



Phosphodiesterase-3 inhibitor (cilostazol) attenuates oxidative stress-induced mitochondrial dysfunction in the heart

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Abstract

Background Cilostazol is a type 3 phosphodiesterase inhibitor which has been previously demonstrated to prevent the occurrence of tachyarrhythmia and improve defibrillation efficacy. However, the mechanism for this beneficial effect is still unclear. Since cardiac mitochondria have been shown to play a crucial role in fatal cardiac arrhythmias and that oxidative stress is one of the main contributors to arrhythmia generation, we tested the effects of cilostazol on cardiac mitochondria under severe oxidative stress. **Methods** Mitochondria were isolated from rat hearts and treated with H₂O₂ to induce oxidative stress. Cilostazol, at various concentrations, was used to study its protective effects. Pharmacological interventions, including a mitochondrial permeability transition pore (mPTP) blocker, cyclosporine A (CsA), and an inner membrane anion channel (IMAC) blocker, 4'-chlorodiazepam (CDP), were used to investigate the mechanistic role of cilostazol on cardiac mitochondria. Cardiac mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane potential change and mitochondrial swelling were determined as indicators of cardiac mitochondrial function. **Results** Cilostazol preserved cardiac mitochondrial function when exposed to oxidative stress by preventing mitochondrial depolarization, mitochondrial swelling, and decreasing ROS production. **Conclusions** Our findings suggest that cardioprotective effects of cilostazol reported previously could be due to its prevention of cardiac mitochondrial dysfunction caused by severe oxidative stress.

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1 Introduction

Cilostazol is a selective type-3 phosphodiesterase inhibitor that has been used to treat claudication. Unlike other type-3 phosphodiesterase inhibitors, such as milrinone and vesnarinone which have been shown to increase cyclic adenosine monophosphate (cAMP) levels, arrhythmogenesis, and mortality rates in heart failure patients,^[1,2] growing evidence suggests that cilostazol could be cardioprotective.^[3–14] This could be due to the fact that cilostazol inhibits not only type 3 phosphodiesterase, but also adenosine uptake, thus reducing the cAMP levels.^[15,16] Previous studies also demonstrated the cardioprotective effects of cilostazol by pre-

venting the occurrence of fatal arrhythmias in Brugada's syndrome.^[17] Our recent study demonstrated that cilostazol could stabilize the cardiac electrophysiology and prevent fatal arrhythmias by increasing the ventricular fibrillation threshold as well as increasing the defibrillation efficacy.^[18] Despite these beneficial effects, the definite mechanism of cilostazol in arrhythmia prevention is still unclear.

Cardiac mitochondrial dysfunction has been shown to play an important role in cardiac arrhythmogenesis.^[19] It has been shown that depolarization of cardiac mitochondrial membrane potential occurring during various stress conditions, including ischemia-reperfusion, could initiate fatal arrhythmia in the heart.^[19] Oxidative stress due to high levels of reactive oxygen species (ROS) produced in the mitochondria during ischemia-reperfusion has been shown to play an important role in triggering the cardiac mitochondrial membrane potential changes.^[19,20] Although several drugs and chemical substances have been demonstrated to attenuate mitochondrial dysfunction caused by oxidative

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stress;^[19,21–23] the role of cilostazol on cardiac mitochondrial function has never been investigated.

In the present study, we tested the hypothesis that cilostazol can prevent cardiac mitochondrial dysfunction caused by H₂O₂-induced severe oxidative stress by attenuating mitochondrial swelling, preventing mitochondrial depolarization, and reducing ROS production in cardiac mitochondria.

2 Methods

2.1 Animal preparation

This study was approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University. Wistar rats (300–350 g) obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand, were housed in a room with temperature 22–25 °C and had a constant 12-h light/dark cycle. All animals received standard pelleted rat diet and water *ad libitum*.

