Very low dose of the Na\(^+\)/Ca\(^{2+}\) exchange inhibitor, KB-R7943, protects ischemic reperfused aged Fischer 344 rat hearts: considerable strain difference in the sensitivity to KB-R7943

Ken Yamamura*, Masato Tani, Hiroshi Hasegawa, Wen Gen

Department of Geriatrics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

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Abstract

Objective: Decreased ischemic tolerance in the aged myocardium is associated with accelerated intracellular Ca\(^{2+}\) overload. However, few drugs have been shown to attenuate reperfusion injury in aged hearts. Because the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1) has been reported to play an important role in Ca\(^{2+}\) overload during reperfusion, we investigated whether KB-R7943 (KB-R), a novel inhibitor of the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange, can protect aged rat hearts against reperfusion injury.

Methods: In the pilot study, isolated hearts from young Sprague–Dawley (SD) rats (12 weeks old), and young (12 weeks old) and aged (78 weeks old) Fischer 344 (F) rats were subjected to 25 min of global ischemia followed by 10 min of reperfusion with various concentrations of KB-R (0, 1 nM, 0.1 \(\mu\)M, 10 \(\mu\)M) and additional 20 min of reperfusion without agent. In subsequent studies with the same protocol in aged F rats, we added a protective dose of KB-R (1 nM) during the initial 10 min of reperfusion. In addition, we compared the amount of NCX1 and the sensitivity of the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange to KB-R under extracellular Na\(^+\)-free condition in F rats with young SD rats.

Results: In the pilot study, protective effects were elicited with 1 nM of KB-R in both young and aged F rats, while 10 \(\mu\)M KB-R was needed for SD rats. In subsequent studies using aged F rats, there was better recovery of LV systolic function and high-energy phosphates with reduced creatine kinase release and the duration of reperfusion arrhythmias. Ca\(^{2+}\) uptake via the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange was also inhibited with 1 nM of KB-R, but not in young SD rats. Although the amount of NCX1 was not different among young SD, young F, and aged F rat hearts. Conclusions: These results demonstrated that KB-R could protect aged F rat hearts as well as young hearts of both strains against ischemia-reperfusion injury. Moreover, the sensitivity to KB-R is very different between these strains, suggesting serious caution when the agent is applied to human beings. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Aging; Calcium (cellular); Ischemia; Na/Ca-exchanger; Reperfusion

1. Introduction

Intracellular Ca\(^{2+}\) overload is thought to be one of the major causes of ischemia-reperfusion injury. Although the mechanisms responsible for Ca\(^{2+}\) overload are still a matter of debate, Ca\(^{2+}\) overload may be caused by Na\(^+\) overload during ischemia. Sodium influx from the extracellular space can occur via the Na\(^+\) channels, the Na\(^+\)/H\(^+\) exchanger or the Na\(^+\)/HCO\(_3\) symporter [1]. Na\(^+\) accumulation during ischemia induces Ca\(^{2+}\) overload during the initial phase of reperfusion by activation of the reverse-mode of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1) [2,3]. Several groups have reported that the hearts of senescent animals are less tolerant to the effects of ischemia-reperfusion injury than are the hearts of young adult animals [4–8]. The decreased tolerance of aged hearts to ischemia can be due to augmentation of the increase in the intracellular Na\(^+\) concentration during ischemia and Ca\(^{2+}\) overload during reperfusion after ischemia. We have previously reported that the increase in the intracellular Na\(^+\) concentration at the end of ischemia was greater in older than in young rats [4]. Therefore, we hypothesized that inhibi-
tion of the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange could provide protection of ischemic cells against Ca\(^{2+}\) overload and reperfusion injury.

Heavy metals (La\(^{3+}\), Cd\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\)) [9], amiloride and its analogues [10,11], and dimethylthiourea [12] are known to block NCX1. However, these cations and drugs are non-selective and inhibit the T-type and L-type Ca\(^{2+}\) channels, and voltage-gated Na\(^{+}\) channels more potently than NCX1. Recently, KB-R 7943 (KB-R) was shown to be a novel inhibitor of NCX1 [13]. KB-R preferentially inhibits the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange, and minimally inhibits other ion transport systems, such as the Na\(^{+}\)/H\(^{+}\) exchanger, L-type Ca\(^{2+}\) channel, voltage-gated Na\(^{+}\) channel, and inward rectifier K\(^{+}\) channel [14].

The aim of the present study was to determine whether inhibition of the reverse-mode of Na\(^+\)/Ca\(^{2+}\) exchange in the early phase of reperfusion provides protection against myocardial injury in aged rat hearts. To this end, we investigated the effects of KB-R on the recovery of cardiac function and metabolites, the release of creatine kinase (CK) in the coronary effluent, and the incidence and duration of reperfusion-induced ventricular tachyarrhythmias in the aged Fischer 344 rats. We found that the beneficial effects of KB-R were obtained at very low concentrations in this strain. Therefore, we analyzed the amount of NCX1 in both Sprague–Dawley (SD) and Fischer 344 rat hearts to exclude strain differences in the expression of the exchanger protein. We also studied the sensitivity of the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange to KB-R by \(^{45}\)Ca\(^{2+}\) uptake under extracellular Na\(^{+}\)-free condition in isolated myocytes of both strains.

2. Methods

2.1. Animals

We studied the Fischer 344 rat, a strain that has been extensively used as a model of aging [5,15,16]. This strain does not exhibit significant coronary, vascular, or valvular abnormalities with aging [15]. We also used male Sprague–Dawley (SD) rats to compare the expression of NCX1. This study conformed to the Guidelines of the Animal Experiment Committee of Keio University.

