ORIGINAL PAPER

Mitochondrial K$^+$ channels are involved in ischemic postconditioning in rat hearts

Chunhong Jin · Jinrong Wu · Makino Watanabe · Takao Okada · Takafumi Iesaki

Abstract The mitochondrial calcium-activated potassium channel (mitoK$_{Ca}$) and the mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$) are both involved in cardiac preconditioning. Here, we examined whether these two channels are also involved in ischemic or pharmacological postconditioning. Using Langendorff perfusion, rat hearts were made hypoxic for 45 min and then reoxygenated for 30 min. Ischemic postconditioning (IPT) was achieved through application of 3 cycles of 10 s of reperfusion and 10 s of ischemia before reoxygenation, with and without paxilline (Pax; a mitoK$_{Ca}$ blocker) or 5-hydroxydecanoate (5-HD; a mitoK$_{ATP}$ blocker). Pharmacological postconditioning was carried out for 5 min at the onset of reoxygenation using NS1619 (a mitoK$_{Ca}$ opener) or diazoxide (Dia; a mitoK$_{ATP}$ opener). Pax and 5-HD abolished IPT-induced cardioprotection from reoxygenation injury, whereas administration of NS1619 or Dia significantly improved cardiac contractile activity and reduced aspartate aminotransferase (an index of myocyte injury) release following reoxygenation. In addition, isolated rat myocytes were loaded with tetramethylrhodamine methyl ester (TMRE; fluorescent mitochondrial membrane potential indicator) and 2′,7′-dichlorofluorescein [DCFH; fluorescent reactive oxygen species (ROS) indicator] or Fluo-4-acetoxymethyl ester (Fluo-4-AM; fluorescent calcium indicator). When TMRE-loaded myocytes were laser illuminated, the DCFH and Fluo-4 fluorescence increased, and TMRE fluorescence decreased. These effects were significantly inhibited by NS1619 and Dia. We therefore conclude that IPT may protect the heart through activation of mitoK$_{ATP}$ and mitoK$_{Ca}$ channels, and that opening of these channels at the onset of reoxygenation protects the heart from reoxygenation injury, most likely by reducing excess generation of ROS and the resultant Ca^{2+} overload.

Keywords Cardioprotection · Hypoxia · Reoxygenation · Mitochondria · K$^+$ channels

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Introduction

Preconditioning is a well-characterized phenomenon in which a brief period of ischemia or addition of a pharmacological agent can protect the myocardium from infarction induced by a subsequent and prolonged ischemic attack [1–5]. More recently, it was reported that postconditioning [6–9]—i.e., repetitive cycles of reperfusion and ischemia or addition of pharmacological agents at the onset of reperfusion—enables substantial salvage of the myocardium and offers a degree of protection similar to that obtained with preconditioning. Cardioprotection through preconditioning and postconditioning can be induced by a variety of stimuli [10, 11], but the opening of mitochondrial ATP-sensitive potassium channels (mitoK$_{ATP}$), which are involved in the regulation of mitochondrial function, appears to be a common step in mediating the cardioprotective effects of ischemic and pharmacological preconditioning and postconditioning [3, 9, 12, 13]. Although the mechanism remains unclear, it is thought that the activity of this

channel may suppress the opening of the mitochondrial permeability transition pore (mPTP).

In addition, recent studies have shown that activation of another potassium channel, the mitochondrial calcium-activated potassium channel (mitoK$_{Ca}$), is also involved in preconditioning [14, 15]. The opening of mitoK$_{Ca}$ channels induces both early and delayed preconditioning in adult mice [16], reduces generation of reactive oxygen species (ROS), and improves mitochondrial bioenergetics after ischemia–reperfusion in guinea pig hearts [4]. However, it is uncertain whether activation of mitoK$_{Ca}$ channels is involved in ischemic postconditioning (IPT) or pharmacological postconditioning.

Events thought to be involved in reperfusion/reoxygenation injury include robust generation of ROS [17–19], Ca^{2+} overload [20, 21] and the opening of mPTP [22]. Our aim was to test the hypothesis that the effects of IPT are associated with activation of mitoK$_{Ca}$ and mitoK$_{ATP}$ channels, and that the activation of these channels at the onset of reoxygenation protects hearts from injury by reducing ROS generation and the accumulation of cytosolic Ca^{2+}. To investigate this issue, we examined the effects of the mitoK$_{Ca}$ channel opener NS1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-trifluoromethyl-2H-benzimidazol-2-one) and the mitoK$_{ATP}$ channel opener diazoxide (Dia) on cardiac function and on ROS generation, mPTP opening, and cytosolic Ca^{2+} levels in isolated cardiac myocytes.