2.2 Isolated cardiac mitochondria preparation

Cardiac mitochondria were isolated based upon the protocol described previously.^[20] In brief, each rat was injected intraperitoneally with thiopental (80 mg/kg), and the heart was removed and homogenized in ice-cold buffer containing sucrose 300 mmol/L, N-(tris (hydroxymethyl) methyl)-2-aminoethanesulfonic acid sodium salt (TES) 5 mmol/L, and ethylene glycol tetraacetic acid (EGTA) 0.2 mmol/L, pH 7.2 at 4 °C. The homogenate was centrifuged at 800 r/min for 5 min and the supernatant was collected and centrifuged at 8,800 r/min for 5 min. Cardiac mitochondrial pellets were resuspended in an ice-cold buffer and centrifuged again at 8,800 r/min for 5 min. Protein concentration was determined according to the bicinchoninic acid (BCA) assay.^[24]

2.3 Experimental protocols

Isolated cardiac mitochondria from rat hearts were used in all study protocols. Identification of cardiac mitochondria was performed using electron microscopy. Application of H₂O₂ at 2 mmol/L with a 5-min incubation time was used to induce oxidative stress in cardiac mitochondria. This concentration and incubation time were chosen as been shown to effectively cause cardiac mitochondrial dysfunction by inducing mitochondrial swelling, increasing mitochondrial ROS production, and depolarizing mitochondrial membrane potential.^[20]

In the first protocol, 20- μ mol/L cilostazol was applied to cardiac mitochondria at various incubation times (5–20 min)

prior to H₂O₂ application. The time in which cilostazol could effectively protect cardiac mitochondrial dysfunction caused by H₂O₂ was chosen for the next protocol. In the second protocol, various concentrations of cilostazol (5–40 μ mol/L) were studied with the incubation time obtained from the first protocol. Vehicle-treated mitochondria were used as a control group.

In the third protocol, the protective mechanism of cilostazol on cardiac mitochondria was investigated using a pharmacological intervention approach. Cyclosporine A (CsA) and 4'-chlorodiazepam (CDP) were used for this purpose. CsA is a blocker of the mitochondrial permeability transition pore (mPTP),^[20,25] whereas CDP is known to inhibit the opening of the inner membrane anion channel (IMAC).^[20] In this protocol, cardiac mitochondria were randomly assigned into 10 treatment groups ($n = 8$ per group), consisting of the control (vehicle); cilostazol (effective dose of cilostazol obtained from the second protocol); CsA (5 μ mol/L); CDP (100 μ mol/L); H₂O₂; H₂O₂ with cilostazol; H₂O₂ with CsA; H₂O₂ with CDP; H₂O₂ with cilostazol + CsA; and H₂O₂ with cilostazol + CDP. In each study protocol, cardiac mitochondria in each group were investigated for mitochondrial swelling, mitochondrial ROS production, and mitochondrial membrane potential changes.

2.4 Determination of cardiac mitochondrial swelling, ROS production and membrane potential changes

Cardiac mitochondrial swelling was determined in all groups by measuring the change in the absorbance of the cardiac mitochondrial suspension at 540 nm (A540) using a microplate reader.^[20,25] Mitochondria (0.4 mg/mL) were incubated in respiration buffer (containing 100 mmol/L KCl, 50 mmol/L sucrose, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 mmol/L KH₂PO₄, pH 7.4 at 37 °C) with the addition of 10-mmol/L pyruvate/malate. Mitochondrial swelling was indicated by a decrease in the absorbance of the suspension.^[20]

ROS production in cardiac mitochondria was determined using dichloro-hydro-fluorescein diacetate (DCFDA),^[26] and measured by a fluorescent microplate reader.^[20] Cardiac mitochondria (0.4 mg/mL) were incubated at 25 °C with 2- μ mol/L of DCFDA for 20 min. The mitochondrial suspension was gently agitated and incubated at room temperature for measurements. DCFDA passed through the mitochondrial membranes where it was oxidized in the presence of H₂O₂ to DCF (a fluorescent form). Fluorescence was determined at λ_{ex} 485 nm and λ_{em} 530 nm according to the spectral characteristics of DCF. The ROS levels were expressed as arbitrary units of fluorescence intensity of DCF.^[20]

