Original Research

Pharmacological activation of mitochondrial BK_{Ca} channels protects isolated cardiomyocytes against simulated reperfusion-induced injury

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Abstract

The aim of this study was to find out whether opening of mitochondrial large-conductance Ca^{2+}-activated potassium channels (BK_{Ca}) protects cardiomyocytes against injury caused by simulated ischemia and reperfusion. This study also aimed to determine whether the protective mechanism involves signaling by reactive oxygen species (ROS) and phosphatidylinositol-3-kinase (PI3K). We used isolated ventricular myocytes, which are believed to contain no functional BK_{Ca} channels in the sarcolemma. Cells were isolated from the left ventricles of adult male Wistar rats and subjected to 25-min metabolic inhibition with NaCN and 2-deoxyglucose followed by 30-min re-energization. NS11021 (0.1 μmol/L), a novel BK_{Ca} channel opener, or hydrogen peroxide (2 μmol/L) added at re-energization, increased cell survival (the number of rod-shaped cells) and markedly reduced the release of lactate dehydrogenase (LDH). These cytoprotective effects of NS11021 were completely abolished by paxilline, a BK_{Ca} inhibitor, or tempol, an antioxidant, but not by wortmannin, an inhibitor of PI3K. NS11021 slightly but significantly increased the fluorescence signal in 2′7′-dichlorodihydrofluorescein diacetate (DCF-DA)-loaded myocytes, indicating an increased ROS formation. The NS11021-induced ROS formation was abolished by paxilline or tempol. NS13558 (0.1 μmol/L), an inactive structural analogue of NS11021, affected neither cell survival/LDH release nor DCF-DA fluorescence. These results suggest that pharmacological activation of mitochondrial BK_{Ca} channels effectively protects isolated cardiomyocytes against injury associated with simulated reperfusion. The mechanism for this form of protection requires ROS signaling, but not the activation of the PI3K pathway.

Keywords: potassium channels, cardiomyocytes, mitochondria, ischemia/reperfusion, cytoprotection, reactive oxygen species

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Introduction

There is a general consensus that mitochondria are key players in various forms of both innate and acquired myocardial tolerance to injury caused by acute ischemia/reperfusion (I/R) insult. Among mitochondrial components involved in cardioprotection, ATP-sensitive potassium (K_{ATP}) channels of the inner mitochondrial membrane are considered to play an important role.[1] Besides K_{ATP}, mitochondria contain large-conductance Ca^{2+}-activated potassium (BK_{Ca}) channels, which can contribute to myocardial salvage against I/R injury. The BK_{Ca} channels are expressed on the surface membrane of many types of cells and contribute to a variety of physiological events. In 1999, they were first identified in mitochondria of glioma cells.[2] Three years later, subunits of the BK_{Ca} channel were found in mitochondria of cardiac myocytes, and the pharmacological activation of the channel was shown to induce cardioprotection.[3] Interestingly, neither the functional BK_{Ca} channel nor its constituents have been detected on the sarcolemma of ventricular myocytes.[4]

In comparison with mitochondrial K_{ATP} channels, the role of BK_{Ca} channels in cardiac protection received much less attention. Nevertheless, several studies using different experimental models indicated that mitochondrial BK_{Ca} channels are involved in both classic ischemic preconditioning[5] and pharmacological preconditioning induced, for example, by adenosine A_1 receptor stimulation[6] or by volatile anesthetics such as desflurane,[4] as well as in protection conferred by sildenafil,[7] adrenomedullin,[8] 17β-estradiol[9] or κ-opioid receptor stimulation.[10] We have shown recently that these channels play a role also in the long-lasting

cardioprotection induced by adaptation to chronic hypoxia.[11] In addition, administration of a BK_{Ca} opener at the onset of reperfusion results in myocardial postconditioning,[12] and the activation of BK_{Ca} channels is involved in postconditioning mediated by adenosine A_1 receptor stimulation.[13] The protective effect of BK_{Ca} opening has been attributed to increased matrix K^+ uptake, increased mitochondrial volume, improved respiratory control,[14] inhibition of mitochondrial Ca^{2+} overload,[7,15] and prevention of permeability transition pore opening,[16] but the detailed mechanisms are not fully understood.

Conclusions about the role of mitochondrial BK_{Ca} channels in cardioprotection are mostly based on pharmacological evidence. However, these results should be interpreted with caution as none of the agents available appears to be strictly specific to BK_{Ca} channels.[17,18] In particular, the conventional opener NS1619 exhibits some BK_{Ca}-independent effects at higher concentrations, such as the inhibition of L-type Ca^{2+} channels,[19] which may contribute to protection. Moreover, this agent has been shown to promote potassium influx into the matrix not solely through BK_{Ca} channels, but also through non-specific ion transport mechanism.[20] In search for better pharmacological tools, the novel compound NS11021 was introduced recently as structurally different, more potent and more specific BK_{Ca} opener when compared with NS1619.[21] This agent was shown to reduce infarct size and improve postischemic recovery of contractile function in isolated rat hearts subjected to I/R.[12]

In this study, we examined the concentration-dependent effect of NS11021 on the resistance of isolated rat ventricular myocytes against injury caused by simulated I/R. The main goal of our experiments was to find out whether the opening of BK_{Ca} channels only at 'reperfusion' will also result in cytoprotection and whether the protective mechanism downstream of BK_{Ca} opening involves reactive oxygen species (ROS) and phosphatidylinositol-3-kinase (PI3K) pathway. In addition, NS13558, the close structural analogue of NS11021, was used as a biologically inactive control agent, which does not open BK_{Ca} channels.[22] In order to target mitochondrial BK_{Ca} channels and avoid potential influence of channels localized in plasma membrane of other types of cardiac cells, we used freshly isolated rat ventricular myocytes, which likely do not contain BK_{Ca} channels in their sarcolemma.