Materials and methods

This investigation conformed to the Guide for the Care and Use of Laboratory Animals [US National Institute of Health publication, DHEW publication No. (NIH) 85-23, 1996] and was approved by the Juntendo University Faculty of Medicine (Tokyo, Japan) animal experimentation committee.

Langendorff perfusion

Male Sprague–Dawley (SD) rats weighing 285–340 g (7–8 weeks) were used. After anesthetizing the rats with pentobarbital sodium (55 mg kg^{-1}, i.p.), they were decapitated and their hearts were quickly excised for Langendorff perfusion [23]. Each heart was perfused with modified Krebs–Henseleit solution (containing in mM: NaCl 116.0, NaHCO$_3$ 25.0, MgSO$_4$ 1.2, KCl 4.7, KH$_2$PO$_4$ 1.2, CaCl$_2$ 2.0, glucose 5.5, pH 7.4) in a retrograde direction at a constant flow rate of 13 ml min^{-1} without recirculation. The perfusate was warmed to 38 °C and oxygenated with a 95 % O$_2$–5 % CO$_2$ gas mixture to maintain the partial pressure of O$_2$ above 400 mmHg. During the period of

hypoxia, glucose in the perfusate was replaced with equimolar sucrose, and the solution was saturated with a 95 % N$_2$–5 % CO$_2$ gas mixture to maintain the partial pressure of O$_2$ at approximately 20 mmHg (hypoxic solution). In addition, a latex balloon was inserted through the mitral annulus into the left ventricular cavity, and distilled water (0.1–0.2 ml) was injected into the balloon until it was inflated to just above the level required to produce a visible (1–2 mmHg) elevation in left ventricular end-diastolic pressure (LVEDP). Left ventricular developed pressure (LVDP), LVEDP, heart rate (HR), and coronary perfusion pressure were monitored throughout the experiments. The extent of the irreversible myocardial damage was assessed by measuring the amount of aspartate aminotransferase (AST) released into the coronary effluent. The AST activity in 10-μl aliquots of coronary effluent was estimated using a dry chemical method with a commercially available kit (Fuji Film, Tokyo, Japan). The released AST activity was normalized to IU g^{-1} min^{-1} (dry tissue).

Perfusion protocols

After stabilization for 20 min, the hearts were subjected to 45 min of hypoxia followed by 30 min of reoxygenation (Fig. 1). IPT was attained by 3 cycles of 10 s of reperfusion and 10 s of ischemia before reoxygenation (by stopping coronary perfusion) in the absence or presence of paxilline (Pax, mitoK$_{Ca}$ blocker; 1 μM) or 5-hydroxydecanoate (5-HD, mitoK$_{ATP}$ blocker; 100 μM). Pharmacological postconditioning was performed for 5 min at the onset of reoxygenation using NS1619 (mitoK$_{Ca}$ opener; 30 μM) or Dia (mitoK$_{ATP}$ opener; 100 μM).
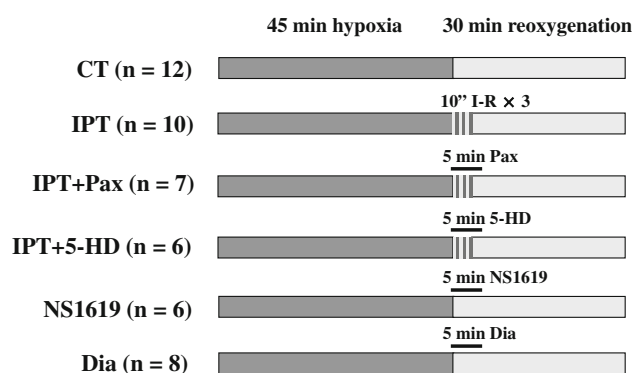

Fig. 1 Protocols for the Langendorff experiment. Hearts were stabilized for at least 20 min, after which they were subjected to 45 min of hypoxic perfusion followed by 30 min of reoxygenation. *CT* control, *IPT* ischemic postconditioning, *I–R* ischemia–reperfusion, *Pax* paxilline (mitoK$_{Ca}$ channel blocker, 1 μM), *5-HD* 5-hydroxydecanoate (mitoK$_{ATP}$ channel blocker, 100 μM), *NS1619* (mitoK$_{Ca}$ channel opener, 30 μM), *Dia* diazoxide (mitoK$_{ATP}$ channel opener, 100 μM), *n* number of hearts

