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Serofendic Acid Protects Against Myocardial Ischemia–Reperfusion Injury in Rats

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Abstract. We previously reported that serofendic acid, a lipophilic extract of fetal calf serum, protects against oxidative stress in primary culture of neonatal rat cardiomyocytes. However, the effect of serofendic acid on myocardial ischemia–reperfusion injury in vivo is yet to be determined. In the present study, we investigated the effect of intravenous administration of serofendic acid on ischemia–reperfusion injury induced by transient occlusion of the left coronary artery in rats. The rat heart was subjected to 25-min ischemia followed by 2-h reperfusion. Bolus intravenous administration of serofendic acid (1 – 10 mg/kg) given twice reduced the infarct volume in a dose-dependent manner. The protective effect of serofendic acid was abolished by pretreatment with 5-hydroxydecanoate, a blocker of mitochondrial ATP-sensitive potassium channels. For further testing of the protective effect of serofendic acid at the subcellular level, we monitored mitochondrial membrane potential (MMP) in individual cells using real-time two-photon imaging of Langendorff-perfused rat heart. A 25-min no-flow ischemia, followed by reperfusion caused progressive MMP loss. Serofendic acid significantly reduced the number of cells undergoing MMP loss. These results suggest that serofendic acid protected cardiac myocytes against myocardial ischemia–reperfusion injury by preserving the functional integrity of mitochondria.

Keywords: serofendic acid, myocardial ischemia, reperfusion, mitochondrial ATP-sensitive potassium channel, cardiac myocyte

Introduction

Ischemic heart disease is the leading cause of morbidity and mortality in all industrialized countries. After acute myocardial ischemia, myocardial reperfusion by thrombolysis, percutaneous coronary intervention or cardiac surgery is the effective treatment for reducing infarct size and improving clinical outcome. However, it is well known that reperfusion can also induce injury called "reperfusion injury" (1 – 3).

Mitochondria play a critical role in cell death in response to a variety of stresses such as myocardial ischemia–reperfusion (4 – 6). Opening of the mitochondrial permeability transition pore (MPTP), which is a non-selective large conductance channel, is a major mechanism of cell death by myocardial ischemia–reperfusion. MPTP opening results in the loss of mitochondrial membrane potential ($\Delta\Psi_m$), matrix swelling, and the release of cytochrome c and other pro-apoptotic factors that lead to cell death (7 – 9). Mitochondrial matrix calcium ($[Ca^{2+}]_m$) overload and increased reactive oxygen species (ROS) favor MPTP opening (10).

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Inhibition of MPTP opening by the prevention of $[Ca^{2+}]_m$ overload and ROS generation should be an effective strategy for the protection of hearts against I/R injury.

We have shown that ATP-sensitive potassium channels located in the inner mitochondrial membrane (mitoK_{ATP} channels) play a central role in the signaling cascade of protection against oxidative stress in cardiac ventricular myocytes (11, 12) and cerebellar granule neurons (13, 14). Opening of mitoK_{ATP} channels prevent $[Ca^{2+}]_m$ overload and ROS generation, thereby inhibiting the MPTP opening in both types of cells (13, 15 – 17). Diazoxide, a selective opener of mitoK_{ATP} channels, has been shown to have protective effects against myocardial ischemia–reperfusion both in vitro (18, 19) and in vivo (20, 21). Unfortunately, the clinical use of this agent has been hampered by unwanted side effects such as excessive hypotension and edema.

We previously found a neuroprotective substance, serofendic acid, in a lipophilic extract of fetal calf serum. Serofendic acid is a 15-hydroxy-17-methylsulfinylatisan-19-oic acid, a sulfur-containing atisan-type diterpenoid (22). It is a low-molecular-weight (MW 382) compound and exhibits a potent protective effect against neurotoxicity induced by glutamate, NO, and oxidative stress without inhibiting glutamate receptors in cultured cortical, striatal, and spinal cord neurons (23 – 26). Moreover, in cardiac myocytes, serofendic acid was found to suppress cell death induced by oxidative stress (27, 28). The mechanism of protection remains unclear, but we have demonstrated that serofendic acid preserves mitochondrial functional integrity and inhibits the generation of hydroxyl radical and caspase-3 activation (23, 24, 26, 27).

