

ORIGINAL

Cardioprotective effects of hyperkalemia during simulated ischemia/reperfusion in neonatal rat cardiomyocytes -Preservation of Na⁺/K⁺-ATPase activity-

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Abstract : Background : Hyperkalemia has multimodal effects on myocardial protection during ischemia/reperfusion. The preservation of Na⁺/K⁺-ATPase activity induced by hyperkalemia may have critical impact on myocardial protection. **Methods :** To elucidate the roles of hyperkalemia (16 mM) and Na⁺/K⁺-ATPase inhibition (100 μM ouabain) in myocardial protection during simulated ischemia (5 mM NaCN and 5.5 mM 2-deoxyglucose)/reperfusion, we measured loss of membrane integrity and bleb formation using a vital dye calcein AM in cultured neonatal rat cardiomyocytes. The control perfusate was switched to treatment solution for 15 min, followed by reperfusion for 30 min. In a second set of experiments, myocardial excitability and diastolic intracellular calcium ion concentration ([Ca²⁺]_i) were measured during a 45-min treatment using a calcium-sensitive fluorescent dye fluo-4 AM. **Results :** Simulated ischemia/reperfusion under ouabain treatment induced loss of membrane integrity, which was suppressed by hyperkalemia. Simulated ischemia/reperfusion induced bleb formation, which was accelerated by ouabain. Hyperkalemia delayed and inhibited the increase in diastolic [Ca²⁺]_i induced by simulated ischemia. Furthermore, hyperkalemia almost completely inhibited the effects of ouabain on the diastolic [Ca²⁺]_i during ischemia. **Conclusions :** These results suggest that hyperkalemia during ischemia is cardioprotective against ischemia/reperfusion insults and that hyperkalemia inhibits the effects of ouabain during ischemia. *J. Med. Invest.* 60 : 66-76, February, 2013

Keywords : myocardial protection, hyperkalemia, ischemia/reperfusion, cardiotonic steroids

INTRODUCTION

Hyperkalemia can have multimodal effect on myocardial protection during ischemia/reperfusion. Extracellular myocardial K⁺ concentration ([K⁺]_o) in ischemic regions increase to 10-20 mM after 3-10

min of ischemia (1, 2). Hyperkalemia may preserve adenosine triphosphate (ATP) by reducing excitability of cardiac cells in energy-limited state. Hyperkalemic cardiac arrest is used as a protective intervention during cardiac surgery (3). In addition, Na⁺/K⁺-ATPase activation induced by hyperkalemia may exert a cardioprotective effect (4). However, membrane depolarization induced by hyperkalemia may cause detrimental effects in myocardial cells by inducing activation of a reversal mode of the Na⁺/Ca²⁺ exchanger and/or slow Ca²⁺ channels (5). Conversely, decreased myocardial Na⁺/K⁺-ATPase

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activity is associated with decreased post-ischemic recovery (6, 7). The Na⁺/K⁺-ATPase activity is inhibited by various factors including the therapeutic use of cardiotoxic steroids such as digoxin (8, 9), endogenous cardiotoxic steroids (10-12), hypothermia (4), and ischemia (13, 14). Cardiotoxic steroids may abolish the protective effects of hyperkalemia. In contrast, [K⁺]_o elevation decreases the affinity of Na⁺/K⁺-ATPase for cardiotoxic steroids (15-17). Hyperkalemia may counteract the inhibition of Na⁺/K⁺-ATPase induced by cardiotoxic steroids during ischemia. However, the effect has not yet been investigated in ischemia/reperfusion studies.

We hypothesized that hyperkalemia during ischemia has a cardioprotective effect during ischemia/reperfusion and that the effects are partly mediated by the preservation of Na⁺/K⁺-ATPase activity. To test this hypothesis, we investigated whether hyperkalemia and ouabain, a synthetic cardiotoxic steroid, modulate the loss of membrane integrity and bleb formation during simulated ischemia/reperfusion in cultured neonatal rat cardiomyocytes. The suggested results were confirmed in a second set of experiments measuring diastolic [Ca²⁺]_i and myocardial excitability during ischemia

MATERIALS AND METHODS

All experimental procedures and protocols used in this study were reviewed and approved by the Animal Investigation Committee of Tokushima University and followed the guidelines of the American Physiological Society (Bethesda, Maryland) for the use of animals in research.

Cell culture

Primary cultures of neonatal rats were prepared from the hearts of 2-day-old Sprague-Dawley rats with a Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp., Lakewood, NJ). This system, which utilizes purified enzyme preparations, provides a reliable and consistent cell isolation method (18). Neonatal rats were anesthetized with sevoflurane and their hearts were quickly removed. Isolation was performed according to the manufacturer's recommendations (18). The cells were maintained in M199 culture medium supplemented with 10% fetal bovine serum, gentamycin (50 µg·ml⁻¹), and amphotericin B (2.5 µg·ml⁻¹) in 35-mm glass-bottomed culture dishes (MacTek Corp, Gates Mills, OH). Experiments were initiated

after 4 days of incubation for calcein studies and 3 days of incubation for fluo-4 studies at 37°C in a 5% CO₂ environment using monolayers of spontaneously beating myocytes.