Isolation of ventricular myocytes

Ventricular myocytes were isolated from adult male SD rats (285–340 g). After anesthetizing the rats with pentobarbital sodium (55 mg/kg, i.p.), the hearts were rapidly excised and perfused using a Langendorff apparatus, first with normal Tyrode solution (in mM: NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 10, glucose 10, pH 7.4) for 5 min, then with Ca^{2+}-free Tyrode for 5 min, and finally with collagenase (0.45 mM; S-1) solution for 5 min. All solutions were saturated with 100 % O_2 and warmed to 37 °C. After digestion, the ventricles were cut into small pieces and dissociated in nominally Ca^{2+}-free Tyrode solution in a shaking water bath. The resultant suspension was filtered through nylon mesh (ϕ 200 μm) to obtain the dissociated myocytes. The Ca^{2+} concentration was then gradually increased to 2.0 mM, and the ventricular myocytes were stored in normal Tyrode solution at room temperature (22–25 °C).

Induction of mPTP opening in ventricular myocytes

Intermittent laser illumination of cardiomyocytes loaded with a mitochondria-specific fluorescent dye, tetramethylrhodamine ethyl ester (TMRE), generates ROS within the mitochondria that provoke the mPTP [24–27]. Oxidative stress generated upon hypoxia/reoxygenation of myocardium also involves the excess production of ROS within the mitochondria, and this model should therefore simulate the events associated with hypoxia/reoxygenation-induced cell injury.

In the present study, isolated myocytes were loaded with TMRE (2 μM) for 10 min, washed, and then loaded with the fluorescent Ca^{2+} indicator Fluo-4 acetoxymethyl ester (Fluo-4-AM, 10 μM) or the ROS-specific indicator 2′,7′-dichlorofluorescein (DCFH; 10 μM) for 30 min. After washout of the fluorescent dyes, the isolated ventricular myocytes were stored at room temperature in normal Tyrode solution. To elicit mPTP opening, TMRE-loaded myocytes were intermittently illuminated (for 4 s with 20-s intervals) at 543 nm for 15 min using a 5-mW He/Ne laser system (Laser-Fertigung, Hamburg, Germany). mPTP opening was detected as a reduction in 574 nm fluorescent emission.

The combination of a 488-nm 200-mW argon laser (Laser-Fertigung) and 520 nm long pass emission filters were used to visualize the Fluo-4 and DCFH fluorescences. In the experiment, the dye-loaded myocytes were perfused with oxygenated Krebs–Henseleit solution, and NS1619 (30 μM) or Dia (100 μM) was administered throughout the laser illumination. In addition, changes in cell length induced by the intermittent laser illumination were measured and expressed as the ratio of final/initial cell length.

To measure time-dependent fluorescence changes, data were acquired every 20 s. Confocal imaging was carried out using a Zeiss laser-scanning microscope (LSM 510 META v.3.2; Carl Zeiss, Germany) equipped with a Zeiss Plan Apochromat 40× immersion objective.

Chemicals

Pax, NS1619, 5-HD and Dia were purchased from Biomol (PA, USA). Collagenase was from Nitta Gelatin (Osaka, Japan). TMRE and Fluo-4-AM were from Molecular Probes (Eugene, OR, USA). DCFH was from Wako Pure Chemicals (Osaka, Japan).

Statistical analysis

Data are presented as mean ± SEM. Differences between means were analyzed using Student's t test or ANOVA and the Bonferroni method, as deemed appropriate. Values of $P < 0.05$ were considered significant.

Results

Blocking of $mitoK_{Ca}$ or $mitoK_{ATP}$ channel inhibits IPT-induced cardioprotection

Figure 2 shows the effects of Pax, a $mitoK_{Ca}$ channel blocker and 5-HD, a $mitoK_{ATP}$ channel blocker on IPT-induced cardioprotection. The changes in HR are shown in Fig. 2a. Recovery of HR at 10 min into reoxygenation was significantly greater ($P < 0.05$) in the IPT group (275 ± 13 bpm, mean ± SEM) compared to the CT group (214 ± 21 bpm), the IPT + Pax group (191 ± 33 bpm), and the IPT + 5HD group (191 ± 34 bpm). After 30 min of reoxygenation, the HR had recovered to the basal level in all four groups, and there was no significant difference among them.

The changes in cardiac contractile activity are shown in Fig. 2b, where it is expressed as a pressure-rate product (PRP = HR × LVDP) and normalized as a percentage of the basal PRP (N). Upon reoxygenation, recovery of PRP was significantly faster and greater in the IPT group (57.9 ± 2.0 %, R30, $P < 0.05$) than in the CT group (47.2 ± 1.8 %, R30). In the IPT + Pax group (51.9 ± 2.9 %, R30) and in the IPT+5-HD (39.4 ± 3.3 %, R30), the changes in PRP upon reoxygenation were similar to those in the CT group, and recovery levels were significantly lower ($P < 0.05$) than in the IPT group.

