Cardioprotective Effect of Diazoxide Is Mediated by Activation of Sarcolemmal but Not Mitochondrial ATP-Sensitive Potassium Channels in Mice

Masashi Suzuki, MD; Tomoaki Saito, MS; Toshiaki Sato, MD; Masaji Tamagawa, BS; Takashi Miki, MD; Susumu Seino, MD; Haruaki Nakaya, MD

Background—We recently demonstrated that the sarcolemmal ATP-sensitive potassium (sarcK\textsubscript{ATP}) channel plays a key role in cardioprotection against ischemia/reperfusion injuries in Kir6.2-knockout (KO) mice. In the present study, we evaluated the effects of diazoxide, a mitochondrial ATP-sensitive potassium (mitoK\textsubscript{ATP}) channel opener, on ischemia-induced myocardial stunning in sarcK\textsubscript{ATP} channel-deficient mice.

Methods and Results—Langendorff-perfused hearts of wild-type (WT) and KO mice were subjected to global ischemia/reperfusion. Diazoxide improved the recovery of contractile function in WT hearts but not in KO hearts. Treatment with HMR1098 (a sarcK\textsubscript{ATP} channel blocker) but not 5-hydroxydecanoate (a mitoK\textsubscript{ATP} channel blocker) abolished the cardioprotective effect of diazoxide in WT hearts. In coronary-perfused WT ventricular muscle preparations, action potential shortening during ischemia was accelerated in the presence of diazoxide.

Conclusions—Diazoxide enhances action potential shortening during ischemia by activating sarcK\textsubscript{ATP} channels and provides cardioprotection in mouse hearts.

Key Words: potassium ■ ischemia ■ reperfusion ■ ion channels

ATP-sensitive potassium (K\textsubscript{ATP}) channels play an important role in cardiovascular system. Our recent study using Kir6.2-deficient (KO) mice revealed that Kir6.2 forms the pore region of the cardiac sarcolemmal K\textsubscript{ATP} (sarcK\textsubscript{ATP}) channel but not that of the vascular sarcK\textsubscript{ATP} channel.\textsuperscript{1,2} In addition, neither Kir6.2 nor Kir6.1 seems to be a constituent of the mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) channel in cardiomyocytes.\textsuperscript{2,3} as assessed by flavoprotein oxidation assay. Recently, it was postulated that activation of mitoK\textsubscript{ATP} channels rather than sarcK\textsubscript{ATP} channels is important in cardioprotection against ischemia/reperfusion injuries, and that diazoxide and 5-hydroxydecanoate (5-HD) are a mitoK\textsubscript{ATP} specific opener and blocker, respectively.\textsuperscript{4-9} This conclusion was based on pharmacological data, however, and the molecular identity of the mitoK\textsubscript{ATP} channel has not been established.

We recently found that ischemic preconditioning is abolished in sarcK\textsubscript{ATP} channel-deficient mice despite intact mitoK\textsubscript{ATP} channel function.\textsuperscript{4} It is unclear whether diazoxide can exert cardioprotective effect, however, even if the sarcK\textsubscript{ATP} channel is negated. In the present study, we performed functional analysis of wild-type (WT) and knockout (KO) mouse hearts subjected to ischemia/reperfusion, and we show that the activity of the sarcK\textsubscript{ATP} channel is essential for diazoxide-induced anti-stunning effect, possibly caused by the reduced cardiac excitability when they are open.

**Methods**

**Kir6.2\textsuperscript{-/-} Mice**

All procedures complied with National Institutes of Health standards for the care and use of animal subjects. A mouse line deficient in K\textsubscript{ATP} channels was generated by targeted disruption of the gene coding for Kir6.2, as described previously.\textsuperscript{10} C57BL/6 mice from our laboratory were used as controls because the knockout animals had been backcrossed to a C57BL/6 strain for 5 generations.

**In Vitro Functional Study Using Langendorff-Perfused Hearts**

Functional study using isolated mouse hearts was performed as previously described.\textsuperscript{1,3} Retrograde perfusion was maintained at a constant flow (3 mL/min) with modified Krebs-Henseleit solution containing (in mmol/L): NaCl 119, KCl 4.8, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 1.2, CaCl\textsubscript{2} 1.0, glucose 10, and NaHCO\textsubscript{3} 24.9, and equilibrated with 95% O\textsubscript{2}/5% CO\textsubscript{2} (pH 7.4, 37°C). A balloon was inserted into the cavity of the left ventricle, and left ventricular pressure and coronary perfusion pressure (CPP) were measured continuously. After stabilization, hearts were subjected to no-flow, global ischemia (20 minutes) and reperfusion (60 minutes). Drug treatment was initiated 15 minutes before the ischemic period and continued throughout the experiments.