In the present study, we investigated whether serofendic acid exerts protective effects against myocardial ischemia–reperfusion injury in vivo, using a rat model of transient left coronary artery occlusion.

Materials and Methods

Animals

Experiments were carried out using 10-week-old male Sprague-Dawley rats weighing 300 – 330 g, which were purchased from Nihon SLC (Shizuoka). The animals were treated in accordance with the guidelines of Kyoto University Animal Experimentation Committee, and the guidelines of The Japanese Pharmacological Society.

Surgical procedure of left coronary artery occlusion and drug administration

Surgical preparation was essentially the same as described in detail previously (29). Briefly, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). Oxygen was supplemented for ventilation to maintain arterial blood gases within the physiological range. Rectal temperature in each rat was maintained within 37°C – 38°C using a heating pad. The heart was exposed via left thoracotomy, and a coronary snare was placed around the left coronary artery by using a 5-0 silk thread. Heparinized saline-filled catheters were placed in the femoral vein and the carotid artery for drug infusion and monitoring arterial blood pressure using a DX-100 pressure transducer (Nihon-Kohden, Tokyo). After a 20-min stabilization period following surgery, rats were administered intravenously vehicle, serofendic acid, or diazoxide. Fifteen minutes after treatment, the left coronary artery was occluded for 25 min and then reperfused. Five minutes before reperfusion, vehicle, serofendic acid, or diazoxide was administered intravenously. After 2 h of reperfusion, the heart was excised and mounted onto a Langendorff apparatus. The heart was perfused with Tyrode's solution containing 134 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 10 mM HEPES, 11 mM D-glucose, and 2 mM $CaCl_2$ (pH = 7.4, adjusted with 1 M NaOH) to wash out the blood, and then the coronary artery was reoccluded. A saline suspension of fluorescent polymer microspheres (Duke Scientific, Palo Alto, CA, USA) was infused into the aorta to mark the area at risk negatively, and the heart was frozen at −20°C. Frozen hearts were sliced into 2-mm sections and stained with triphenyltetrazolium chloride (TTC). Area of infarct and area at risk were determined using image analysis software (Image J).

Two-photon laser-scanning microscopy imaging of Langendorff-perfused rat hearts

Surgical preparation was essentially the same as described in detail previously (30). Rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and the heart was excised. The ascending aorta was cannulated with a customized needle and hearts were perfused in the Langendorff mode. Perfusion was carried out at a constant mean perfusion pressure with oxygenated (95% O_2) Tyrode's solution. After an initial perfusion period of approximately 10 min, the buffer was switched to oxygenated Tyrode's solution containing 100 nM tetra-methyl rhodamine ethyl ester (TMRE) and this was followed by a 10-min washout with dye-free solution. The temperature was maintained at 37°C using a solution heater and a platform heater (Warner Instruments, Hamden, CT, USA) installed on the microscope stage. After dye loading and washout periods, the hearts were placed in a circular glass-bottomed dish (35-mm diameter) and subjected to myocardial ischemia–reperfusion (25 min of global ischemia followed by 60 min of reperfusion). Ischemia was achieved by clamping the perfusion line and reperfusion by releasing the clamp. During the reperfusion period, the hearts were perfused with Tyrode's solution containing 10 mmol/L 2,3-butanedione monoxime to eliminate contraction-induced movement of the heart during image

acquisition. Images were recorded with a Zeiss LSM510 laser scanning microscope modified for TPLSM. Illumination for two-photon excitation was provided by a mode-locked Ti:Sapphire laser (Spectra-Physics, Irvine, CA, USA); the excitation wavelength was 810 nm. Hearts were imaged through a Zeiss 40 × 1.30 numerical aperture oil-immersion objective with a working distance of 200 μm. Emitted light was collected by two photo-multiplier tubes fitted with bandpass filters for 565 – 615 nm (for TMRE). The images shown are representative of at least three independent experiments, and we confirmed the reproducibility of the responses. Post-acquisition image analysis was performed using software (Image J). From the image sequences, regions of interest were drawn over a part of an individual cell, and fluorescence signals within these regions were collected over time. $\Delta\Psi_m$ was monitored using mean TMRE brightness within the regions.