Changes in the cardiac mitochondrial membrane potential were determined using the dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1).^[20,27,28] The isolated cardiac mitochondria (2 mg/mL) were stained with JC-1 (310 nmol/L) at 37 °C for 30 min. The intensity of fluorescence was determined using a fluorescent microplate reader. JC-1 monomer fluorescence (green) was excited at 485 nm and the emission was detected at 530 nm. JC-1 aggregate fluorescence (red) was excited at 485 nm and the emission fluorescence was recorded at 590 nm. Cardiac mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio.^[20]

2.5 Statistical analysis

All data were expressed as means \pm SE. Comparisons among groups were determined by one-way ANOVA, followed by the Fisher post-hoc test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Effects of cilostazol on cardiac mitochondrial swelling, ROS production and membrane potential changes

Application of H₂O₂ at 2 mmol/L caused cardiac mitochondrial dysfunction as represented by decreased absorbance indicating mitochondrial swelling (Figure 1A), increased ROS levels (Figure 1B), and a decreased red/green fluorescent ratio indicating mitochondrial membrane depolarization (Figure 1C), in comparison to the control group. When 20- μ mol/L cilostazol was applied to cardiac mitochondria, there was no change in absorbance, ROS levels, or mitochondrial membrane potential in cardiac mitochondria at any of the incubation times. However, the application of cilostazol to cardiac mitochondria for 5, 10, and 20 min prior to H₂O₂ application could prevent mitochondrial swelling, decrease ROS levels, and prevent mitochondrial depolarization caused by H₂O₂ (Figure 1). From this