Materials and methods

Materials

NS11021 and NS13558 were a generous gift from NeuroSearch A/S (Ballerup, Denmark). Collagenase was obtained from Yakult (Tokyo, Japan). Lactate dehydrogenase (LDH) Liqui-UV kit was from Stanbio (Boerne, TX, USA), 2′7′-dichlorodihydrofluorescein diacetate (DCF-DA) was from AATBioquest (Sunnyvale, CA, USA), and SYTOX green from Invitrogen (Eugena, OR, USA). Paxilline, wortmannin, tempol, and all remaining reagents and chemicals were purchased from Sigma (Hamburg, Germany).

Animals

Adult male Wistar rats (250–300 g body weight) were used. The animals were housed in a controlled environment (23°C; 12:12-h light-dark cycle; light from 5:00) with free access to water and standard chow diet. The study was conducted in accordance with the procedures established by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). Experimental protocol was approved by the Animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic (Permit number: 140/2011).

Isolation of cardiomyocytes

Isolation was performed as described previously.[11] Rats were heparinized and killed by cervical dislocation. The heart was quickly excised and perfused via the aorta with Tyrode solution (10 mL/min) for five minutes followed by perfusion with nominally Ca^{2+}-free Tyrode for eight minutes. Tissue digestion was initiated by adding 14,000 U collagenase and 7 mg proteinase type XIV into 30 mL of Ca^{2+}-free Tyrode containing 50 mg bovine serum albumin. After 9–12 min, the collagenase–protease cocktail was washed out by 10-min perfusion with Ca^{2+}-free Tyrode. The free wall of the left ventricle was dispersed mechanically, myocytes solutions were adjusted to a standard cell density and transferred to culture medium (50% Dulbecco's Modified Eagle's Medium and 50% Nutrient Mixture F12HAM, containing 0.2% bovine serum albumin, 100 U/mL penicillin, and 100 μg/mL streptomycin). Cells were kept in a CO_2 incubator (95% air, 5% CO_2, 28 °C) for 1-h stabilization period.

Assessment of cell viability with SYTOX green

Myocytes were subjected to NS11021 or NS13558 at concentrations ranging from 0.01 to 3 μmol/L for up to 20 h to test these agents for potential toxicity. Control cells were treated with 0.1% dimethyl sulfoxide (DMSO) as a vehicle. Cell viability was assessed with SYTOX green as a probe using a Synergy HT Mutlidetection Microplate Reader (Biotek Instruments, Winooski, VT, USA) at an excitation wavelength of 485 nm and emission wavelength of 528 nm. The fluorescence signal of SYTOX green is proportional to the number of dead cells.[23] At the end of the experiments, all cells were lysed with 1% triton X100 to get the signal for 100% dead cells. From this number, the signal observed before lysis was subtracted to obtain the percentage of surviving cells for each group. Survival of the treated cells was calculated by dividing the number of surviving myocytes in the treated group by the number of surviving myocytes in the DMSO group and expressed as a percentage.

Simulated ischemia/reperfusion

Myocytes were subjected to metabolic inhibition (MI) and re-energization (MI/R) to simulate I/R insult as described recently.[11] Cells from each heart were divided into the required number of treatment groups (see below) and cells

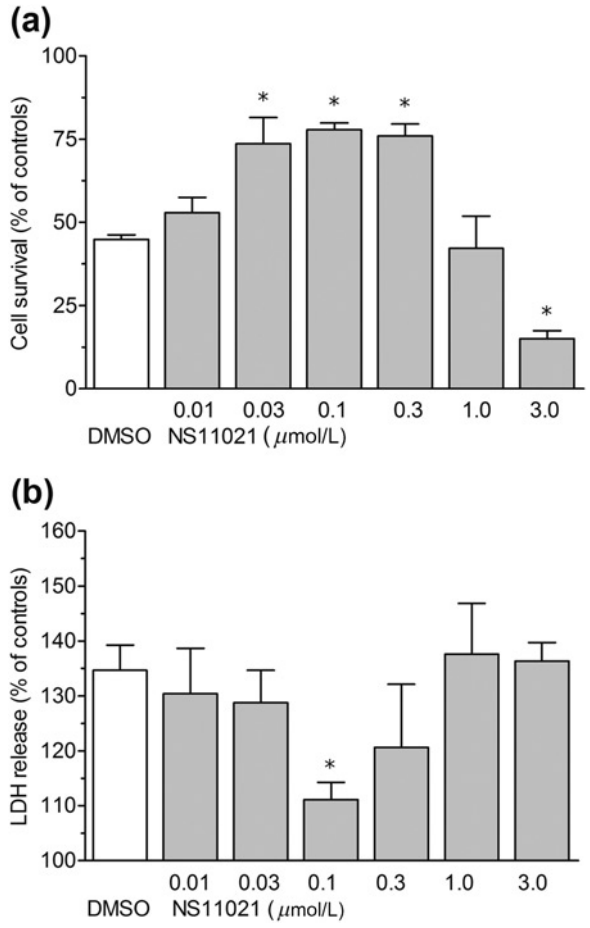

Figure 1 Cytoprotective effects of NS11021 against simulated ischemia/reperfusion injury. NS11021 (0.01–3 μmol/L) was added to isolated ventricular myocytes 25 min before metabolic inhibition. Cell survival (a) and total lactate dehydrogenase (LDH) release (b) during metabolic inhibition and re-energization are expressed as a percentage of control values. Significance was tested using Bonferroni *post hoc* test after analysis of variance. Values are mean $\pm$ SEM from six to eight hearts in each group; $^{*}P < 0.05$ versus dimethyl sulfoxide (DMSO)-treated group

from each treatment group were split into two parts of equal volumes. Control cells were incubated in normal Krebs solution and not exposed to MI/R. Experimental cells were subjected to 25 min of MI followed by 30 min of re-energization. MI was induced by exposing cells to the modified Krebs solution containing 1.5 mmol/L NaCN and 20 mmol/L 2-deoxyglucose instead of glucose. The re-energization was achieved by removing the metabolic inhibitors and replacing the MI solution with the normal cell culture medium (the same medium was applied to control cells).