Measurement of loss of membrane integrity and bleb formation during simulated ischemia/reperfusion

Simulated ischemia using NaCN, an inhibitor of mitochondrial cytochrome oxidase, and 2-deoxyglucose, an inhibitor of glycolysis, has been used in ischemia/reperfusion or ischemia studies (5, 19). The vital dye calcein-AM was used to assess membrane integrity (20-22). Necrotic blebs form in cells exposed to intense noxious stimuli such as hypoxia/reoxygenation and appear prior to cell permeabilization (20, 23). We selected an ouabain concentration (100 µM) that resulted in 50% inhibition of Na⁺/K⁺-ATPase but produces no loss of viability in neonatal rat cardiomyocytes (24).

Loading with the vital dye calcein AM (Molecular Probes, Eugene, OR) was accomplished by incubation with calcein AM (200 nM in phosphate-buffered saline) for 20 min. The cells were maintained at 37°C for another 20 min to ensure complete hydrolysis after washing with phosphate-buffered saline.

A cell perfusion system was designed to measure cell fluorescence in response to rapid solution changes. The system used an inverted epifluorescence microscope (Eclipse TS100, Nikon, Tokyo, Japan). A culture dish incubation system (Harvard Apparatus, DH-35i, Holliston, MA) was placed on the microscope stage. In addition, 35-mm glass-bottomed culture dishes and a chamber insert closed for 35-mm dishes (Harvard Apparatus, RC-37FC) were mounted on the incubation system. Cell perfusion solutions were infused using an infusion pump at a rate of 2 ml·min⁻¹. Solutions were maintained at a constant temperature by a 35-mm tissue culture dish heater (Harvard Apparatus, DH-35), solution in-line heater (Harvard Apparatus, SH-27B), and chamber system heater controller (Harvard Apparatus, TC-344B).

Excitation of calcein was obtained using a Xenon lamp (50 W ; Nikon) filtered at 450-490 nm and reflected to the microscope objective (×20, CFI Plan Fluor ELWD 20×C ; Nikon, Tokyo, Japan) by a dichroic mirror centered at 505 nm. Cell fluorescence was collected by the objective, passed through a 520-nm-long path filter, and directed to a cooled digital B/W CCD camera (ORCA, Hamamatsu Photonics, Hamamatsu, Japan).

Relative changes in the calcein fluorescence intensity in each region of interest (ROI) relative to the baseline (%) were used as an index of membrane integrity. The times until bleb appearance in the ROIs were also recorded. The ROI included almost all cells in a single field of view of the microscope, which included more than fifty cells. Fluorescence images with an exposure time of 555 msec were taken every 30 seconds using a computer-controlled shutter. The cells were initially perfused with “normal” perfusate i.e., Earle’s balanced salt solution (EBSS), containing (in mM) 116.4 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgSO₄, 26.2 NaHCO₃, 1 NaH₂PO₄, and 5.6 D-glucose, which was titrated to pH 7.40. After equilibration (approximately 10 min), the perfusate was randomly switched to one of treatment solutions: EBSS (CON; n=6), EBSS containing 100 μM ouabain (OUA; n=5), modified EBSS containing NaCN and 2-deoxyglucose (ISC; n=6), ISC+HIK (n=6), ISC+OUA (n=6) or ISC+HIK+OUA (n=6), and measurements were performed for 15 min. The perfusate was then switched back to normal perfusate to measure reperfusion injury for an additional 30 min at 37°C.

Measurement of myocardial excitability and diastolic [Ca²⁺]_i during ischemia

Cultured neonatal rat cardiomyocytes have been used to study spontaneous beating (18, 25). A phasic rise and fall in fluorescence is well-established as representing the systolic rise and diastolic fall of [Ca²⁺]_i upon which contraction and relaxation depend (25, 26). Therefore, we used the rhythmical

fluorescence changes as an index of myocardial excitability in the fluorescence images. Loading with the Ca²⁺ indicator, fluo-4 AM (Molecular Probes, Eugene, OR), was accomplished by incubation with fluo-4 AM (5 μM in phosphate-buffered saline) for 20 min at room temperature; the cells were maintained at 37°C for another 20 min to ensure complete hydrolysis after washing with phosphate-buffered saline. We used the same cell perfusion and epifluorescence system as the calcein study. Fluorescence images with an exposure time of 334 msec were taken for 15 sec at 2 frames·s⁻¹ at 5 minute intervals using a computer-controlled shutter and an image processing system.

The cells were initially superfused with control perfusate. After equilibration (approximately 10 min), the perfusate was randomly switched to one of treatment solutions: CON (n=5), HIK (n=5), OUA (n=5), ISC (n=5), ISC+HIK (n=5), ISC+OUA (n=5) or ISC+HIK+OUA (n=5). Measurements were taken for 45 min at 37°C.