Statistics

Data are expressed as means ± S.E.M. Statistical analyses were performed using GraphPad Instat (Graph Pad Software, San Diego, CA, USA). The effects on the infarct volume and $\Delta\Psi_m$ were analyzed using one-way analysis of variance followed by Tukey's test. The effect of serofendic acid on the infarct volume in a dose-dependent manner was analyzed with Dunnett's two-tailed test. Two-way analysis was used for mean blood pressure. Statistical significance was defined as a probability value of less than 5%.

Results

Dose-dependent protective effect of serofendic acid against myocardial ischemia–reperfusion

We examined the protective effect of serofendic acid on infarct size induced by myocardial ischemia–reperfusion by TTC staining. Bolus intravenous administration of serofendic acid was given twice: 15 min before ischemia and 5 min before reperfusion. Figure 1A shows representative images of perfused fluorescent polymer microspheres and TTC staining. The area that was not perfused with fluorescent polymer microspheres indicated the ischemic area (area at risk) during left coronary artery occlusion. There was no difference in the size of the area at risk between vehicle- and serofendic acid–treated groups (Fig. 1B). The infarct area indicated by negative staining by TTC was smaller in the serofendic acid (10 mg/kg)-treated group than in the vehicle-treated group. The protective effect of serofendic acid was dose-dependent in the range of 1 – 10 mg/kg (Fig. 1C). Myocardial sections in the vehicle-treated group were more likely to have intramyocardial bleeding (brownish area inside the infarct zone), indicating greater myocardial damage (arrows in lower panels in Fig. 1A).