Seven series of experiments were performed with the following cell treatment groups:

Series I: NS11021 (0.01– 3 μmol/L);
Series II: NS11021 (0.1 μmol/L), NS13558 (0.1 μmol/L), paxilline (0.1 μmol/L) + NS11021 (0.1 μmol/L), or paxilline (0.1 μmol/L);
Series III: NS11021 (3 μmol/L), paxilline (0.1 μmol/L) + NS11021 (3 μmol/L), or paxilline (1 μmol/L) + NS11021 (3 μmol/L);
Series IV: NS11021 (0.1 μmol/L), NS13558 (0.1 μmol/L), paxilline (0.1 μmol/L) + NS11021 (0.1 μmol/L), or paxilline (0.1 μmol/L);
Series V: NS11021 (0.1 μmol/L), tempol (100 μmol/L) + NS11021 (0.1 μmol/L), or tempol (100 μmol/L);
Series VI: NS11021 (0.1 μmol/L), wortmannin (0.1 μmol/L) + NS11021 (0.1 μmol/L), wortmannin (0.5 μmol/L) + NS11021 (0.1 μmol/L), wortmannin (0.1 μmol/L), or wortmannin (0.5 μmol/L);
Series VII: hydrogen peroxide (0.5–100 μmol/L), or paxilline (0.1 μmol/L) + hydrogen peroxide (2.0 μmol/L).

In experimental series I–III, the treatments with NS11021 or NS13558 started 25 min before MI and continued till the end of re-energization; paxilline was applied 25 min before adding NS11021. In experimental series IV–VI, NS11021 or NS13558 were added only at the end of MI to target BK_{Ca} channels at re-energization. Paxilline, tempol or wortmannin were applied five minutes before the end of MI and all agents were present throughout the re-enerization period. In experimental series VII, hydrogen peroxide was added at the end of MI and the treatment continued till the end of re-energization. DMSO (0.1%) served as a vehicle in all series of experiments.

Cell viability and LDH release were evaluated at the beginning of the experiments (after stabilization), after MI (LDH release only) and after re-energization. The number of viable and dead (stained) myocytes was determined by Trypan blue exclusion.[24] Typically, 50–100 myocytes were counted in duplicates from six to eight independent experiments. Viability after MI/R was expressed as a percentage of rod-shaped cells (the cell length-to-width ratio $> 3{:}1$) that survived the MI/R insult, normalized to the appropriate control group not exposed to MI/R. Supplementary Figures S1a–c (see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1177/1535370212474596/-/DC1), respectively, shows example images of myocytes after the isolation, after the control experiment and after MI/R. LDH was determined spectrophotometrically[25] using the LDH Liqui-UV kit. LDH released during MI and during re-energization, reflecting the rate of cell death, was expressed as a percentage of the appropriate control value. Data on myocyte survival (rod-shaped cells) and LDH release do not necessarily correlate due to different proportion of still viable (Trypan blue-negative) non-rod-shaped cells, which do not contribute to LDH release (data not shown).

Measurement of ROS formation

Myocytes in the culture medium (5 mL) were loaded with the ROS-sensitive dye 2′7′-dichlorodihydrofluorescein diacetate (DCF-DA; 20 μmol/L) for one hour at 28°C. Control cells were incubated with cell culture medium only. After loading, myocytes were washed twice with Tyrode and 100 μL of the cell suspension was placed into each well of a 96-well plate containing 100 μL of the agents to be tested. The DCF-DA fluorescence was measured at 485/528 nm at selected time intervals and background fluorescence was deducted for each agent used. First, the concentration dependence of the