Irreversible cardiac damage was estimated based on the amount of AST released into the coronary effluent (Fig. 2c). After the first 2 min of reoxygenation, the AST release was significantly lower ($P < 0.05$) in the IPT
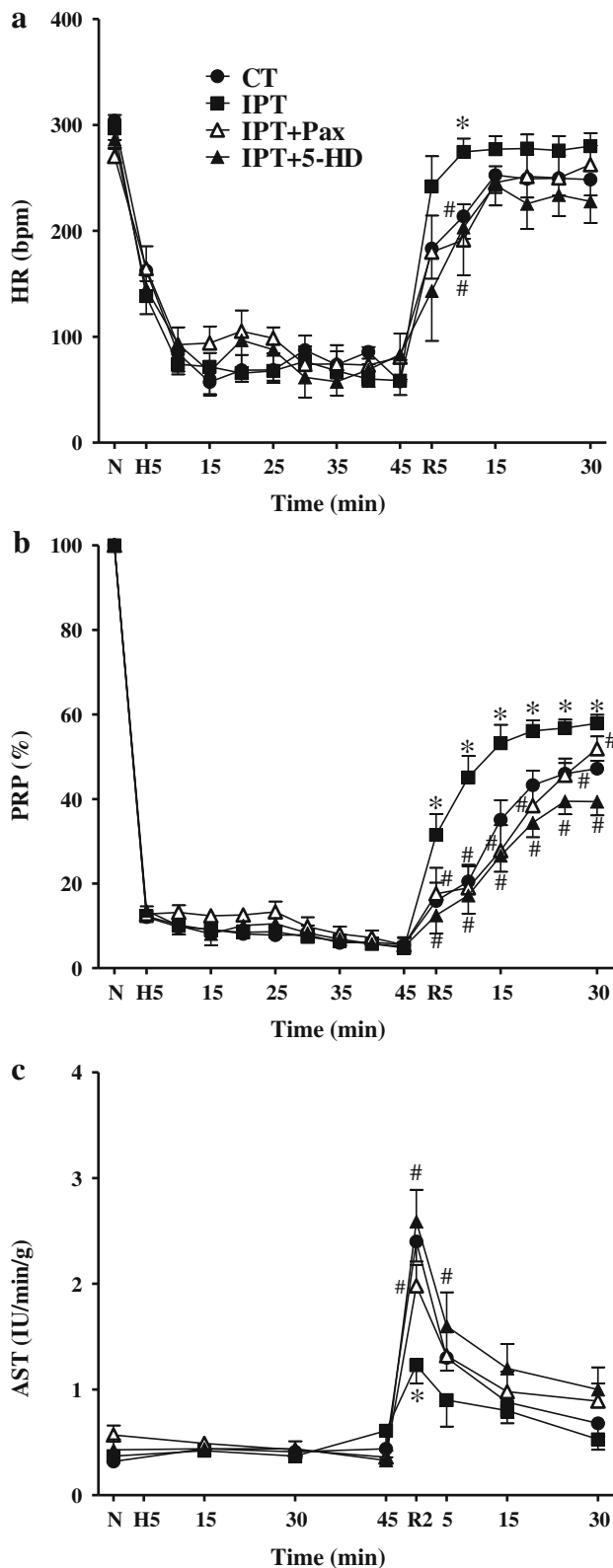
Fig. 2 Effects of $mitoK_{Ca}$ and $mitoK_{ATP}$ channel inhibition on IPT-induced cardioprotection. *Filled circles*, *filled squares*, *open triangles* and *filled triangles* indicate the control (*CT*), IPT, *IPT + Pax*, and *IPT + 5-HD* groups, respectively. **a** Heart rate, **b** pressure-rate product (*PRP*), **c** released *AST*. *$P < 0.05$ versus CT; #$P < 0.05$ versus IPT

group $(1.2 \pm 0.2$ IU g^{-1} min$^{-1})$ than in the CT group $(2.4 \pm 0.2$ IU g^{-1} min$^{-1})$, IPT + Pax group $(2.0 \pm 0.2$ IU g^{-1} min$^{-1})$, and IPT + 5-HD $(2.6 \pm 0.3$ IU g^{-1} min$^{-1})$ group.

Pax $(n = 6)$ or 5-HD $(n = 6)$ were also given for 5 min without any other treatment at the onset of reoxygenation. There were no significant differences in HR, PRP, and AST release among the CT, Pax, and 5-HD groups (data not shown).