Data analysis was performed as follows. We chose 3-7 ROIs, which contained a cell or small clusters of cells and met the criteria for selection, at random from a single field of view of the microscope. The criteria for selection were that the fluorescences of ROI changed rhythmically and that the diastolic fluorescence did not increase until the intervention. Representative traces of fluo-4 fluorescence value are presented in Figure 1. Background values (minimum value in the windows of the ROIs that included the area outside of the cells) were always subtracted. Diastolic fluo-4 fluorescence values in

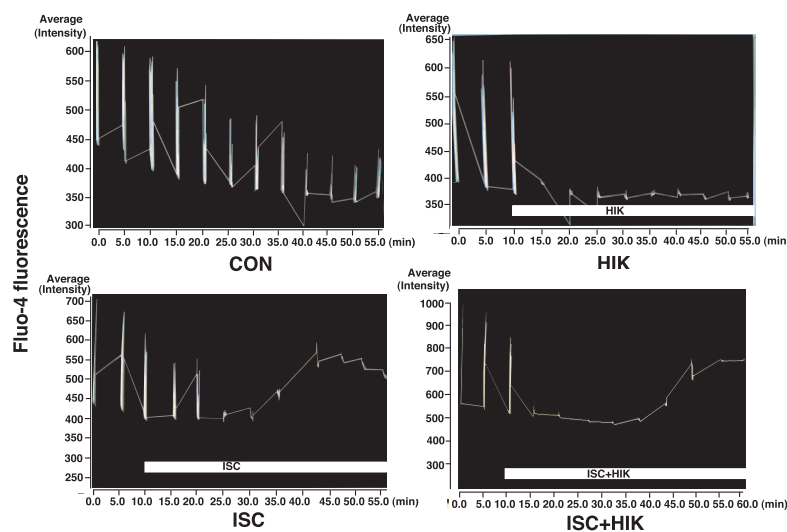


Figure 1. Graph showing representative traces of fluo-4 fluorescence in the CON, HIK, ISC, and ISC+HIK groups. After equilibration with “normal” perfusate (Earle’s balanced salt solution EBSS) for approximately 10 min, the perfusate was switched to either EBSS (CON), modified EBSS containing 16 mM KCl (HIK), modified EBSS containing 5 mM NaCN and 5.5 mM 2-deoxyglucose (ISC), or modified EBSS containing 5 mM NaCN, 5.5 mM 2-deoxyglucose, and 16 mM KCl (ISC+HIK).

the ROIs were evaluated by calculating the mean value of the bottom five average pixel intensities in the 30 fluorescence images. Fluo-4 fluorescence was expressed relative to the baseline value (%). If at least one rhythmical increase in fluorescence was observed over 15 seconds, we considered the cell or cell mass to have retained excitability. Changes in excitability of cardiomyocytes were expressed as percentages of excitable myocardial cells (Figure 2).

Statistical analysis

Data are expressed as the mean ± SD. Comparison of several means of diastolic intracellular [Ca²⁺]_i, calcein fluorescence intensities, and the times until bleb appearance were performed using a two-way analysis of variance followed by a Student-Newman-Keuls post-hoc test for multiple comparisons. All P-values are two-tailed. Comparisons of myocardial excitability were performed using Fisher's exact test. A P-value of less than 0.05 was considered significant. Statistical analysis of the data was performed using the commercially available software program StatView 5.0 (SAS Institute Inc., Cary, NC).

RESULTS

Effects of hyperkalemia in the presence or absence of ouabain on membrane integrity and bleb formation during simulated ischemia/reperfusion

Three patterns were observed in the loss of calcein fluorescence : gradual loss, abrupt loss with bleb formation, and abrupt loss without bleb formation (Figure 3). The latter two patterns were observed to have similar timings in this study to those reported in another study (22). The times until bleb appearance in the CON, OUA ISC, ISC+HIK, ISC+OUA ISC+OUA+HIK group were 35 ± 12 min, 29 ± 14 min, 20 ± 13 min, 27 ± 11 min, 13 ± 4 min*, and 24 ± 12 min, respectively (*P<0.05 vs. CON). During control perfusion at 37°C, gradual decreases in calcein fluorescence were observed (Figure 4, 5). However, appearance of bleb delayed until 35 min. Thus, although some cells in the CON group suffered a loss of membrane integrity, the majority of the decrease in calcein fluorescence was likely induced by dye leakage or photobleaching. Ouabain itself did not produce a significant difference from control. Abrupt losses of calcein fluorescence were