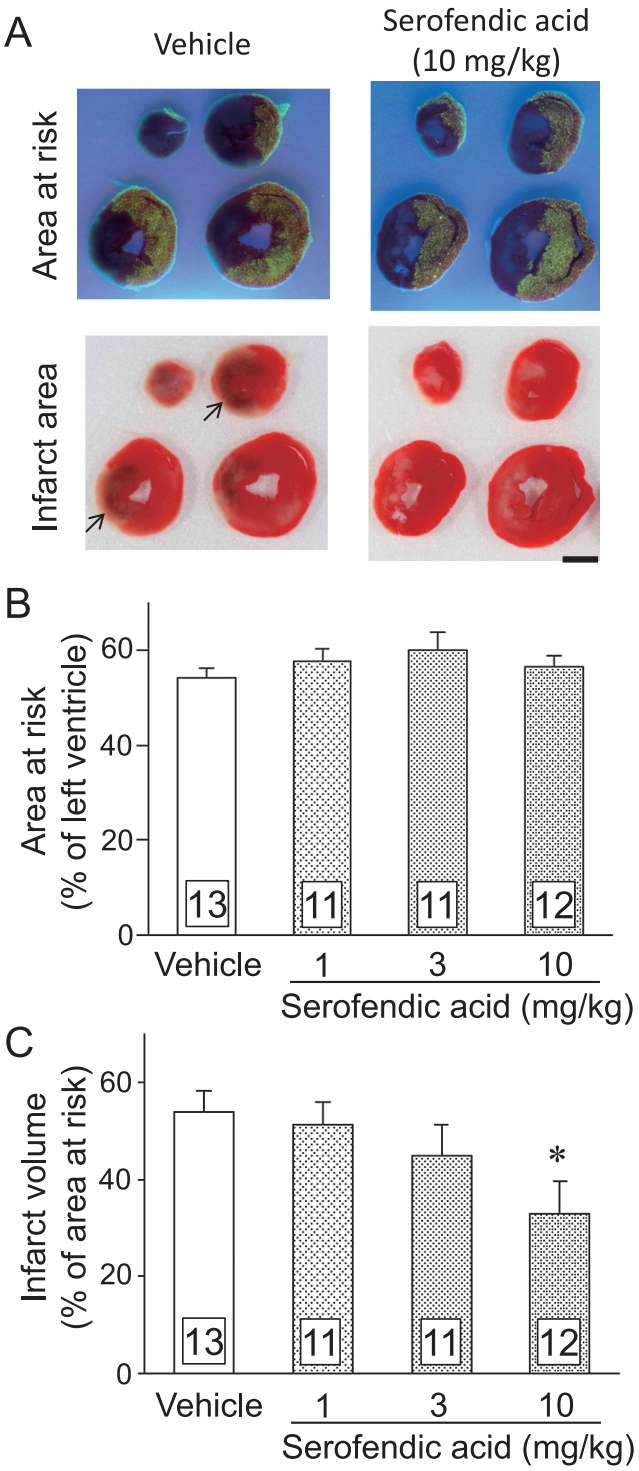

Fig. 1. Dose-dependent effect of serofendic acid against myocardial ischemia–reperfusion injury. The left coronary artery was occluded for 25 min with subsequent reperfusion. After 2 h of reperfusion, the hearts were sliced into coronal sections for TTC staining. Serofendic acid was administered intravenously 15 min before ischemia and 5 min before reperfusion. A) Representative images showing the effect of vehicle or serofendic acid (10 mg/kg) on the area at risk and the infarct area. Arrows indicate intramyocardial bleeding. B) Effects of serofendic acid on the area at risk. C) Dose-dependent effect of serofendic acid on infarct size. The numbers in the columns indicate the numbers of experiments performed. Scale bar: 3 mm. *$P < 0.05$ vs. vehicle-treated group.

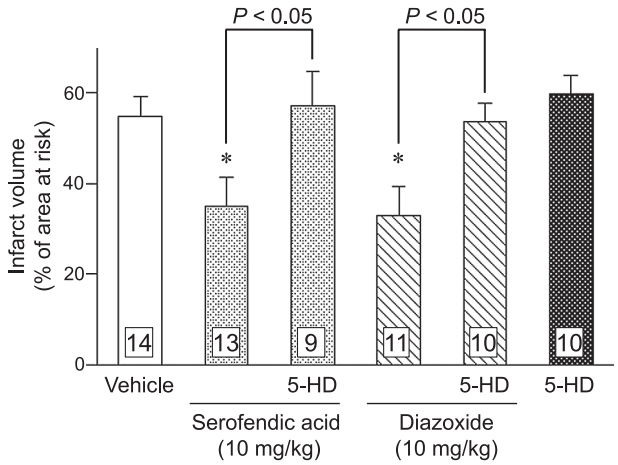

Fig. 2. Effect of 5-HD, a mitoK$_{ATP}$-channel blocker, on the protective effect of serofendic acid. The left coronary artery was occluded for 25 min with subsequent reperfusion. After 2 h of reperfusion, the hearts were sliced into coronal sections for TTC staining. Serofendic acid and diazoxide were each administered intravenously 15 min before ischemia and 5 min before reperfusion. 5-HD was administered 5 min before the respective drugs. The numbers in the columns indicate the numbers of experiments performed. *$P < 0.05$ vs. vehicle-treated group.

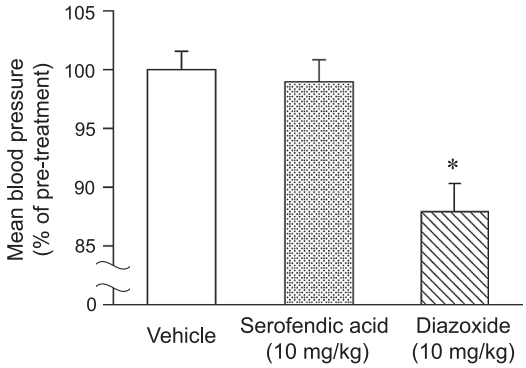

Fig. 3. Effect of serofendic acid and diazoxide on blood pressure. Arterial blood pressure was measured 15 min after the administration of drugs. *$P < 0.05$ vs. vehicle-treated group.