Pharmacological postconditioning with a $mitoK_{Ca}$ or $mitoK_{ATP}$ channel opener mitigates hypoxia-reoxygenation injury

Our observation that blocking of $mitoK_{Ca}$ and $mitoK_{ATP}$ channels inhibited the cardioprotective effects of IPT prompted us to assess the efficacy of pharmacological postconditioning with a $mitoK_{Ca}$ or $mitoK_{ATP}$ channel opener, NS1619 or Dia, respectively (Fig. 3). We found that HR recovered to a similar level in the three groups tested (Fig. 3a). On the other hand, NS1619 improved the recovery of PRP. At the end of the reoxygenation, PRP had recovered to 59.8 ± 6.0 % in the NS1619 group, which was significantly higher $(P < 0.05)$ than in the CT group $(46.6 \pm 2.8$ %, R30) (Fig. 3b). Dia had a similarly beneficial effect on PRP, so that the recovery of PRP $(56.8 \pm 2.6$ %, R30) was significantly better $(P < 0.05)$ in the Dia group than the CT group (Fig. 3b). Finally, after the first 2 min of reoxygenation, significantly less $(P < 0.05)$ AST were released in the NS1619 $(1.0 \pm 0.3$ IU g^{-1} min$^{-1})$ and Dia $(1.4 \pm 0.1$ IU g^{-1} min$^{-1})$ groups than in the CT group $(2.9 \pm 0.5$ IU g^{-1} min$^{-1})$ (Fig. 3c).

$MitoK_{Ca}$ and $mitoK_{ATP}$ channel openers inhibit ROS production and Ca^{2+} accumulation in cardiac myocytes

We next assessed the ability of NS1619 and Dia to protect against laser-induced cardiac myocyte damage. Figure 4a shows representative sequential confocal images of TMRE/DCFH-loaded (left panel) and TMRE/Fluo-4-loaded (right panel) cardiomyocytes before and after 15 min of intermittent laser illumination. Increases and decreases in the levels of intracellular ROS and Ca^{2+} are reflected by corresponding changes in the fluorescence intensity of DCFH (yellow) and Fluo-4 (green), respectively, while mPTP openings are indicated by reductions in TMRE fluorescence (red). After 15 min of intermittent laser illumination, DCFH intensity had increased to 1.69 ± 0.19-fold (mean $\pm$ SEM, expressed as fold changes of intensity before laser illumination) in the CT group (Fig. 4b). This increase in the relative DCFH intensity was significantly $(P < 0.05)$ diminished to 1.13 ± 0.12-fold in the NS1619 group and to 1.26 ± 0.10-fold in the Dia group (Fig. 4b), indicating a reduction in ROS generation.
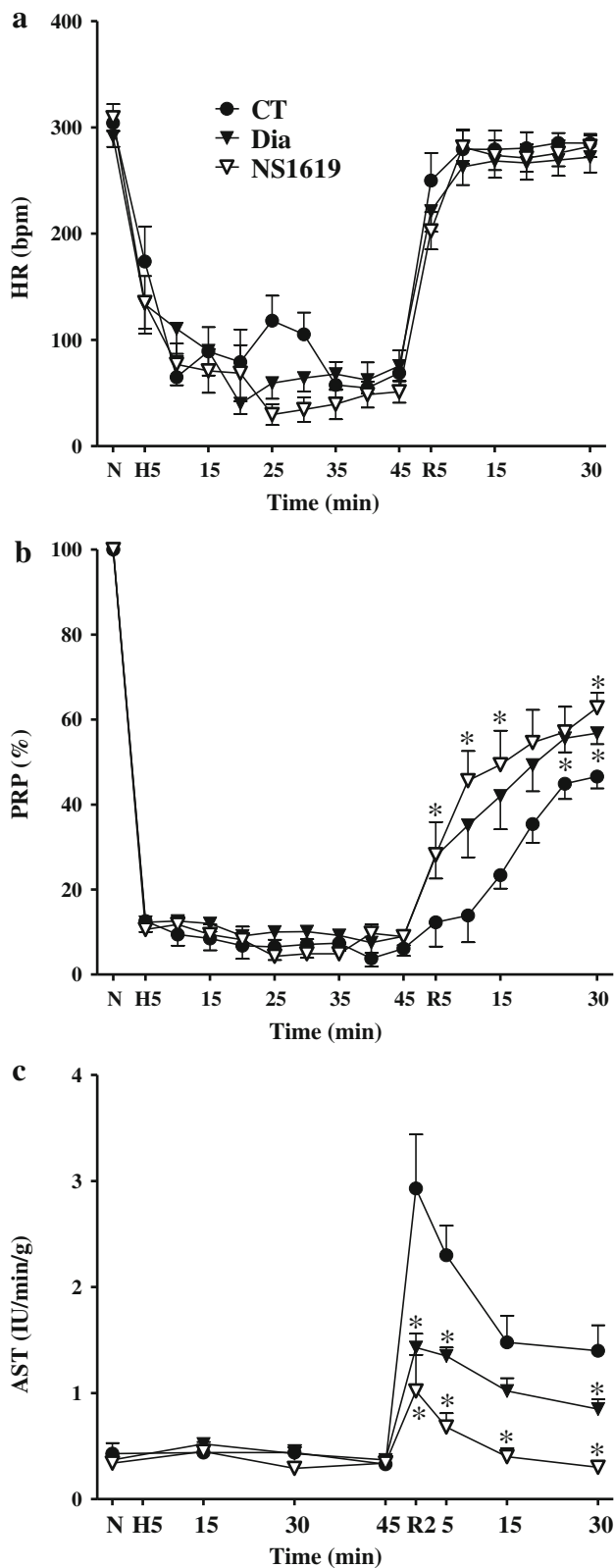
Fig. 3 Effects of pharmacological postconditioning using a mitoK$_{ATP}$ or mitoK$_{Ca}$ channel opener. *Filled circles, open inverted triangles* and *filled inverted triangles* indicate the control (*CT*), *NS1619* and diazoxide (*Dia*) groups, respectively. **a** Heart rate, **b** pressure-rate product (*PRP*), **c** released *AST*. *$P < 0.05$ versus CT