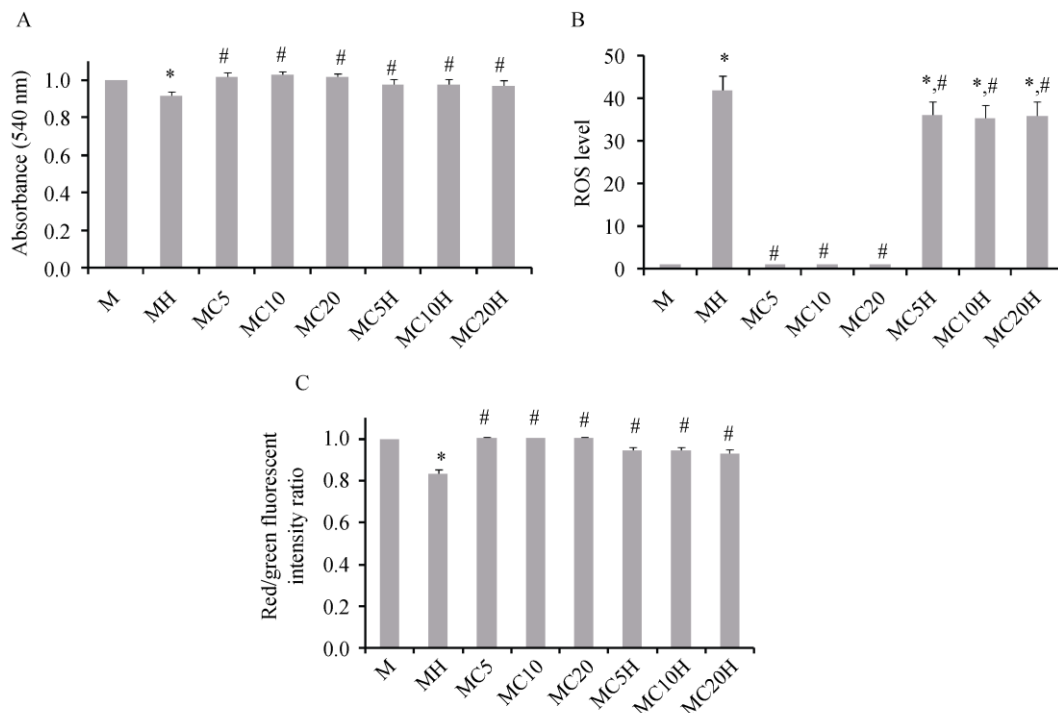


Figure 1. Effects of incubation times of 20- μ mol/L cilostazol on cardiac mitochondrial swelling (A), ROS levels (B), and membrane potential change (C) after 4-min H₂O₂ application. Without H₂O₂, cilostazol applied to cardiac mitochondria for 5, 10, and 20 min did not alter the absorbance, ROS levels, or membrane potential of cardiac mitochondria, in comparison with the control group. Application of H₂O₂ to cardiac mitochondria caused marked cardiac mitochondrial swelling, increased ROS levels, and a decreased red/green fluorescent intensity ratio (i.e., mitochondrial depolarization). When cilostazol was applied to cardiac mitochondria for 5, 10 and 20 min prior to H₂O₂ application, it could prevent mitochondrial swelling, decrease ROS levels, and prevent mitochondrial depolarization, in comparison with the H₂O₂ treated group. M: cardiac mitochondria without any treatment; MH: cardiac mitochondria treated with H₂O₂; MC5/MC10/MC20: cardiac mitochondria treated with 20 μ mol/L cilostazol for 5, 10, and 20 min without H₂O₂ application, respectively; MC5H/MC10H/MC20H: cardiac mitochondria treated with 20 μ mol/L cilostazol for 5, 10, and 20 min prior to H₂O₂ application, respectively. * $P < 0.05$ vs. M group, # $P < 0.05$ vs. H₂O₂ treated mitochondria. ROS: reactive oxygen species.