Involvement of mitochondrial ATP-sensitive potassium channels in myocardial protection of serofendic acid

We next investigated the mechanism by which serofendic acid protects against myocardial ischemia–reperfusion. We previously suggested the involvement of activation of mitoK$_{ATP}$ channels in the protective effect of serofendic acid against H_2O_2 injury in neonatal rat cardiomyocytes (27). Therefore, we examined whether 5-hydroxydecanoate (5-HD), a mitoK$_{ATP}$-channel blocker, affects the protective effect of serofendic acid in the in vivo model (Fig. 2). We administered 5-HD 5 min before the administration of serofendic acid. It abolished the protective effects of serofendic acid, whereas 5-HD alone did not affect the infarct size. Moreover, the effect of serofendic acid on infarct size reduction was comparable to that of diazoxide, a well-known mitoK$_{ATP}$-channel opener. These results suggest that myocardial protection of serofendic acid is mediated by opening mitoK$_{ATP}$ channels.

Effects of serofendic acid on mean blood pressure

Previous reports showed that diazoxide exhibits side effects: reduction of blood pressure or increase of blood glucose, because of the action on the plasma membrane ATP-sensitive potassium channels (31, 32). We measured the rate of reduction of mean blood pressure at 15 min after first administration to clarify whether serofendic acid affects blood pressure (Fig. 3). Blood pressure dropped to about 90% after the administration of diazoxide. Unlike diazoxide, serofendic acid did not change the blood pressure. Moreover, treatment with serofendic acid did not affect the blood glucose level (data not shown). These results indicate that serofendic acid may have fewer side effects than diazoxide.

Effect of serofendic acid on mitochondrial membrane potential loss induced by ischemia–reperfusion

The opening of mitoK$_{ATP}$ channels leads to the prevention of $\Delta\Psi_m$ loss induced by several stresses (33). For further testing of the protective effect of serofendic acid at the subcellular level, we monitored $\Delta\Psi_m$ in individual cells using real-time two-photon imaging. Time-lapse confocal analysis of Langendorff-perfused rat heart was performed at 5-min intervals. At first, we confirmed that TMRE fluorescence did not change during the 85 min of observation in the sham group (Fig. 4A, sham). In contrast, no-flow ischemia followed by reperfusion caused progressive loss of the red fluorescence intensity, indicating $\Delta\Psi_m$ loss (Fig. 4A, vehicle). Pretreatment of serofendic acid remarkably prevented the $\Delta\Psi_m$ loss (Fig. 4A, serofendic acid). Remarkably, none of the cells in the serofendic acid group lost $\Delta\Psi_m$ during the ischemic period. Moreover, serofendic acid robustly delayed the onset of $\Delta\Psi_m$ loss during the reperfusion period. Ten cells were randomly selected in each group, and the TMRE fluorescence intensity from each individual cell was plotted, as shown in Fig. 4B. Serofendic acid decreased the number of cells undergoing the dissipation of $\Delta\Psi_m$. Figure 4C shows the average TMRE fluorescence intensity from 10 randomly selected cells in each group and the mean TMRE fluorescence intensity at the end of the experimental period (85 min), indicating the significant protective effects of serofendic acid.