**Action Potential Recordings in Coronary-Perfused Ventricular Preparations**

The heart was isolated and perfused at a constant flow (2 mL/min) with the Tyrode solution containing (in mmol/L): NaCl 125, KCl 4,
NaH₂PO₄ 1.8, MgCl₂ 0.5, CaCl₂ 2.7, glucose 5.5, and NaHCO₃ 25, and gassed with 95% O₂/5% CO₂. The right and left atri, right ventricular free wall, and septum were removed. Coronary-perfused ventricular muscle preparations stimulated at 5 Hz were subjected to no-flow global ischemia. Action potentials (APs) were recorded with conventional microelectrode. Diazoxide was applied 10 minutes before the ischemic period.

Drugs
The drugs used were 5-HD, diazoxide (Sigma), and HMR1098 (1-[5-[2-(5-chloro-6-aminophenyl)sulfonyl]-3-methylthiourea, Aventis Pharma, Tokyo, Japan). Diazoxide was dissolved in DMSO, and the final concentration of solvent was 0.01%. HMR1098 and 5-HD were dissolved in the perfusate.

Statistics
All data are presented as mean±SEM. Statistical analyses of the data were performed using Student’s t test or ANOVA. Probability values less than 0.05 were considered significant.

Results
There were no significant differences in baseline values of heart rate, left ventricular developed pressure (LVDP), and CPP among the 6 experimental groups: control WT (WT-CON, n=9), diazoxide (100 μmol/L)-treated WT (WT-DZ, n=7), diazoxide (100 μmol/L) plus 5-HD (500 μmol/L)-treated WT (WT-DZ+5HD, n=6), diazoxide (100 μmol/L) plus HMR1098 (30 μmol/L)-treated WT (WT-DZ+HMR, n=7), control KO (KO-CON, n=9), and diazoxide (100 μmol/L)-treated KO hearts (KO-DZ, n=7). Treatment with diazoxide significantly decreased CPP in WT (107±5 mm Hg to 73±4 mm Hg) and KO hearts (108±7 mm Hg to 70±6 mm Hg). The drug concentration used in this study was reported to activate or block mitoK₄ATP or sarcK₄ATP channels.⁸

During global ischemia, left ventricular developed pressure gradually declined and the left ventricular end-diastolic pressure (EDP) increased (Figure 1a). It took significantly more time for KO-CON (7.7±1.2 minutes, P<0.05) and WT-DZ+HMR hearts (8.0±2.5 minutes, P<0.05) to cease contracting during ischemia compared with WT-CON hearts (3.5±0.5 minutes). Ischemic contracture appeared more rapidly in WT-DZ+HMR (4.9±0.5 minutes, P<0.05), KO-CON (6.2±0.8 minutes, P<0.05), and KO-DZ hearts (4.2±1.1 minutes, P<0.05) than in WT-CON hearts (9.8±1.0 minutes).³ The increase in EDP at 15 minutes of ischemia was significantly greater in KO than WT hearts (Figure 1b). Diazoxide slightly attenuated the increase in EDP in WT (WT-DZ, not significant) but not KO hearts (KO-DZ). Coadministration of HMR1098 abolished the lessening effect of diazoxide on the increase in EDP in WT hearts (P<0.05), whereas addition of 5-HD to diazoxide slightly and insignificantly enhanced the increase in EDP (Figure 1b).

After reperfusion, LV contractile function recovered gradually. Treatment with diazoxide significantly improved the recovery of LV function in WT hearts (Figure 1c). However, diazoxide did not improve the recovery of LV function in KO hearts. In addition, the recovery of LV function in WT-DZ+HMR hearts was similar to that in both KO groups (KO-CON, KO-DZ). Coadministration of 5-HD (WT-DZ+5HD) did not significantly affect the diazoxide-induced protective effect (Figure 1c). These findings indicate that activation of sarcK₄ATP rather than mitoK₄ATP channels is necessary for diazoxide-induced cardioprotection.

To determine if diazoxide activates sarcK₄ATP channels during ischemia in WT hearts, we measured APs in coronary-perfused ventricular muscle preparations. The preischemic values of AP duration at 90% repolarization (APD₉₀) and resting membrane potential in control (untreated) WT hearts (n=5) and diazoxide-treated WT hearts (n=5) were 61.4±3.7 ms and −71.6±2.4 mV and 58.0±4.4 ms and −73.7±3.4 mV, respectively. There were no significant differences between the baseline values. After induction of global ischemia, APD gradually decreased in untreated WT hearts (Figure 2a). Treatment with diazoxide significantly