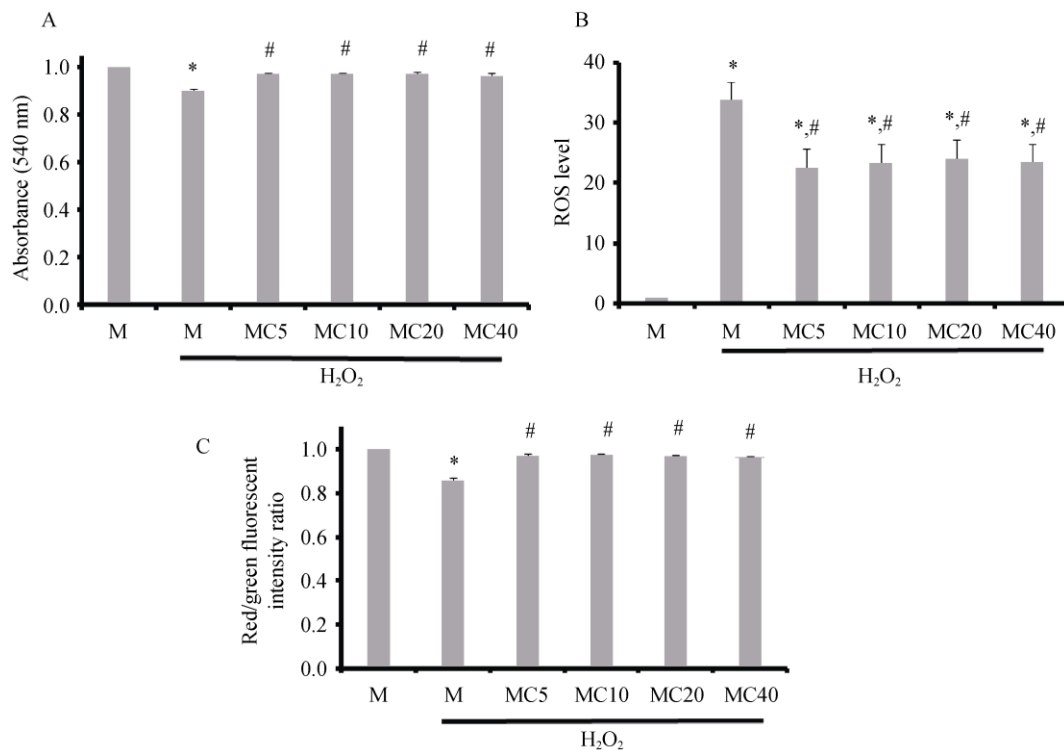


Figure 2. Effects of different concentrations of cilostazol on cardiac mitochondrial swelling (A), ROS levels (B), and membrane potential changes (C) after 4-min H₂O₂ application. Cilostazol at 5, 10, and 20 $\mu\text{mol/L}$ with a 10 min incubation period could effectively prevent cardiac mitochondrial swelling, decrease ROS levels, and prevent mitochondrial membrane depolarization, in comparison with the H₂O₂ treated group. M: cardiac mitochondria without any treatment; MC5/MC10/MC20/MC40: cardiac mitochondria treated with cilostazol at 5, 10, 20, and 40 $\mu\text{mol/L}$ for 5 min, respectively. * $P < 0.05$ vs. M group; # $P < 0.05$ vs. H₂O₂ treated mitochondria. ROS: reactive oxygen species.

protocol, a 10-min incubation time was chosen for cilostazol as the standard period for subsequent protocols in this study.

To investigate the dose-dependent effect of the drug, cilostazol at 5, 10, 20, and 40 $\mu\text{mol/L}$ was tested for its protective effect, and the results are shown in Figure 2. All tested concentrations of cilostazol could significantly prevent mitochondrial swelling caused by H₂O₂ (Figure 2A). Furthermore, all tested concentrations of cilostazol could decrease the ROS levels (Figure 2B) and prevent cardiac mitochondrial depolarization induced by H₂O₂ application (Figure 2C). From this protocol, 5- $\mu\text{mol/L}$ cilostazol was chosen as the standard concentration for subsequent protocols in this study.

3.2 Effects of cilostazol, CsA, and CDP on cardiac mitochondrial swelling, mitochondrial ROS levels and mitochondrial membrane potential changes

The protective effects of cilostazol, CsA and CDP on cardiac mitochondria were tested, and the results are shown in Figure 3. cilostazol, CsA and CDP did not alter the

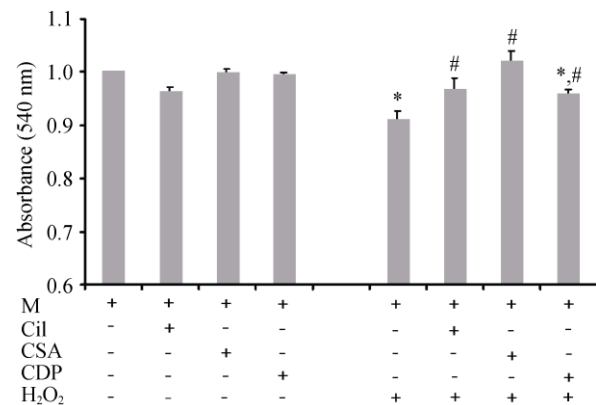


Figure 3. Effects of cilostazol, CsA, and CDP on cardiac mitochondrial swelling. Cardiac mitochondria were pretreated with cilostazol (5 $\mu\text{mol/L}$, 10 min incubation), CsA, or CDP prior to the administration of H₂O₂. Cilostazol and CsA completely protected cardiac mitochondrial swelling caused by H₂O₂, whereas CDP could only partially protect cardiac mitochondrial swelling. * $P < 0.05$ vs. M (control group), # $P < 0.05$ vs. H₂O₂ treated group. CDP: 4'-chlorodiazepam; Cil: cilostazol; CsA: cyclosporine A; M: cardiac mitochondria without any treatment.

absorbance in cardiac mitochondria. In H₂O₂-treated cardiac mitochondria, the groups that were pretreated with Cilostazol and CsA did not show mitochondrial swelling caused by H₂O₂. Although CDP could attenuate cardiac mitochondrial swelling caused by H₂O₂, it could not restore the absorbance to the baseline level (Figure 3).