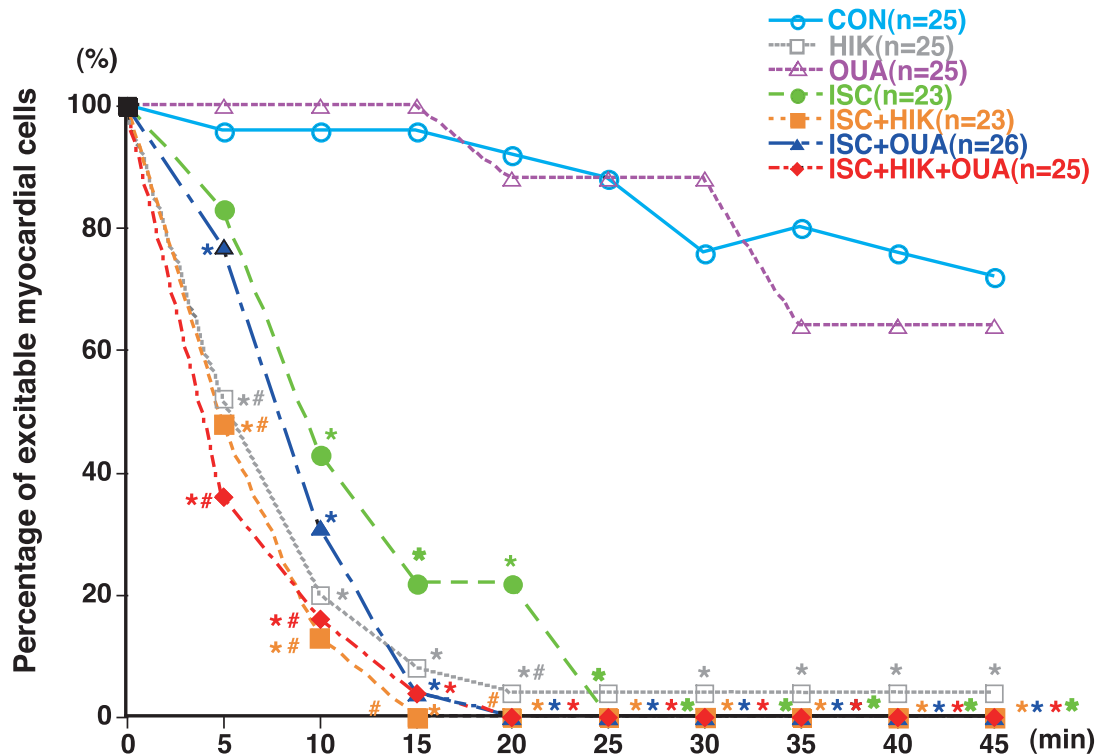


Figure 2. Time course of myocardial excitability in neonatal rat cardiomyocytes during treatment : control (CON), 16 mM KCl (HIK), 100 μM ouabain (OUA), simulated ischemia (ISC), simulated ischemia plus 16 mM KCl (ISC+HIK), simulated ischemia plus 100 μM ouabain (ISC+OUA), and simulated ischemia plus 16 mM KCl plus 100 μM ouabain (ISC+HIK+OUA). Simulated ischemia was induced by 5 mM NaCN and 5.5 mM 2-deoxyglucose. If at least one rhythmical increase in fluorescence was observed over 15 seconds, we considered the cell or the cell mass to have retained excitability. Myocardial excitability was expressed as the percentage of excitable cells in each group at every measured point (%). *P<0.05 vs. CON. #P<0.05 vs. ISC.

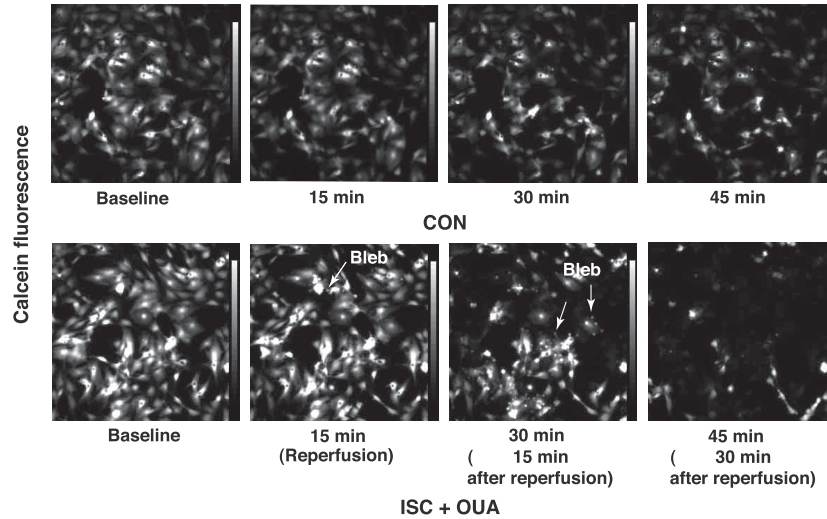


Figure 3. Figure showing representative changes in calcein fluorescence in the CON and ISC+OUA group. After equilibration with “normal” perfusate (Earle’s balanced salt solution EBSS) for approximately 10 min, the perfusate was switched to either EBSS (CON) or modified EBSS containing 5 mM NaCN, 5.5 mM 2-deoxyglucose, and 100 μM ouabain (ISC+OUA). Figures were obtained at baseline, 15, 30, and 45 min. Arrows indicate blebs.