In untreated cells, TMRE fluorescence remained unchanged through a 15-min observation period (data not shown). By contrast, the relative TMRE intensity had declined to 0.48 ± 0.07-fold after 15 min of intermittent laser illumination in the CT group (Fig. 4c), indicating a loss of mitochondrial membrane potential due to the opening of mPTP. NS1619 and Dia significantly mitigated this reduction in the TMRE fluorescence, such that the relative TMRE intensity was 0.86 ± 0.05-fold in the NS1619 group and 0.84 ± 0.12-fold in the Dia group, both of which were significantly ($P < 0.05$) higher than in the CT group (Fig. 4c).

Fluo-4 fluorescence increased during the intermittent laser illumination, indicating a rise in cytosolic Ca^{2+} concentration, and NS1619 and Dia attenuated the laser-induced increase in the Fluo-4 intensity. The relative Fluo-4 intensity was 1.28 ± 0.14-fold in the NS1619 group and 1.15 ± 0.17-fold in the Dia group, which was significantly ($P < 0.05$) lower than in the CT group (1.75 ± 0.17-fold) (Fig. 4d).

Intermittent laser illumination also induced cell shortening (final/initial cell length in the CT group: 0.52 ± 0.07-fold), and those were significantly ($P < 0.05$) mitigated by NS1619 (0.90 ± 0.07-fold) and by Dia (0.80 ± 0.08-fold) (Fig. 4e).

Discussion

In this study, we found that (1) the recovery of LVDP and PRP upon reoxygenation was facilitated by IPT, and that the effect of IPT was inhibited by blockade of the mitoK$_{Ca}$ or mitoK$_{ATP}$ channels; (2) pharmacological postconditioning using a mitoK$_{Ca}$ or mitoK$_{ATP}$ channel openers improved recovery of PRP and inhibited AST release upon reoxygenation; and (3) pharmacological postconditioning inhibited the ROS production, cytosolic Ca^{2+} accumulation, and depolarization of mitochondrial membrane potential caused by laser-induced mPTP opening in isolated myocytes.

The recovery of cardiac contractile activity upon reoxygenation was improved by IPT, and that beneficial effect was antagonized by mitoK$_{Ca}$ channel blockade and mitoK$_{ATP}$ channel blockade (Fig. 2), which indicates IPT is mediated, at least in part, by activation of mitoK$_{Ca}$ channels and mitoK$_{ATP}$ channels. MitoK$_{ATP}$ channels has been reported to play a role in mediating IPT-induced cardioprotection [7, 9], and our finding that 5-HD administered at the onset of reoxygenation inhibited IPT-induced cardioprotection is consistent with those earlier reports (Fig. 2). In the present study, we also demonstrated IPT-mediated cardioprotection is abolished by not only mitoK$_{ATP}$ channel blockade but also by mitoK$_{Ca}$ channel