For cardiac mitochondrial ROS production, Cilostazol and CDP did not alter the ROS level, whereas CsA caused a slight increase in ROS levels in cardiac mitochondria without H₂O₂ application (Figure 4). In H₂O₂-treated cardiac mitochondria, the groups that were treated with cilostazol, CDP and CsA had decreased ROS levels, compared to the H₂O₂ treated group. However, the amount of ROS reduction was the greatest in the CsA, cilostazol + CDP, and cilostazol + CsA groups.

For cardiac mitochondrial membrane potential changes, cilostazol, CsA and CDP did not alter cardiac mitochondrial membrane potential (Figure 5). While H₂O₂ caused cardiac mitochondrial depolarization, pretreatment with cilostazol, CsA, CDP, cilostazol + CDP, and cilostazol + CsA could prevent cardiac mitochondrial depolarization caused by H₂O₂.

4 Discussion

The major finding of the present study is that cilostazol prevents cardiac mitochondrial dysfunction caused by severe oxidative stress due to H₂O₂ application. The protective

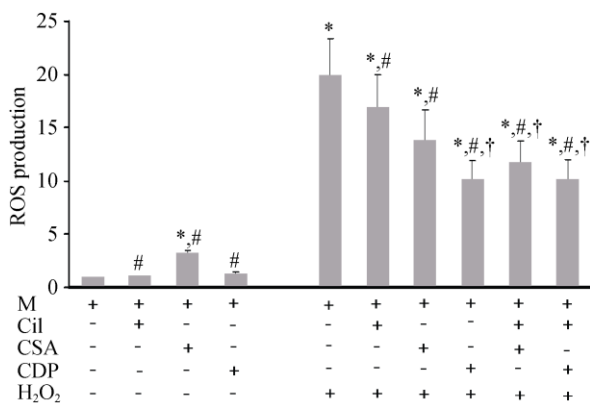


Figure 4. Effects of cilostazol, CsA, and CDP on ROS production in cardiac mitochondria. Without H₂O₂, cilostazol and CDP did not alter the ROS levels, whereas CsA caused a slight increase in ROS levels in cardiac mitochondria. While H₂O₂ caused a marked increase in ROS levels, cilostazol, CsA, and CDP could significantly decrease the ROS levels created by H₂O₂. **P* < 0.05 vs. M (control group), #*P* < 0.05 vs. H₂O₂ treated group, †*P* < 0.05 vs. (CsA + H₂O₂) treated group. CDP: 4'-chlorodiazepam; Cil: cilostazol; CsA: cyclosporine A; M: cardiac mitochondria; ROS: reactive oxygen species.

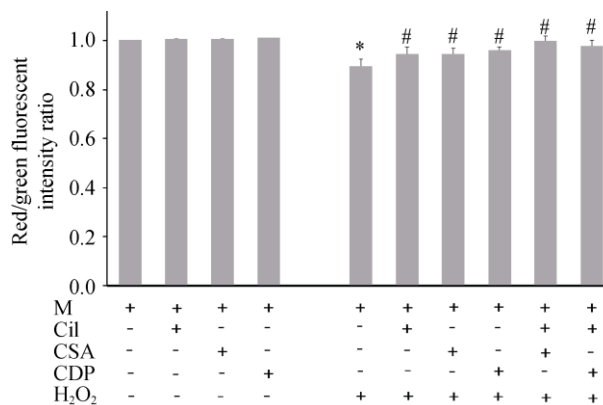


Figure 5. Effects of cilostazol, CsA, and CDP on cardiac mitochondrial membrane potential changes. Without H₂O₂, cilostazol, CsA and CDP did not alter the cardiac mitochondrial membrane potential. While H₂O₂ caused mitochondrial depolarization, cilostazol, CsA and CDP could completely prevent cardiac mitochondrial membrane depolarization caused by H₂O₂. **P* < 0.05 vs. M (control) group, #*P* < 0.05 vs. H₂O₂ treated group. CDP: 4'-chlorodiazepam; Cil: cilostazol; CsA: cyclosporine A; M: cardiac mitochondria.