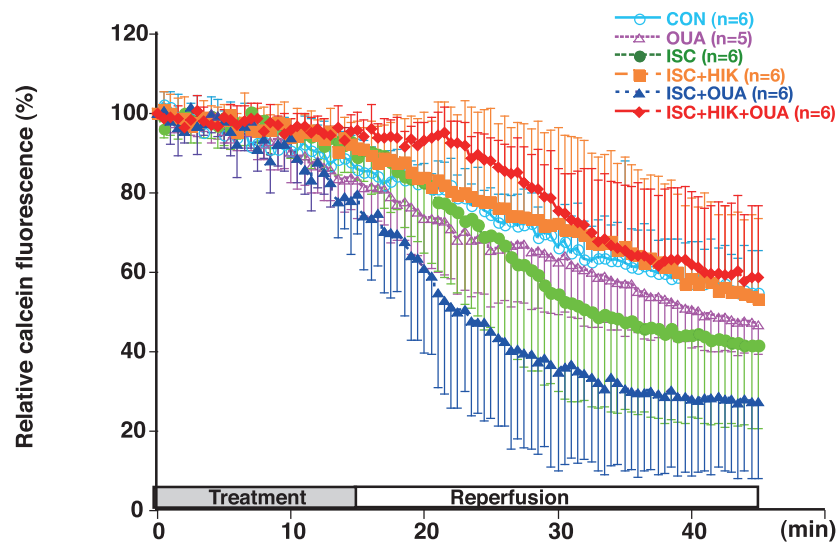


Figure 4. Time course of calcein fluorescence during and after treatment in time control (CON), 100 μM ouabain (OUA), simulated ischemia (ISC), simulated ischemia plus 16 mM KCl (ISC+HIK), simulated ischemia plus 100 μM ouabain (ISC+OUA), or simulated ischemia plus 16 mM KCl plus 100 μM ouabain (ISC+HIK+OUA) groups. Simulated ischemia was induced by 5 mM NaCN and 5.5 mM 2-deoxyglucose. Calcein fluorescence was measured relative to baseline (%). Data are represented as the mean ± SD.

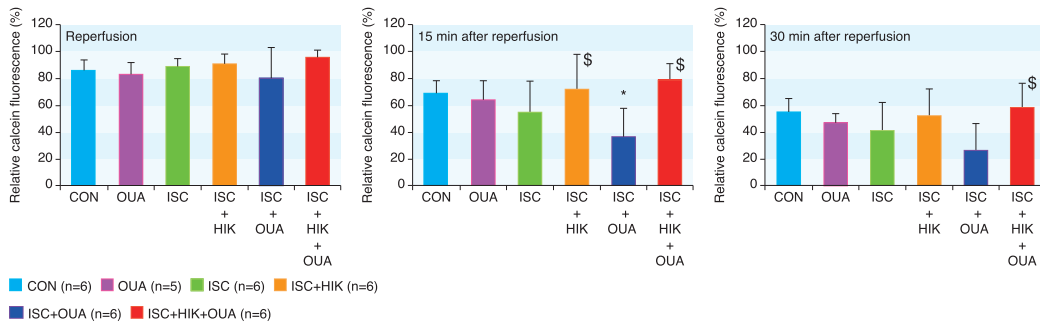


Figure 5. Effects of treatment/reperfusion on calcein fluorescence in cultured neonatal rat cardiomyocytes : control (CON), 100 μM ouabain (OUA), simulated ischemia (ISC), simulated ischemia plus 16 mM KCl (ISC+HIK), simulated ischemia plus 100 μM ouabain (ISC+OUA), and simulated ischemia plus 16 mM KCl plus 100 μM ouabain (ISC+HIK+OUA). Simulated ischemia was induced by 5 mM NaCN and 5.5 mM 2-deoxyglucose. Evaluation was performed at reperfusion, 15 min after reperfusion, and 30 min after reperfusion. Values are expressed as the mean ± SD. *P<0.05 vs. CON. #P<0.05 vs. ISC+OUA.

observed after reperfusion in many cells in the ISC group (Figure 4). The timing of appearance of the bleb is consistent with the abrupt loss of calcein fluorescence, suggesting reperfusion injury.

Perfusion with ouabain during simulated ischemia induced bleb formation in few cells (Figure 3, 4). Abrupt losses of calcein fluorescence were observed in most cells after reperfusion (Figure 4), although significant differences were not observed between the ISC and ISC+OUA groups. Difference with the CON group became significant in the times until bleb appearance. Thus, ouabain may accelerate and potentiate the membrane injury induced by simulated ischemia/reperfusion. Perfusion with hyperkalemia during simulated ischemia/reperfusion did not induce any difference in calcein fluorescence relative to the CON group, suggesting protective effects (Figure 5).

Hyperkalemia during simulated ischemia/reperfusion inhibits the decrease in calcein fluorescence induced by ischemia/reperfusion even in the presence of ouabain (Figure 4). Significant differences were observed in the calcein fluorescence between the ISC+OUA and the ISC+OUA+HIK group (Figure

5). Thus, the preventive effect became obvious in the presence of ouabain. Hyperkalemia nearly completely inhibited the effects of ouabain during ischemia/reperfusion.

Effects of hyperkalemia on myocardial excitability and diastolic $[Ca^{2+}]_i$ during simulated ischemia in the presence or absence of ouabain

The change in myocardial excitability and diastolic $[Ca^{2+}]_i$ in the ouabain group occurred over a similar time course as that of the control (Figure 6). Hyperkalemia induced diastolic arrest within approximately 5 min (Figure 6). Simulated ischemia in the absence and presence of ouabain also induced diastolic arrest within approximately 10 min and 8 min, respectively (Figure 6). Significant differences were observed between hyperkalemia groups and ischemia group at 5 min.