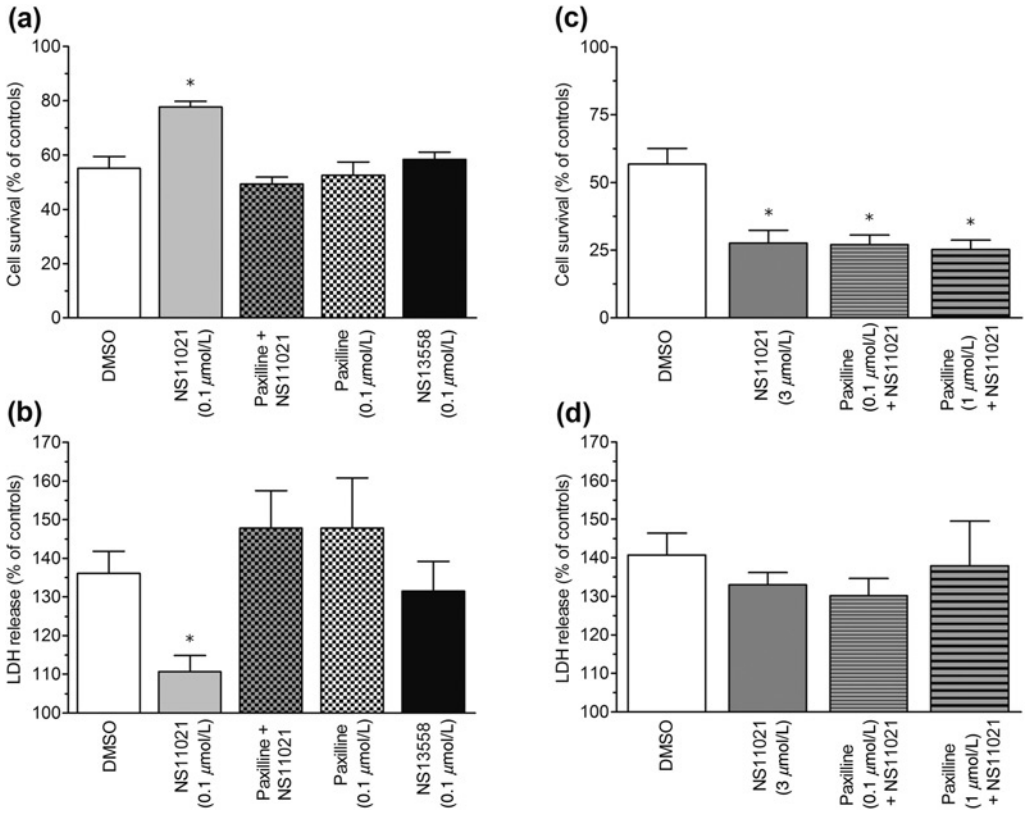

Figure 2 Role of BK_{Ca} channels in the cytoprotective effect of NS11021 against simulated ischemia/reperfusion injury. (a,b) NS11021 (0.1 μmol/L), NS11021 (0.1 μmol/L) + paxilline (0.1 μmol/L), paxilline alone, NS13558 (0.1 μmol/L), or (c,d) NS11021 (3 μmol/L) and NS11021 (3 μmol/L) + paxilline (0.1 μmol/L or 1 μmol/L) were added to isolated ventricular myocytes 25 min before metabolic inhibition. Cell survival (a,c) and total lactate dehydrogenase (LDH) release (b,d) during metabolic inhibition and re-energization are expressed as a percentage of control values. Significance was tested using Bonferroni *post hoc* test after analysis of variance. Values are mean ± SEM from six hearts in each group; $^*P < 0.05$ versus dimethyl sulfoxide (DMSO)-treated group

effects of NS11021 (0.1–3 μmol/L) and hydrogen peroxide (0.5–100 μmol/L) was determined. Then the following treatment groups were examined: NS11021 (0.1 μmol/L), paxilline (0.1 μmol/L) + NS11021 (0.1 μmol/L), paxilline (0.1 μmol/L), tempol (100 μmol/L) + NS11021 (0.1 μmol/L), tempol (100 μmol/L), NS13558 (0.1 μmol/L), or DMSO (0.1%).

Statistical analysis

Data are expressed as mean ± SEM from the indicated number of experiments. One-way analysis of variance with Bonferroni *post hoc* means comparison was used. Differences were considered statistically significant when $P < 0.05$.

Results

Cell viability after 20-h exposure to NS11021 or NS13558

Viability of myocytes assessed by SYTOX green ranged between 86% and 91% after 1-h stabilization. Culturing the cells for 20 h in the presence of 0.1% DMSO decreased the number of viable rod-shaped cells to 60 ± 5% of that counted after stabilization; the effect of DMSO was not significant compared with the untreated group. NS11021 did not significantly influence cell viability at any concentration ranging from 0.01 to 3 μmol/L, while NS13558 at the highest concentration of 3 μmol/L reduced significantly the number of surviving cells to 39 ± 1%.

Concentration-dependent effect of NS11021 on myocytes subjected to MI/R

Exposure of cells to MI/R decreased their survival; when expressed as a percentage of control group (not subjected to MI/R), only about 45% of rod-shaped myocytes survived this insult in the vehicle-treated group. The response of cell viability to increasing concentrations of NS11021 applied before MI/R exhibited a bell-shaped pattern (Figure 1a). While concentrations of 0.03, 0.1 and 0.3 μmol/L significantly improved survival to more than 70%, 1 μmol/L had no effect and 3 μmol/L even significantly promoted cell injury. Figure 1b shows effects of NS11021 on total LDH release from myocytes during MI/R, expressed as percentage of the appropriate control values. While only the concentration of 0.1 μmol/L reduced total LDH release and LDH release during MI, both 0.1 and 0.3 μmol/L significantly protected the cells during the re-energization phase (Supplementary Figures S2a and b; see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1177/1535370212474596/-/DC1). Based on these results, the concentration of 0.1 μmol/L NS11021 was chosen as the most effective one for further experiments focused on cell protection.

Paxilline blunts the protective effects of NS11021

Figures 2a and b, respectively, illustrates effects of 0.1 μmol/L NS11021, paxilline and NS13558 applied before MI/R