effects of cilostazol include prevention of cardiac mitochondrial swelling, decreased mitochondrial ROS production, and prevention of cardiac mitochondrial depolarization due to oxidative stress induced by H₂O₂.

Previous studies demonstrate that cilostazol can have antiarrhythmic effects in both animal models and patients.^[18,29] However, its effects on cardiac mitochondria have never been investigated. Since cardiac mitochondrial dysfunction has been shown to be associated with cardiac arrhythmias,^[21] it is possible that the antiarrhythmic effect of cilostazol could, in part, be due to its protective effects regarding cardiac mitochondria. We previously demonstrated that oxidative stress in cardiac mitochondria caused by H₂O₂ can cause cardiac mitochondrial dysfunction, and that this model can be used to study the efficacy of drugs for their potential cardioprotective effects.^[20] As shown in our study, cilostazol demonstrated its effects in preventing cardiac mitochondrial swelling, decreasing mitochondrial ROS production, and preventing cardiac mitochondrial membrane depolarization due to H₂O₂ application, indicating that this drug can assist in the prevention of cardiac mitochondrial dysfunction caused by severe oxidative stress.

In the present study, the mechanism of cardiac mitochondrial protection by cilostazol was tested using a pharmacological approach. It is known that oxidative stress with increased ROS production can lead to an opening of the mPTP in cardiac mitochondria, resulting in mitochondrial swelling and membrane potential depolarization.^[30,31] In our study, when CsA was applied to cardiac mitochondria prior

to H₂O₂ application, it was possible to prevent cardiac mitochondrial swelling, decrease ROS production, and prevent cardiac mitochondrial membrane depolarization. Although cilostazol offers effects that prevent cardiac mitochondrial dysfunction similar to those found with CsA, the reduction of mitochondrial ROS production in the cilostazol-treated group was less than that of the CsA-treated mitochondria. Nevertheless, cilostazol could still prevent mitochondrial depolarization similar to that of the CsA-treated group, suggesting that the effects of cilostazol on ROS reduction were already sufficient to bring the ROS levels down to a level below the critical threshold that causes mitochondrial depolarization.^[20] The prevention of cardiac mitochondrial dysfunction including the restoration of mitochondrial membrane potential by cilostazol could be responsible for its previously reported antiarrhythmic effects.^[3,18] There are several limitations in the present study. It is well known that the roles of PDE-3 involve both cAMP and cyclic guanosine monophosphate (cGMP), however, it has been demonstrated that PDE-3 is functionally a cAMP-hydrolyzing enzyme.^[32] In the present study, we did not determine the effects of cilostazol on cAMP and cGMP. Moreover, it has been shown that PDE-3 inhibitor could exert cardioprotective benefits via its activation of cAMP-dependent protein kinase A (PKA) and potentiating the opening of mitochondrial Ca²⁺-activated K⁺ channels.^[33] In the present study, the roles of cilostazol on the PKA and mitochondrial Ca²⁺-activated K⁺ channels were not investigated. Future studies are needed to determine the effects of cilostazol on these signaling cascades.

In conclusion, under oxidative stress, cilostazol prevents cardiac mitochondrial dysfunction by attenuating cardiac mitochondrial swelling, ROS production, and mitochondrial membrane potential changes in cardiac mitochondria. These effects, particularly those regarding the prevention of cardiac mitochondrial depolarization, may explain its previously found antiarrhythmic effect.

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