Hyperkalemia and ouabain did not significantly increase diastolic $[Ca^{2+}]_i$ in the absence of ischemia (Figure 6, 7). Simulated ischemia with and without hyperkalemia induced significant increases in diastolic $[Ca^{2+}]_i$ after 25 min and 15 min, respectively. Significant differences between the ISC+HIK and

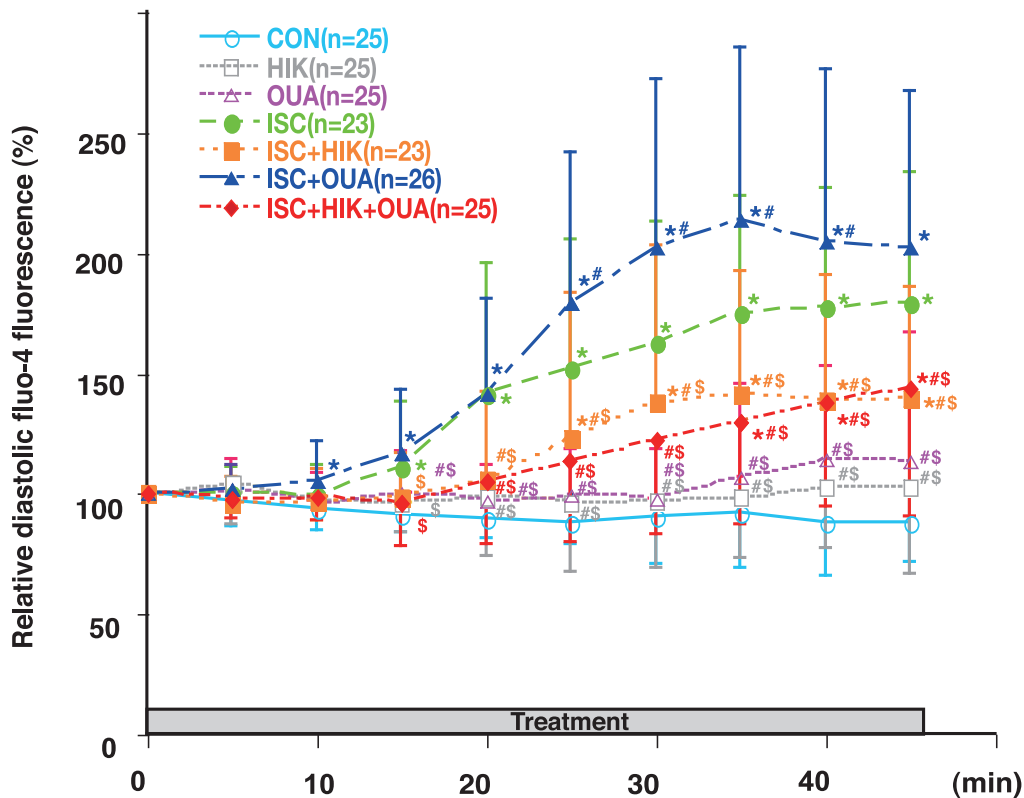


Figure 6. Time course of diastolic fluo-4 fluorescence in neonatal rat cardiomyocytes during treatment: control (CON), 16 mM KCl (HIK), 100 μ M ouabain (OUA), simulated ischemia (ISC), simulated ischemia plus 16 mM KCl (ISC+HIK), simulated ischemia plus 100 μ M ouabain (ISC+OUA), and simulated ischemia plus 16 mM KCl plus 100 μ M ouabain (ISC+HIK+OUA). Simulated ischemia was induced by 5 mM NaCN and 5.5 mM 2-deoxyglucose. Fluo-4 fluorescence was measured relative to baseline (%). Data are presented as the mean \pm SD. *P < 0.05 vs. CON. #P < 0.05 vs. ISC. \$P < 0.05 vs. ISC+OUA