Figure 1. Summarized data of mechanical function of isolated hearts during ischemia/reperfusion. a, Representative left ventricular pressure changes in control WT (WT-CON), diazoxide (100 μmol/L)-treated WT (WT-DZ), diazoxide (100 μmol/L) plus 5-HD (500 μmol/L)-treated WT (WT-DZ+5HD), diazoxide (100 μmol/L) plus HMR1098 (30 μmol/L)-treated WT (WT-DZ+HMR), control KO (KO-CON), and diazoxide (100 μmol/L)-treated KO hearts (KO-DZ) are shown. Arrows with full line and those with dashed line show the time points of cessation of contraction and onset of contracture, respectively. b, Left ventricular end-diastolic pressure (EDP) at 15 minutes after ischemia and c, recovery rate of left ventricular developed pressure at 30 minutes of reperfusion are summarized. Values are expressed as mean±SEM. *P<0.05 versus WT-CON; #P<0.05 versus WT-DZ.
accelerated the action potential shortening during ischemia (Figure 2b). APD90 at 2 minutes after ischemia in untreated and diazoxide-treated WT hearts were 50.4±4.1 ms and 17.2±2.5 ms, respectively (P<0.05) (Figure 2c). These findings indicate that diazoxide activates sarcKATP channels and enhances AP shortening during ischemia.

Discussion

Diazoxide is generally thought to be a mitoKATP channel-specific opener in cardiomyocytes. In addition, diazoxide induces no sarcolemmal KATP currents using nystatin-perforated patch-clamp techniques in isolated mouse ventricular cells (Suzuki et al, unpublished observations, 2001), as has been reported also in other species.4,8,11 Because diazoxide produces a flavoprotein oxidation that reflects similar openings of mitoKATP channels in ventricular cells of both WT and Kir6.2-deficient mice,3 diazoxide should have a similar mitoKATP channel-mediated cardioprotective effect. However, diazoxide significantly improved the recovery of left ventricular function after ischemia/reperfusion in WT but not in KO hearts in this study. These diazoxide-induced cardioprotective effects were abolished by coadministration of HMR1098 but not 5-HD, further indicating that the cardioprotective effect is mediated by sarcKATP channels but not mitoKATP channels. In addition, diazoxide shortened APD during myocardial ischemia in coronary-perfused ventricular muscle preparations of WT mice. These findings strongly suggest that in mice hearts, diazoxide activates sarcKATP channels and shortens the action potential duration during ischemia, thereby blunting intracellular Ca2+ overload.

Recently, Matsuoka et al12 and D’hahan et al13 reported that diazoxide activates sarcKATP channels during simulated ischemia or when ADP is added in reconstituted channels (Kir6.2/SUR2A) or guinea pig ventricular myocytes, in accord with our findings. During myocardial ischemia, the intracellular ADP level might increase sufficiently to allow diazoxide to activate the sarcKATP channels. Because ischemia-induced action potential shortening occurred more rapidly in the presence of diazoxide (Figure 2), intracellular Ca2+ overload might well be lessened and the recovery of left ventricular function after reperfusion improved.

It should be noted that in our studies contractile function but not infarct size was measured, we did not give any mitoKATP openers other than diazoxide, and direct assessment of mitoKATP channel activity was not performed, although our previous study showed that mitoKATP channel activity, indirectly assessed by flavoprotein fluorescence measurement, was preserved.3 These facts potentially limit the interpretation of our findings. In addition, role of sarcKATP against ischemia/reperfusion injuries might be exaggerated in mouse relative to larger animals, as mouse is a species in which the physiological heart rate is approximately 10-fold higher than in humans. In this context, it is noteworthy that in rat, cardioprotective doses of diazoxide did not shorten APD during ischemia, and the cardioprotection by diazoxide was blocked by 5-HD but not by HMR1098.4,7 Therefore, the findings obtained from mouse hearts cannot be directly extrapolated to clinical settings.

In conclusion, diazoxide-induced protective effect on the ischemia-induced contractile dysfunction of mouse heart is mediated by accelerated activation of sarcKATP channels during ischemia, suggesting that activation of sarcKATP channels rather than of mitoKATP channels is important for diazoxide-induced anti-stunning effect.

Acknowledgments

This work was supported by a Scientific Research Grant and a Grant-in-Aid for Creative Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Mitsui Life Insurance Research Foundation; and K. Watanabe Research Fund. HMR1098 was kindly provided by Aventis Pharma, Tokyo, Japan. We thank Drs H. Uemura and T. Ogura for helpful discussion. We are grateful to Y. Reien and I. Sakashita for excellent technical and secretarial assistance.

References


Cardioprotective Effect of Diazoxide Is Mediated by Activation of Sarcolemmal but Not Mitochondrial ATP-Sensitive Potassium Channels in Mice
Masashi Suzuki, Tomoaki Saito, Toshiaki Sato, Masaji Tamagawa, Takashi Miki, Susumu Seino and Haruaki Nakaya

_Circulation_. 2003;107:682-685; originally published online February 3, 2003; doi: 10.1161/01.CIR.0000055187.67365.81
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/107/5/682

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/