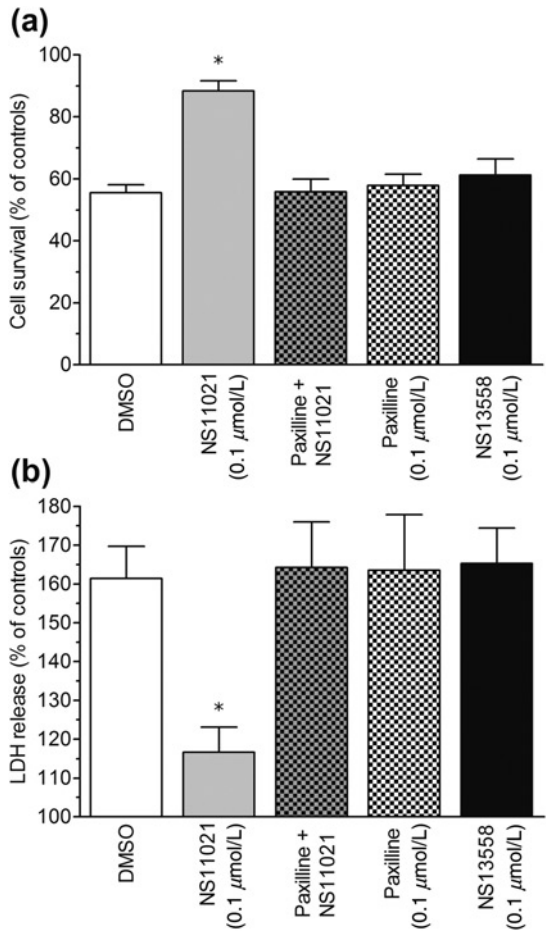

Figure 3 Role of BK_{Ca} channels in the cytoprotective effect of NS11021 against simulated reperfusion injury. NS11021 (0.1 μmol/L), NS11021 (0.1 μmol/L) + paxilline (0.1 μmol/L), paxilline alone, or NS13558 (0.1 μmol/L) were added to isolated ventricular myocytes at the end of metabolic inhibition. Cell survival (a) and lactate dehydrogenase (LDH) release (b) during re-energization are expressed as a percentage of control values. Significance was tested using Bonferroni *post hoc* test after analysis of variance. Values are mean ± SEM from six hearts in each group; $^{*}P < 0.05$ versus dimethyl sulfoxide (DMSO)-treated group

on myocyte viability and total LDH release during MI/R. The protective effects of NS11021 were completely blocked by the BK_{Ca} inhibitor paxilline (0.1 μmol/L). Supplementary Figures S3a and b (see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1177/1535370212474596/-/DC1), respectively, show that the blocking effect of paxilline on the NS11021-induced drop of LDH release occurred both during MI and during re-energization. Neither paxilline alone nor the inactive analogue of the BKc_a opener, NS13558 (0.1 μmol/L), had any influence on cell survival and LDH release (Figures 2a and b and Supplementary Figure S3). These results suggest that protection triggered by nanomolar concentrations of NS11021 can be attributed to BK_{Ca} channel opening.

In contrast, Figure 2c shows that the detrimental effect of 3 μmol/L NS11021 on rod-shaped cell survival following MI/R was not abolished by paxilline, suggesting a mechanism of action independent of BK_{Ca} channels at higher concentrations. LDH release during MI/R was not significantly affected by 3 μmol/L NS11021 (Figures 1b and 2d).

NS11021 protects myocytes when applied at re-energization

Figures 3a and b, respectively, show effects of 0.1 μmol/L NS11021 applied only at the end of MI on myocyte viability and LDH release during re-energization. NS11021 significantly improved cell survival and reduced LDH release, and paxilline again completely abolished these effects. Neither paxilline alone nor NS13558 affected the extent of injury (Figure 3). These results show that pharmacological opening of BK_{Ca} channels can effectively protect myocytes against injury associated with re-energization after MI.

Protective mechanism of NS11021 requires ROS signaling but not activation of PI3K pathway

In order to find out whether the cytoprotective effects of NS11021 at re-energization depend on ROS formation, the antioxidant tempol (100 μmol/L) was applied to myocytes before adding NS11021. Figures 4a and b, respectively, show that tempol abolished both the improved cell viability and the reduced LDH release in the NS11021-treated group. Moreover, the application of hydrogen peroxide at re-energization mimicked the protective effects of NS11021 on cell survival (2 μmol/L) and LDH release (2 and 10 μmol/L) as shown in Figures 4c and d, respectively. These effects were not inhibited by paxilline (Figures 4e and f). Higher concentrations of hydrogen peroxide had no influence (50 μmol/L) or tended to promote cell injury (100 μmol/L).

Unlike tempol, the specific inhibitor of PI3K, wortmannin, applied at concentrations of 0.1 or 0.5 μmol/L before adding NS11021 did not affected the NS11021-induced protection against cell death and LDH release during re-energization, as shown in Figures 5a and b, respectively. These results suggest that ROS formation, but not PI3K activity, is involved in the protective mechanism triggered by BK_{Ca} channel opening after MI.

NS11021 but not NS13558 stimulates ROS formation by myocytes

Figure 6 shows quantified DCF-DA fluorescence signals obtained in cell suspensions after 5-min treatments with the respective agents, expressed as a percentage of DMSO-treated control cells. Whereas 0.1 μmol/L NS11021 increased fluorescence reflecting ROS formation, the effect was lost at higher concentrations (1 and 3 μmol/L). Although the increase of fluorescence by 0.1 μmol/L NS11021 was very slight, it occurred in all independent experiments and reached statistical significance in both experimental series (Figures 6a and b). Both paxilline and tempol completely abolished the effect of NS11021. Neither paxilline alone, tempol alone, nor NS13558 exhibited any effect on DCF-DA fluorescence (Figure 6b).

The addition of hydrogen peroxide to the cells resulted in a concentration-dependent increase of DCF-DA fluorescence. Quantitatively, signals produced by 2 μmol/L hydrogen peroxide and 0.1 μmol/L NS11021 were comparable (Figure 6a).