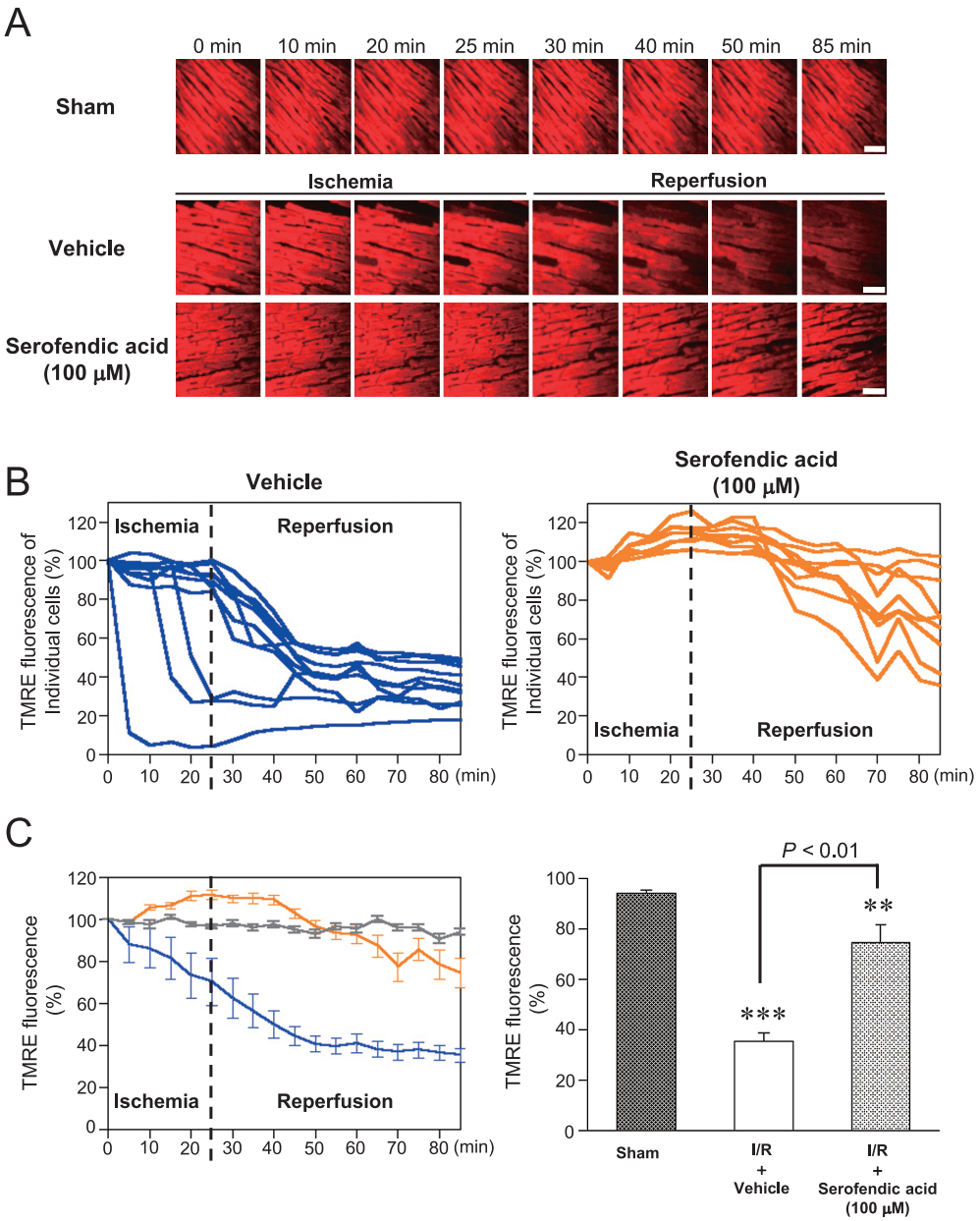

Fig. 4. Effect of serofendic acid on $\Delta\Psi_m$ loss induced by ischemia–reperfusion. A) Time-lapse imaging taken every 5 min. Sham indicates intact heart. Vehicle and serofendic acid indicate infusion of vehicle and serofendic acid, followed by 25 min of ischemia and 60 min of reperfusion. Data are from a single experiment, representative of at least 3 independent experiments. Scale bar = 50 μm. B) Time course of TMRE fluorescence in each individual cell (left panel) and mean fluorescence from 10 cells monitored at 5-min intervals. C) Mean fluorescence intensity at the end of the experimental period from 10 cells randomly and prospectively selected in each group. I/R: ischemia–reperfusion. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. sham.

Discussion

The major finding of this study is that serofendic acid reduced infarct size in a rat model of left coronary artery occlusion followed by reperfusion and that the protective effect was mediated by the opening of mitoK$_{ATP}$ channels. This is the first report on the protection against myocardial ischemia–reperfusion injury.