Fig. 4 Effects of mitoK$_{Ca}$ and mitoK$_{Ca}$ channel openers on mitochondrial membrane potential, ROS production, cytosolic Ca^{2+} concentration and cell length. **a** Representative sequential confocal images of TMRE (*red*)/DCFH (*yellow*)-loaded and TMRE/Fluo-4 (*green*)-loaded myocytes before and after 15 min of intermittent laser illumination. **b** Changes in ROS production (*DCFH* fluorescence). **c** Changes in mitochondrial membrane potential (*TMRE* fluorescence). **d** Changes in the cytosolic Ca^{2+} concentration (*Fluo-4* fluorescence). **e** Changes in cell length (final/initial of cell length). *N* before laser illumination, *CT* control, *Dia* diazoxide, *n* number of cells. *$P < 0.05$ versus CT (color figure online)
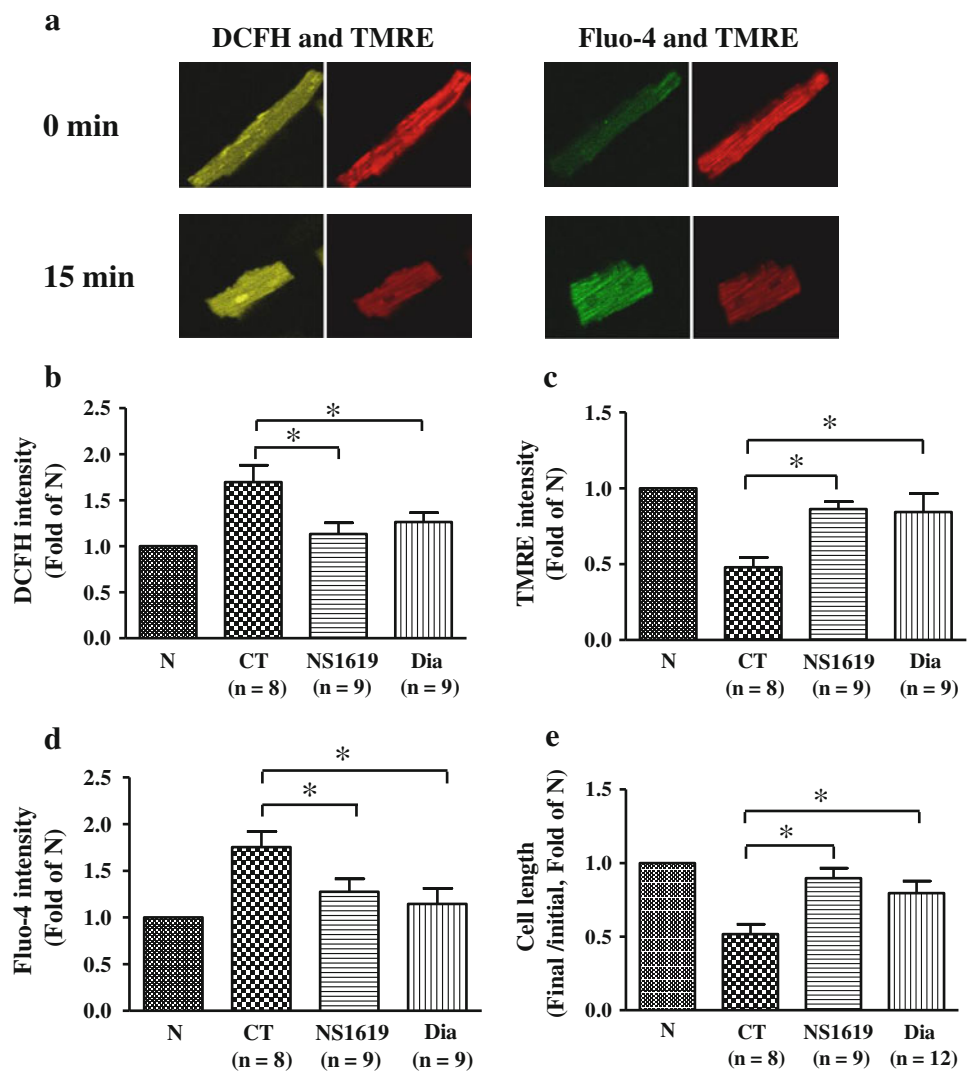
blockade. This is the first study which examined two types of mitochondrial K$^+$ channels on IPT induced cardiac protection in one experiment. Conversely, direct activation of mitoK$_{Ca}$ channel with the opener NS1619 (Fig. 3) or direct activation of mitoK$_{ATP}$ channel with the opener Dia (Fig. 3) at the onset of reoxygenation have cardioprotective effects like that of IPT. Taken together, these findings suggest that activation of mitochondrial K$^+$ channels is a key cardioprotective event, and that IPT activates both mitoK$_{Ca}$ and mitoK$_{ATP}$ channels.

