0.05). In contrast, carvedilol treatment significantly decreased the inducibility of ventricular tachyarrhythmias compared with vehicle- and cilostazol-treated infarcted rats (both P<0.05).

Discussion
In the present study, we demonstrated that despite similar ven-

NGF Protein and mRNA Expression
Western blotting showed that NGF levels were significantly upregulated 2.02-fold in the vehicle-treated infarcted rats than in sham (P<0.01; Figure 5). When compared with vehicle- and cilostazol-treated infarcted rats, milrinone-treated infarcted rats had significantly higher NGF levels. To elucidate the role of the adenosine A1 receptor and antioxidant in modulating NGF, DPCPX and tempol were assessed in an in vitro model. Figure 5 also shows that DPCPX significantly increased the expression of NGF compared with cilostazol alone, confirming a role of adenosine in mediating NGF expression. Furthermore, the increased levels of milrinone-induced NGF were significantly attenuated by administering tempol, to a degree similar to the combination of cilostazol and tempol.

PCR amplification of cDNA revealed 2.14-fold upregulation of NGF mRNA levels in the vehicle-treated infarcted rats compared with sham (P<0.001; Figure 6). In cilostazol-treated infarcted rats, the NGF mRNA levels were significantly decreased compared with those in milrinone-treated infarcted rats.

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Figure 6. Left ventricular expression of nerve growth factor (NGF) mRNA. Each mRNA was corrected for the level of GAPDH mRNA. Each column and bar represents mean±SD. *P<0.01, compared with sham; †P<0.05, compared with vehicle and cilostazol.

Figure 7. Inducibility quotient of ventricular arrhythmias by programmed electrical stimulation. *P<0.001, compared with sham; †P<0.05, compared with vehicle and cilostazol; ‡P<0.05 compared with vehicle, milrinone and cilostazol.
tricular remodeling after MI in rats treated with either milrinone or cilostazol, cilostazol was associated with neutrality in the development of sympathetic reinnervation after MI; however, milrinone was associated with a worsening of the incidence of fatal ventricular arrhythmias. The neutral effect of cilostazol on arrhythmia was attributable to an increase in the adenosine level in the remote myocardium, which counterbalanced cAMP-induced arrhythmias. This further supports the idea that the pharmacodynamic effects of all PDE-3 inhibitors may not be alike. Our results were demonstrated by the effects of PDE-3 inhibitors, as documented structurally by a reduction in cardiac nerve sprouting, molecularly by myocardial NGF protein and mRNA levels, biochemically by tissue superoxide, cAMP, NE and interstitial adenosine levels, and functionally by ventricular remodeling and fatal ventricular tachyarrhythmias. Furthermore, the role of sympathetic hyperinnervation in arrhythmic vulnerability was confirmed by the result that β-blockade by carvedilol attenuated pacing-induced ventricular arrhythmias. β-blockers have been shown to inhibit NE’s interactions with β-adrenergic G protein-coupled receptors, thus preventing formation of second messenger cAMP and reactive oxygen species, and attenuating sympathetic hyperinnervation. Indeed, our results were consistent with the findings of Wen et al, who showed that excessively increased post-injury sympathetic nerve density was responsible for the genesis of ventricular arrhythmia and that carvedilol inhibited sympathetic nerve sprouting and decreased the inducibility of ventricular arrhythmias after MI.

The neutral effect of cilostazol on fatal arrhythmias is supported by 3 lines of evidence from the present study.

(1) Compared with milrinone, cilostazol significantly increased interstitial adenosine levels and reduced the production of myocardial cAMP. MI is associated with increased myocardial cAMP levels. Our results are consistent with those from previous studies showing that cilostazol inhibits adenosine uptake with an IC50 of 5 μmol/L, whereas milrinone has no significant inhibitory effect at concentrations as high as 100 μmol/L. Inhibition of cellular adenosine uptake increases the interstitial adenosine concentration, which in turn results in activation of Gi-coupled A1 receptors and a decrease in the cAMP level. Thus, in the heart, inhibition of adenosine uptake by cilostazol may serve to attenuate the increase in cAMP level caused by the inhibition of PDE-3. Furthermore, milrinone has greater inhibition of PDE-4 activity than cilostazol. Given that degradation of cAMP by PDE-3 is crucial in the regulation of cAMP and that PDE-4 activity is more important in regulating the cAMP level than PDE-3 during angiotensin II stimulation, a condition similar to postinfarction remodeling, cAMP is expected to be higher with milrinone than with cilostazol.

(2) We demonstrated that the interstitial adenosine concentration increased 2-fold in the hearts of cilostazol-treated infarcted rats. This is believed to confer attenuated oxidative stress. Previous studies have shown that adenosine exerts as an antioxidant through activation of the A1 receptor in ischemic myocardium. Our results were not consistent with the previous findings of Park et al, who showed that the increased cAMP level with cilostazol, but not adenosine, is directly coupled to the reduction in NAD(P)H-dependent superoxide production in human endothelial cells. If cAMP plays a role in attenuating superoxide production, rats treated with milrinone should have had lower levels of superoxide compared with cilostazol-treated rats, which was not the case. The discrepancy can be explained by different models, tissues and species, which appear to show differences in the cAMP-PDE system. Indeed, our results were consistent with a recent study showing that cilostazol at 5 mg·kg⁻¹·day⁻¹ can attenuate oxidative stress.