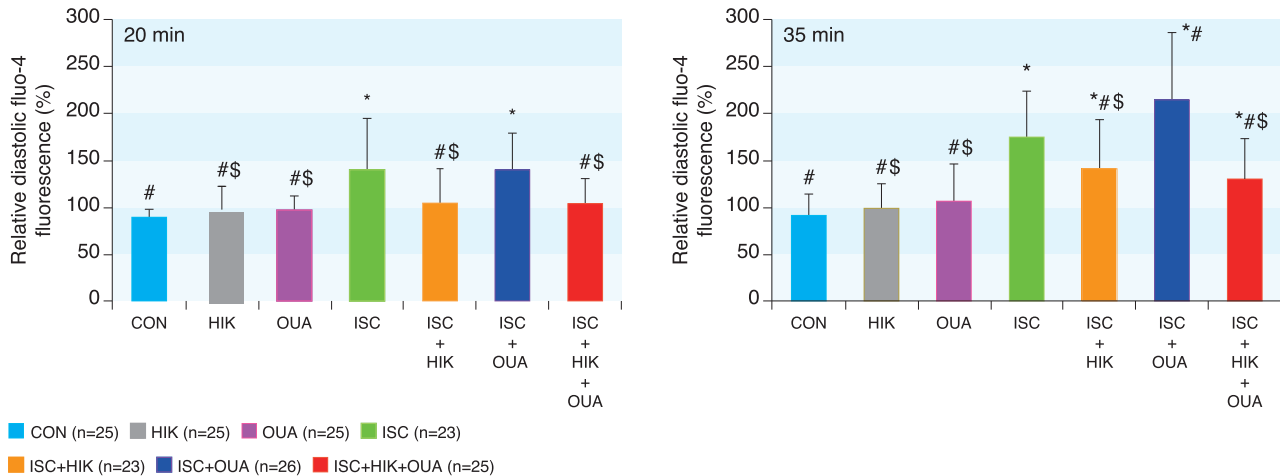


Figure 7. Effects of treatment on diastolic fluo-4 fluorescence in cultured neonatal rat cardiomyocytes: control (CON), 16 mM KCl (HIK), 100 μ M ouabain (OUA), simulated ischemia (ISC), simulated ischemia plus 16 mM KCl (ISC+HIK), simulated ischemia plus 100 μ M ouabain (ISC+OUA), and simulated ischemia plus 16 mM KCl plus 100 μ M ouabain (ISC+HIK+OUA). Simulated ischemia was induced by 5 mM NaCN and 5.5 mM 2-deoxyglucose. Evaluation was performed at 20 min and 35 min. Values are expressed as mean \pm SD. * $P < 0.05$ vs. CON. # $P < 0.05$ vs. ISC. \\$ $P < 0.05$ vs. ISC+OUA

ISC group and between the ISC+HIK and HIK group were observed after 20 min and 30 min, respectively. Thus, hyperkalemia delayed and inhibited the increase in diastolic $[Ca^{2+}]_i$ induced by simulated ischemia, suggesting cardioprotective effects, although hyperkalemia did not fully abolish the effects of ischemia (Figure 6, 7).

Simulated ischemia with ouabain induced significant increases in diastolic $[Ca^{2+}]_i$ after 10 min. Significant differences between the ISC+OUA and ISC group were observed between 25 min and 40 min. Thus, ouabain accelerated and potentiated the increase in diastolic $[Ca^{2+}]_i$ induced by simulated ischemia (Figure 6, 7).

Simulated ischemia under hyperkalemia conditions with and without ouabain induced significant increases in diastolic $[Ca^{2+}]_i$ after 35 min and 25 min, respectively. Thus, hyperkalemia almost completely inhibited the effects of ouabain during simulated ischemia (Figure 6, 7).

DISCUSSION

Cardiotonic steroids and effects of hyperkalemia on myocardial protection during ischemia/reperfusion

Our results suggest that hyperkalemia during ischemia is cardioprotective against ischemia/reperfusion insult. One of possible mechanisms is the antagonistic effect of hyperkalemia on cardiotonic steroids. Ouabain itself did not alter membrane integrity, bleb formation, myocardial excitability, or

diastolic $[Ca^{2+}]_i$ in this study. Although nontoxic concentrations of ouabain (up to 100 μ M) cause a less than twofold increase in diastolic $[Ca^{2+}]_i$ (27), it does not cause arrhythmic contraction and have no effect on cell viability in neonatal rat cardiomyocytes (24, 27). Our results suggest that ouabain accelerated and potentiated the increase in diastolic $[Ca^{2+}]_i$ during ischemia and possibly the membrane injury during ischemia/reperfusion. These data suggest that myocardial tolerance to ischemia is reduced during partial inhibition of Na^+/K^+ -ATPase.

Hyperkalemia almost completely inhibited the effects of ouabain during ischemia/reperfusion in this study. These results confirmed by the increase in diastolic $[Ca^{2+}]_i$ during ischemia. Extracellular K^+ is known to counteract the inhibition of Na^+/K^+ -ATPase by cardiotonic steroids (16, 17), including a neonatal rat study (15). Hermans *et al.* reported a possible mechanism of the antagonism (16). K^+ and ouabain bind to different conformational states of the Na^+/K^+ -ATPase (E_2-P and $E_2-P Na_2$, respectively), which are transiently exposed to the external side of the sarcolemma. Upon K^+ binding the state ($E_2-P K_2$) is removed from the extracellular side. If the K^+ concentration is increased, more $E_2-P K_2$ is formed and, consequently, active Na^+-K^+ transport is enhanced. At the same time, less $E_2-P Na_2$ is available for ouabain binding (16). Many studies have demonstrated that serum (11) and myocardial (12, 28) cardiotonic steroid activity levels are significantly increased during acute myocardial ischemia and that anti-digoxin antiserum has a

protective effect against myocardial ischemia-reperfusion injury (11, 12). We did not provide direct evidence that simulated ischemia induces the release of cardiotonic steroids or hyperkalemia enhance myocardial membrane Na^+/K^+ -ATPase activity in the present study. In addition, released cardiotonic steroids could have been washout because myocardial cells were continuously superfused in this study. Therefore, whether this mechanism was responsible for the results of the ISC group is not clear. However, it is clear that hyperkalemia strongly inhibited the effects of ouabain during ischemia. Myocardial cells may be exposed to the condition such as that of the ISC+HIK+OUA group at the site of myocardial ischemia. Therefore, we suppose that the cardioprotective effects of hyperkalemia may be induced by the inhibition of cardiotonic steroids.