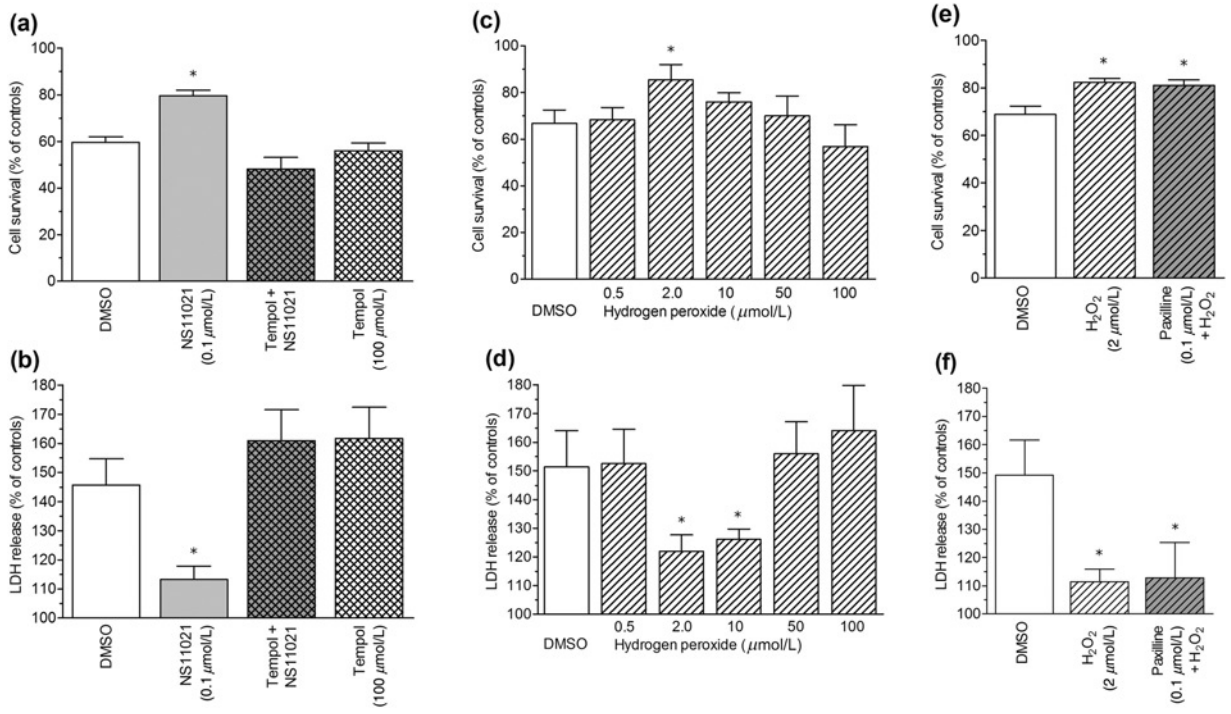

Figure 4 Role of reactive oxygen species in the cytoprotective effect of NS11021 against simulated reperfusion injury. (a,b) NS11021 (0.1 μmol/L), NS11021 (0.1 μmol/L) + tempol (100 μmol/L), tempol alone, or (c,d) hydrogen peroxide (0.5–100 μmol/L), or (e,f) hydrogen peroxide (2.0 μmol/L) + paxilline (0.1 μmol/L) were added to isolated ventricular myocytes at the end of metabolic inhibition. Cell survival (a,c,e) and lactate dehydrogenase (LDH) release (b,d,f) during re-energization are expressed as a percentage of control values. Significance was tested using Bonferroni *post hoc* test after analysis of variance. Values are mean ± SEM from six hearts in each group; $^{*}P < 0.05$ versus dimethyl sulfoxide (DMSO)-treated group

Discussion

This study resulted in four major findings. First, the novel BK_{Ca} opener NS11021 administered prior to MI protected isolated rat left ventricular myocytes against injury only in a rather narrow nanomolar concentration range, and its salutary effects were abolished by inhibition of BK_{Ca} channels with paxilline. Second, similar protection was conferred by NS11021 and low micromolar concentration of hydrogen peroxide administered at the end of MI. This postconditioning-like effect of NS11021 was abolished by paxilline or by the antioxidant tempol, but not by PI3K inhibitor wortmannin. Third, NS11021 slightly, but significantly increased fluorescence of DCF-DA-loaded myocytes reflecting ROS formation, which was eliminated by paxilline or tempol. Finally, neither myocyte tolerance to MI/R injury, nor DCF-DA fluorescent signal was affected by NS13558, the inactive close structural analogue of NS11021, which does not open BK_{Ca} channels.

Our initial experiments with SYTOX green showed that NS11021 in the concentration range up to 3 μmol/L did not affect cell viability during 20-h incubation. However, its effect on myocyte tolerance to injury caused by MI/R exhibited strong concentration dependence. While the concentration of 0.1 μmol/L was most effective in terms of improved cell survival and reduced LDH release, the highest concentration of 3 μmol/L increased cell injury. This is in contradiction with a recent report, which demonstrated protective effects of 3 μmol/L NS11021 on infarct size and recovery of contractility in isolated perfused rat hearts subjected to global I/R.[12,22] The explanation for the higher sensitivity of isolated myocytes to NS11021 compared with perfused hearts is not clear and may reflect a number of essential differences between these experimental models. However, our results are in full agreement with data reported by Aon *et al.*,[14] indicating that NS11021 in the nanomolar concentration range accelerated the rate of potassium uptake by isolated myocardial mitochondria and potentiated matrix swelling in the presence of permeable anions without affecting the inner membrane potential. They concluded that these effects are likely required to confer protection without compromising oxidative phosphorylation during recovery from metabolic stress. Moreover, when mitochondria were exposed to NS11021 at concentrations higher then 1 μmol/L, the membrane potential collapsed leading to energetic deterioration.[14] This effect, unlike those elicited by nanomolar NS11021, is likely to be non-specific as it was insensitive to BK_{Ca} inhibitors. Similarly, our observation that the impaired tolerance to MI/R injury in cells treated with 3 μmol/L NS11021 was not affected by paxilline implies the involvement of other mechanism independent of BK_{Ca} channels.