(3) Decreased superoxide production was associated with attenuated NGF expression. Proposed mechanisms for NGF expression include cAMP and interstitial adenosine concentrations. Blocking the adenosine A1 receptor with DPCPX significantly elevated the cilostazol-induced cAMP effect to levels similar to those with vehicle. Furthermore, the addition of tempol was shown to similarly attenuate the NGF level in the cilostazol-treated group as in the milrinone-treated group. These data suggest that A1 receptor activation-mediated antioxidation following treatment with cilostazol plays an integral role in the signaling cascade responsible for the attenuated NGF expression in this model.

**Mechanisms**

Differences in the sympathetic reinnervation with cilostazol and milrinone are not only related to their differential effects on the cAMP level, but may also result from the greater increase in the interstitial adenosine level by cilostazol than by milrinone. Previous studies have shown that cilostazol increases NGF mRNA expression. The NGF promoter contains activator protein-1, and cAMP has been shown to enhance activator protein-1 activation in PC12 cells. It is not surprising that milrinone more significantly increased NGF expression than cilostazol, probably through enhanced cAMP levels. Besides, the present study results hint that the mechanism by which cilostazol potently attenuates NGF expression may be related to adenosine. Adenosine has been shown to attenuate superoxide stress. Exactly how the activation of superoxide leads to attenuated sympathetic hyperinnervation can not be determined from this study. Inhibition of free radicals decreases tissue nitrotyrosine formation, a marker of peroxynitrite specific cellular injury. Peroxynitrite has been shown to activate activator protein-1. Our present results confirmed that cilostazol has a neutral effect on sympathetic innervation possibly by counterbalancing the PDE-3 inhibition-induced cAMP and adenosine-related antioxidation pathways.

**Other Mechanisms**

Although the present study results suggest that the mechanism of PDE-3 inhibition-induced arrhythmias may be related to cAMP and adenosine levels, other potential mechanisms need to be studied. First, previous studies have shown that milrinone exerts greater inhibition of PDE-4 activity than cilostazol. Increased PDE-4 activity has been shown to have an antiarrhythmic effect. Previous studies have also shown that mice with PDE-4D deficiency show accelerated progression of heart failure following MI and that pharmacological PDE-4 inhibition was associated with exercise-induced cardiac arrhythmias. Thus, it is possible, at least in theory, that the greater selectivity of cilostazol for PDE-3 may help prevent cardiac arrhythmias. Second, cilostazol has been shown to inhibit recombiant human PDE-5, a major cGMP hydrolyzing enzyme, and correspondingly increases cGMP. Cilostazol has been shown to downregulate NGF expression. However, PDE-5 inhibition does not appear to play a significant role in the NGF expression associated with cilostazol because cilostazol as a PDE-5 antagonist requires a higher therapeutic concentration (10 μmol/L) than reported in this study. Third, cilostazol appears to possess an additional effect that does not require inhibition of PDEs. Cilostazol may directly inhibit secretion of catecholamine through its blocking action on Ca²⁺ movement in adrenal chromaffin cells.
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Clinical Implications
Cilostazol is an alternative therapy to thienopyridines in patients with coronary artery disease.1-37 but to date, no studies have directly addressed the question of whether or not long-term treatment with different PDE-3 inhibitors may influence susceptibility to ventricular arrhythmias after MI. Our results show that the association between PDE-3 inhibitors and ventricular arrhythmias is anatomically and functionally linked. After an acute MI, patients remain at high risk for recurrent cardiovascular events and mortality.1-3 Although both milrinone and cilostazol are used as PDE-3 inhibitors, our study demonstrated that cilostazol has a neutral effect on the severity of experimentally-induced ventricular arrhythmias, whereas milrinone may increase the susceptibility to ventricular arrhythmias. Chronic use of milrinone in heart failure patients has been associated with decreased survival, primarily as a result of arrhythmias and sudden death.38 Despite excluding all but a small number of patients with mild heart failure from interim claudication clinical trials, cilostazol has not been associated with increased mortality in clinical trials for intermittent claudication.48 Our finding that milrinone increased sympathetic reinnervation may provide an explanation for the increased arrhythmias seen with chronic PDE-3 inhibitor therapy.

Conclusions
The present results suggest that despite similar beneficial ventricular remodeling after MI in rats treated with either cilostazol or milrinone, cilostazol, but not milrinone, had a neutral effect on NOF expression and sympathetic reinnervation after MI. Inhibition of adenosine uptake is involved in the mechanism underlying the pharmacological action of cilostazol. The present evidence at least did not suggest a proarrhythmic effect of cilostazol. These effects of cilostazol could be advantageous from a therapeutic point of view, because patients with coronary artery disease are at risk of arrhythmias.

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References
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