The effects of activation of Na^+/K^+ -ATPase induced by hyperkalemia on myocardial protection during ischemia/reperfusion

Activation of Na^+/K^+ -ATPase is another possible mechanism of hyperkalemia-induced myocardial protection (4). Although Na^+/K^+ -ATPase is activated to a considerable extent under normal $[\text{K}^+]_o$ conditions (29), Terkildsen *et al.* suggested that Na^+/K^+ pump amplitude declines rapidly during phase one of simulated ischemia to a level ~65% of normoxic pump current and the increases in $[\text{K}^+]_o$ are suggested to stimulate pump activity during phase one and phase two (the plateau phase) (30). Ouabain did not abolish the protective effects of hyperkalemia in this study. Indeed, dihydro-ouabain did not affect the activation of Na^+/K^+ pump current on $[\text{K}^+]_o$ in cardiac ventricular myocytes, although Na^+/K^+ pump current decreased in the presence of dihydro-ouabain (16). Yamamoto *et al.* reported that metabolic inhibition abolished the hyperkalemia-induced cardioprotective effects induced by Na^+/K^+ -ATPase activation during normothermic reperfusion (4). Therefore, activation of Na^+/K^+ -ATPase may not be the main mechanism of the cardioprotective effects of hyperkalemia during ischemia, although we cannot deny the mechanism.

Membrane potential and the effects of hyperkalemia on myocardial protection during ischemia/reperfusion

Diastolic arrest may have beneficial effects in myocardial cells under an energy-limited condition and could be another possible mechanism. A previous report suggested that 15 mM hyperkalemic

solutions induce a depolarization of the resting membrane potential (V_m) to -66 mV from a resting V_m of -87 mV in adult rat cardiomyocytes (5). At this depolarized potential, the fast Na^+ channels are inactivated because the threshold is -70 to -65 mV, resulting in diastolic arrest (3). Thus, high $[\text{K}^+]_o$ may preserve ATP by reducing excitability of cardiac cells. However, ischemia also reduces the excitability of cardiac cells, although differences of several minutes were observed. The impact of the energy saving effects on the results is unclear. Baczko *et al.* reported that increased $[\text{K}^+]_o$ during ischemia and reperfusion can lead to increased Ca^{2+} overload and cell hypercontracture/death, which increases in parallel with depolarization (5). However, in their study, hyperkalemic conditions were maintained at the time of reperfusion, in contrast with our study. Reversal of increased $[\text{K}^+]_o$ after reperfusion could decrease membrane potential, which was suggested in our preliminary study using a voltage-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol bis-oxonol (31) (Data not shown).

Experimental model and limitations of this study

Modeling ischemia/reperfusion is difficult because of complicated and interdependent processes (32). We determined minimum exposure time for the induction of reperfusion injury using bis-oxonol in a preliminary study (data not shown). And, reperfusion-induced membrane injury and reperfusion-induced membrane depolarization observed in this study and in preliminary studies using bis-oxonol (data not shown) suggest successful simulation of reperfusion injury. Although relatively large dish-to-dish or cell-to-cell variation was observed, heterogeneity of the myocardial cell response to ischemia is well documented (32-34). To simplify the model, we investigated ischemia only and selected relatively intact cells in the second series of study. Ca^{2+} overload increased prior to reperfusion and the determinants of Na^+ overload are important determinants of Ca^{2+} overload in reperfused myocytes (14). Calpain activation, which occurs when reperfusion is induced by elevated diastolic $[\text{Ca}^{2+}]_i$, induces inactivation of Na^+/K^+ -ATPase and further calpain activation, resulting in sarcolemmal rupture and cell death (14). In fact, we observed that hyperkalemia applied only during the ischemic period affected membrane injury and bleb formation after reperfusion.

In our study, we used cultured neonatal rat cardiomyocytes as a model. Several studies have suggested

that immature myocytes have poorly developed sarcoplasmic reticulum and lack fully developed Ca^{2+} channels (35). There is a slight developmental change in α_2 and α_3 isoforms 14-21 days after birth (36). However, neonatal and adult cells have comparable time courses of the rise in $[Ca^{2+}]_i$, development of sarcolemmal blebs and cumulative enzyme release during energy depletion. (19). Although we must explore this result to adult heart carefully, myocardial protection of juvenile heart is itself clinically important (37). Eliminations of spontaneous activity were observed in some cells during fluo-4 loading and control perfusion in this study, suggesting damage caused by these procedures (38).

Although hyperkalemic cardioplegic solutions have a number of problems in cardiac surgery (3), hyperkalemia may counteract the effects of cardiotoxic steroids if applied under normothermia.

In conclusion, the data obtained in the present study suggest three important findings. First, 15 mM hyperkalemia during ischemia may be cardioprotective against ischemia/reperfusion insult in neonatal cardiomyocytes if applied to intact cells under normothermia. Second, inhibition of Na^+/K^+ -ATPase activity during ischemia accelerates and potentiates myocardial injury induced by ischemia/reperfusion. Third, hyperkalemia during ischemia almost completely inhibits the effects of cardiotoxic steroids during ischemia/reperfusion. We propose that preservation of Na^+/K^+ -ATPase activity induced by the antagonistic effect of $[K^+]_o$ on endogenous or therapeutic cardiotoxic steroids may play a role in the cardioprotective effects of hyperkalemia.

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