Based on the data summarized above, the concentration of 0.1 μmol/L NS11021 was used in subsequent experiments. This concentration resulted in similar and well-reproducible protective effects on cell survival and LDH release when administered either before MI or only during re-energization. We also showed that, unlike the harmful effect of 3 μmol/L NS11021, the protection triggered by 0.1 μmol/L NS11021 in both pre- and post-MI settings was fully abolished by BK_{Ca} inhibitor paxilline at 0.1 μmol/L, which is much below the concentration

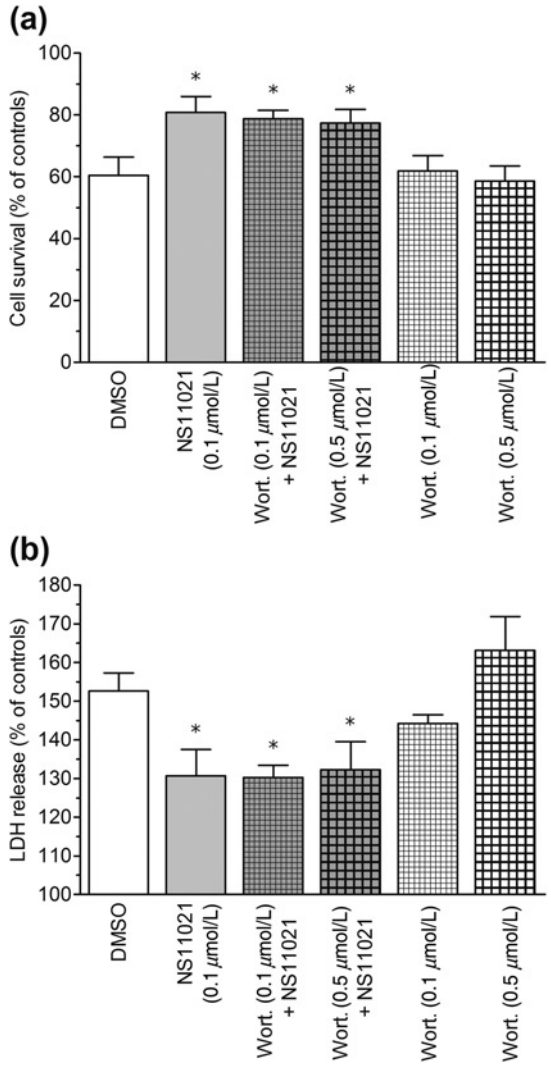

Figure 5 Role of phosphatidylinositol-3-kinase in the cytoprotective effect of NS11021 against simulated reperfusion injury. NS11021 (0.1 μmol/L), NS11021 (0.1 μmol/L) + wortmannin (0.1 μmol/L or 0.5 μmol/L), or wortmannin alone were added to isolated ventricular myocytes at the end of metabolic inhibition. Cell survival (a) and lactate dehydrogenase (LDH) release (b) during re-energization are expressed as a percentage of control values. Significance was tested using Bonferroni *post hoc* test after analysis of variance. Values are mean $\pm$ SEM from six hearts in each group; $^{*}P < 0.05$ versus dimethyl sulfoxide (DMSO)-treated group

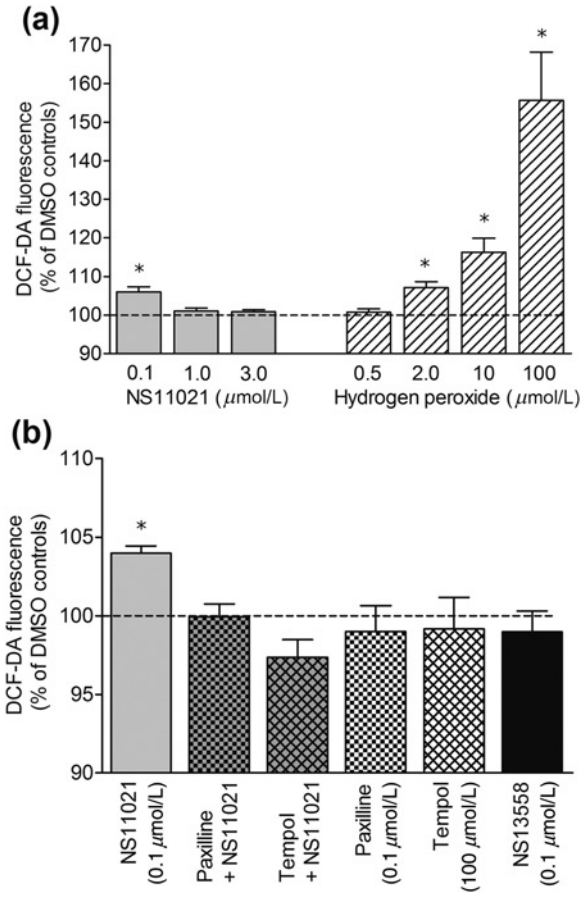

Figure 6 Effects of NS11021 and hydrogen peroxide on 2′7′-dichlorodihydrofluorescein diacetate (DCF-DA) fluorescence. (a) NS11021 (0.1–3 μmol/L) or hydrogen peroxide (0.5–100 μmol/L), or (b) NS11021 (0.1 μmol/L), NS11021 (0.1 μmol/L) + paxilline (0.1 μmol/L), paxilline alone, NS11021 (0.1 μmol/L) + tempol (100 μmol/L), tempol alone, or NS13558 (0.1 μmol/L) were added to isolated ventricular myocytes. DCF-DA fluorescence signal was obtained in cell suspensions after 5-min treatments and expressed as a percentage of the dimethyl sulfoxide (DMSO)-treated group. Significance was tested using Bonferroni post hoc test after analysis of variance. Values are mean $\pm$ SEM from six to nine independent experiments in each group; $^{*}P < 0.05$ versus dimethyl sulfoxide (DMSO)-treated group

causing potential side effects, such as the inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase[26] or inositol 1,4,5-trisphosphate receptors.[27] Moreover, NS13558 obtained by methylation of the terminal tetrazolic ring of NS11021 without affecting its overall structural conformation[22] did not improve myocyte resistance to MI/R injury in our study, consistent with the absence of stimulatory effect on mitochondrial potassium uptake and matrix volume.[14] This compound retains comparable biological activity toward other ion channels as NS11021 at higher concentrations, but it lacks BK_{Ca} activator properties and is not cardioprotective.[22] Taken together, these results suggest that the cytoprotective effects of NS11021 demonstrated in this study can be most likely ascribed to the opening of mitochondrial BK_{Ca} channels.