There have been a number of studies implicating mitoK$_{ATP}$ channels in cardioprotection [28–30], and more recently the involvement of mitoK$_{Ca}$ channels in preconditioning [1, 14, 15] or postconditioning [31] have also been suggested. For example, it was recently shown that treatment with the mitoK$_{Ca}$ channel opener NS1619 can induce cardioprotection [14, 15, 31, 32]. Similarly, application of NS11021, a novel large-conductance Ca^{2+}-activated K$^+$ channel activator, prior to or at the

onset of reperfusion protects the heart from ischemia–reperfusion injuries [1]. On the other hand, it has also been reported that NS1619 failed to exert a postconditioning effect in an earlier study [16]. This discrepancy may be due to the differences in the experimental model between studies in the previous study and in the present study.

IPT reportedly protects the heart by inhibiting ROS generation [19]. We therefore examined the effect of mitoK$_{Ca}$ and mitoK$_{ATP}$ channel activation on ROS generation induced by intermittent laser illumination. Previous reports have shown that both ROS generation and intracellular Ca^{2+} overload play crucial roles in the induction of cell death during reperfusion/reoxygenation [33, 34]. A burst of ROS increases Ca^{2+} influx through L-type Ca^{2+} channels, increasing the cytosolic Ca^{2+} concentration [35, 36]. In addition, ROS reportedly increase the activity of the Na$^+$/H$^+$ exchanger, which further increases cytosolic Ca^{2+} by stimulating reverse mode Na$^+$/Ca^{2+} exchange [37].

We found that, in the CT group, intermittent laser illumination of TMRE-loaded myocytes consistently resulted in mPTP opening, as indicated by the loss of TMRE fluorescence (Fig. 4a, c), which is well known to reflect collapse of the mitochondrial membrane potential [24–27]. This model is a widely used and reliable means of reproductively inducing a loss of mitochondrial membrane potential, which has been identified unequivocally as a reflection of the mPTP opening, and therefore simulates the events associated with reoxygenation-induced cell injury. In fact, mitochondrial Ca^{2+} overload and ROS generation are the major triggers of mPTP opening [14, 38]. Evidence suggests that IPT may mitigate mPTP opening, and that activation of the salvage kinase cascade and mitochondrial K_{ATP}/ROS/PKC pathway may be crucial for cardioprotection [8, 38]. Our observation that NS1619 and Dia each inhibited the ROS generation and accumulation of cytosolic Ca^{2+} (Fig. 4b, d) induced by intermittent laser illumination indicates that ROS generation and cytosolic Ca^{2+} overload can be mitigated by activation of mitoK_{Ca} and mitoK_{ATP} channels. In addition, NS1619 and Dia delayed the onset of cell shortening by the intermittent laser illumination, and thus delayed the cell death (Fig. 4e). We therefore suggest that activation of mitoK_{Ca} and mitoK_{ATP} channels, which mitigates ROS generation, cytosolic Ca^{2+} overload, and depolarization of mitochondrial membrane potential, is an important component of IPT- and drug-induced cardioprotection.

Although neither NS1619 nor Pax are mitoK_{Ca} channel specific, since large-conductance Ca^{2+}-activated K^+ channels are absent from the myocardial sarcolemmal membrane [15], it would be reasonable to assume that they affected the mitoK_{Ca} channel. However, we cannot completely exclude the possibility that the protection of NS1619 is due to mechanisms other than the activation of mitoK_{Ca} channels. Possible upstream mechanisms of mitoK_{Ca} channel activation remains to be elucidated. Recently, it has also been reported that NS1619 could inhibit the L-type Ca^{2+} channel, which may protect myocardium from hypoxia-reoxygenation injury by reducing Ca^{2+} influx [39].

In the present study, we found neither Pax nor 5-HD affect the protection of IPT when it is given at the onset of reoxygenation. Pax [16, 32] and 5-HD [7, 9] are often used in Langendorff perfusion studies investigating preconditioning signaling mechanisms. It has been reported that Pax does not act on mitoK_{ATP} channels [32], and that 5-HD alone does not alter hypoxia/reoxygenation-induced changes in mitochondrial membrane potential and mPTP opening [7]. Therefore, we can exclude the possibilities that Pax or 5-HD is potentially harmful to the myocardium.

In summary, this is the first study demonstrating that the IPT protects the heart through activation of not only the mitoK_{ATP} but also the mitoK_{Ca} channels. At the onset of reoxygenation, activation of these channels can inhibit the opening of mPTP caused by ROS production and cytosolic Ca^{2+} overload, thereby preventing hypoxia-reoxygenation injury.

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