We previously reported that serofendic acid protected cardiac myocytes against oxidative stress by preventing $\Delta\Psi_m$ loss, calcium overload, and ROS accumulation (27, 28). The cardioprotective effect can be mediated by mitoK$_{ATP}$-channel openers, and mitoK$_{ATP}$-channel blockers prevent both preconditioning and pharmacological cardio-

protection (34, 35). MitoK_{ATP}-channel activation induces partial and modest mitochondrial membrane potential depolarization, thereby reducing the driving force for calcium uptake by mitochondria and preventing mitochondrial [Ca^{2+}] elevation (15). In the present study, we showed that 5-HD, a mitoK_{ATP}-channel blocker, abolished the protective effect of serofendic acid. This result suggests that the activation of mitoK_{ATP} channels plays a principal role in infarct size limitation of serofendic acid in vivo. However, we did not yet elucidate whether serofendic acid activates mitoK_{ATP} channels directly or indirectly. To reveal the target of serofendic acid in the activation of mitoK_{ATP} channels, we need further experiments. Serofendic acid possesses hydroxyl radical–scavenging activity (24). It may partially contribute to myocardial protection because ROS is an important inducer of collapsing mitochondrial integrity followed by cell death. In fact, serofendic acid prevented ROS accumulation induced by H_2O_2 in neonatal rat cardiac myocytes (27). Moreover, serofendic acid inhibited caspase-3 activation and reduced TUNEL-positive cells in cardiac myocytes and cortical neurons (26, 27). Thus, serofendic acid may exert myocardial protection through the combination of these effects.

The opening of MPTP leads to $\Delta\Psi_m$ loss and cell death (9). A growing body of evidence supports the concept that the inhibition of MPTP is an effective and promising strategy to prevent ischemia–reperfusion injury of the heart (5, 36, 37). One of the pharmacological targets to inhibit the opening of MPTP is an activation of mitoK_{ATP} channels. Opening of mitoK_{ATP} channels suppresses excessive elevation of mitochondrial [Ca^{2+}] and ROS, which are the most important inducers of MPTP opening (13, 15 – 17). MPTP opening leads to a sudden dissipation of $\Delta\Psi_m$ followed by mitochondrial dysfunction (17). Serofendic acid decreased the number of cells undergoing $\Delta\Psi_m$ loss and also delayed the onset of $\Delta\Psi_m$ loss induced by ischemia–reperfusion in Langendorff-perfused rat hearts. Our results suggest that serofendic acid attenuates myocardial ischemia–reperfusion injury by the prevention of MPTP opening mediated by the activation of mitoK_{ATP} channels.

Diazoxide causes hypotension because it acts not only on mitoK_{ATP} channels but also on membrane ATP-sensitive potassium channels of vascular smooth muscle cells (31, 32, 38). A highly selective agent for mitoK_{ATP} channels is preferred to avoid unwanted side effects. In fact, serofendic acid affected neither blood pressure nor individual death (data not shown). These results suggest that serofendic acid is free from unwanted side effects, in terms of the absence of the reduction of blood pressure and sudden death, which are among the major obstacles to clinical application. This result suggests that serofendic acid may selectively act on mitoK_{ATP} channels. Further experiments are essential to confirm whether serofendic acid acts on mitoK_{ATP} channels selectively.

In conclusion, our findings suggest that serofendic acid could be a novel candidate for cardioprotection against ischemia–reperfusion injury. As an endogenous substance, serofendic acid could be expected to have minimal, if any, unpredictable side effects. In fact, in the present study, we did not detect any effects of serofendic acid on blood pressure. This result further suggests that serofendic acid is free from unwanted side effects, which are among the major obstacles to clinical application. Therapeutic interventions designed to prevent MPTP opening during ischemia–reperfusion hold major promise as novel strategies for reducing cardiac injury from ischemia–reperfusion. Although further study should be performed in order to determine whether serofendic acid could improve the prognosis and left ventricular functions, serofendic acid or its analogs are potential therapeutic agents for cardiac ischemia–reperfusion injury.

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