Our observation of salutary effects of NS11021 associated with re-energization is in line with recent findings that BK_{Ca} openers NS11021 and NS1619, albeit used at higher concentrations, triggered postconditioning-like effects when administered at reperfusion to globally ischemic rat hearts[12] and at reoxygenation of hypoxic rat embryonic cardiomyoblast-derived H9c2 cells,[13] respectively. However, the mechanism underlying this form of protection is unclear. Stowe *et al.*[28] showed that myocardial preconditioning by NS1619 depends on the formation of ROS, as the bracketing of the opener with a dismutator of superoxide antagonized its protective effect in guinea pig hearts subjected to global I/R. In our experiments, the antioxidant tempol also completely abolished the NS11021-induced cytoprotection, suggesting that a similar ROS-dependent mechanism may play a role in myocytes salvation conferred by BK_{Ca} opening at re-energization. This view is further supported by our observation that 2 μmol/L hydrogen peroxide, but not 50 or 100 μmol/L, provided paxilline-insensitive salutary

effects comparable to that of NS11021. It is apparently in contrast with the common image of ROS produced in large quantities by reperfused myocardium as an important component of post-ischemic damage. However, several recent reports provided convincing evidence that ROS-dependent signaling in the early reperfusion appears crucial to cardioprotection induced by both preconditioning and postconditioning.[29–31] Interestingly, ROS generated at reperfusion play a role also in the mechanism of long-lasting protection of chronically hypoxic hearts,[32] which involves mitochondrial BK_{Ca} channels.[11]

Studies on mitochondrial ROS production in response to BK_{Ca} openers provided rather controversial results.[33] A decreased rate of hydrogen peroxide was found in rat brain mitochondria exposed to NS1619 or CGS7184; these effects were sensitive to BK_{Ca} blockers iberiotoxin and charybdotoxin.[34] Either increased or decreased generation of superoxide was reported in heart mitochondria treated with NS1619 depending on substrate and energetic conditions.[35,36] It has been proposed that the openers may inhibit complex I-dependent ROS production in the matrix and stimulate complex III-dependent formation of superoxide, which is responsible for the cytoprotective signal transduction to the cytosol,[33] but more research is needed to resolve this issue. Nevertheless, our data show that 0.1 μmol/L NS11021 added to energized myocytes very slightly but significantly increased ROS formation detected by DCF-DA fluorescence. Interestingly, the quantitatively similar DCF-DA signal was recorded after the addition of 2 μmol/L hydrogen peroxide, which was also cytoprotective, whereas 50 or 100 μmol/L hydrogen peroxide resulted in much higher fluorescence and the loss of protection. This is in line with the assumption that only trace amounts of ROS are needed to activate protective pathways. The NS11021-induced ROS signal was fully blocked by paxilline or tempol in agreement with their blunting effects on cytoprotection. Moreover, both the increased DCF-DA fluorescence and cytoprotection were lost at micromolar concentrations of NS11021 and the inactive analogue NS13558 affected neither ROS formation nor cell survival. These results suggest that the cytoprotective ROS signal occurred downstream of BK_{Ca} opening and not as a consequence of any potential side effect of NS11021 unrelated to BK_{Ca} channels.

A variety of ROS-sensitive protein kinases can play a role in mediating the pro-survival signaling of postconditioning.[37] PI3K and its downstream target protein kinase B (Akt) are key components of the RISK (reperfusion injury signaling kinase) pathway, which alleviates myocardial injury associated with reperfusion. A number of studies showed that inhibitors of PI3K eliminated the phosphorylation of Akt and cardioprotective effects of postconditioning (e.g. refs.[38–40]). We used the specific inhibitor of PI3K wortmannin to find out whether the protective mechanism triggered by BK_{Ca} opener at re-energization involves PI3K/Akt pathway. These experiments failed to demonstrate any inhibition of NS11021-induced salutary effects by wortmannin. Consistent with our results, the infarct size-limiting effect of adrenomedulin, mediated by BK_{Ca} channels, was not blocked by PI3K inhibitor LY294002.[8] Thus, it seems that PI3K is not linked to mitochondrial BK_{Ca} channels and does not play a role in protective signal transduction downstream of BK_{Ca} opening.

In conclusion, we have shown that the activation of mitochondrial BK_{Ca} channels by NS11021 slightly increased ROS formation and effectively protected isolated ventricular myocytes against injury caused by MI/R, while NS13558 had no effect. Similar degree of protection, sensitive to paxilline, was achieved when the opener was administered before MI or only at re-energization. The salutary effects of NS11021 at re-energization were abolished by tempol but not by wortmannin, and the ROS signal was blocked by paxilline or tempol. These results suggest that specific activation of mitochondrial BK_{Ca} channels by NS11021 can be a promising approach in mitigating myocardial reperfusion injury. Further studies are needed to delineate the protective mechanism downstream of BK_{Ca} channels, which likely involves ROS signaling but not the activation of the PI3K/Akt pathway.

Author contributions: GHB and FK participated in the design, interpretation of the studies and writing of the manuscript; GHB and MH conducted the experiments and analyzed the data.

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