Following the intraperitoneal injection of sodium pentobarbital (40 mg/kg), hearts were removed from young male SD (SD, 12 weeks, 330 to 380 g, n=12), and young (Y, 12 weeks, 200 to 220 g, n=27) or aged (A, 78 weeks, 350 to 400 g, n=72) male Fischer 344 rats.

2.2. Experimental protocols

2.2.1. Pilot study

Isolated hearts (young SD n=9, young Fischer n=12, aged Fischer n=12) were perfused at 37°C according to the Langendorff technique. The coronary perfusion pressure was set at 70 mmHg. The perfusate consisted of 118 mmol/l NaCl, 25 mmol/l NaHCO\(_3\), 4.7 mmol/l KCl, 1.2 mmol/l MgSO\(_4\), 1.2 mmol/l KH\(_2\)PO\(_4\), 1.75 mmol/l CaCl\(_2\), 11 mmol/l glucose, and 5 mmol/l pyruvate, and was equilibrated with O\(_2\):CO\(_2\) (95:5%).

Control hearts were subjected to 10 min of recirculating perfusion followed by 25 min of sustained global ischemia and 30 min of reperfusion while various concentrations of KB-R (0, 1 nM, 0.1 μM, 10 μM) were administered during the initial 10 min of reperfusion in KB-R-treated hearts (Fig. 1). After 30 min of reperfusion, hearts were frozen and stored in liquid nitrogen until the time of assay. In this pilot study, recovery of left ventricular (LV) function and energy metabolites were analyzed according to the methods described in Sections 2.3 and 2.4.

2.2.2. Studies in aged Fischer 344 rats

Hearts of aged Fischer 344 rats were divided into control (C, n=24) or KB-R (1 nM) groups (K, n=24). Perfusion protocol was the same as that in the pilot study except that \(^{45}\)Ca\(^{2+}\) was present throughout the reperfusion period.

2.3. Analysis of LV function

LV pressure was recorded using a plastic catheter with a latex balloon tip placed through the left atrium into the left ventricle. The LV end-diastolic pressure (LVEDP) was adjusted to 10 mmHg (9 to 11 mmHg) by filling the balloon with fluid. Hearts were paced at 5 Hz for 5 min by attaching platinum electrodes to the right atrium and the aorta. LV function (LV systolic pressure, LVSP; LV developed pressure, LVEDP=LVSP−LVEDP; and the maximum and minimum values of the first derivative of LV pressure, LV peak positive dP/dt and LV peak negative dP/dt, respectively) was measured before inducing sustained global ischemia. Pacing was terminated during sustained global ischemia to avoid inducing ventricular tachyarrhythmias during reperfusion [17]. The pacing was reinitiated and the LVEDP readjusted to 10 mmHg (9 to 11 mmHg) after 30 min of reperfusion to measure the post-ischemic recovery of LV function.

2.4. Analysis of myocardial energy metabolites

Frozen hearts were weighed and pulverized with a mortar and pestle that were cooled in liquid nitrogen. An aliquot of the frozen tissue powder was extracted with ice-cold perchloric acid (6% w/v). The neutralized perchloric acid extracts were assayed for ATP, creatine phosphate, and lactate using standard enzymatic proce-


Fig. 1. Perfusion protocols. Hearts from rats in each age group were subjected to 20 min of recirculating perfusion followed by 25 min of sustained global ischemia and 30 min of reperfusion. ‘Control groups’ indicate hearts perfused without any drug ( ) in young SD, young Fischer, and aged Fischer groups, respectively; ‘KB-R7943 groups’ indicate hearts treated with KB-R7943 at concentration of 1 nM, 0.1 μM, or 10 μM ( ) in young SD, young Fischer, and aged Fischer groups, respectively. The coronary perfusion pressure was kept constant at 70 mmHg. Arrows indicate the time of measurement of LV function ( ), CK activity in the coronary effluent ( ), myocardial Ca uptake ( ), and energy metabolite concentrations ( ).


dures [18]. Data are expressed as μmol/g dry weight of tissue.

2.5. Analysis of CK release

The coronary perfusate (50 ml for each heart) used during the pre-ischemic perfusion or during the 10 min of recirculating reperfusion was stored for each heart (n=24 for each group). The CK activity was determined from an aliquot of the perfusate. CK activity was measured by the adenosine diphosphate-dependent dephosphorylation method using creatine phosphate as the substrate [19]. Activity is expressed as IU/g dry weight of tissue.

2.6. Analysis of myocardial $^{45}$Ca$^{2+}$ uptake after reperfusion as an index of the intracellular Ca$^{2+}$ content

We measured myocardial $^{45}$Ca$^{2+}$ uptake as an index of intracellular Ca$^{2+}$ overload during reperfusion [2,20]. Myocardial $^{45}$Ca$^{2+}$ uptake increased rapidly during 10 to 20 min of reperfusion, reaching a plateau after 30 min of reperfusion [21]. For this reason, 30 min of reperfusion was selected for the measurement of reperfusion $^{45}$Ca$^{2+}$ uptake. Aliquots of neutralized perchloric acid extracts and coronary effluent from each heart were used to determine $^{45}$Ca$^{2+}$ radioactivity using a liquid scintillation spectrometer (LS3801, Beckman Instruments, Fullerton, CA). The $t_{1/2}$ for $^{45}$Ca$^{2+}$ radioactivity in the coronary effluent was 4 s during washout [2]. $^{45}$Ca$^{2+}$ uptake was calculated by dividing the residual tissue radioactivity after 3 min of washout by the specific radioactivity of $^{45}$Ca$^{2+}$ in the perfusate. The myocardial $^{45}$Ca$^{2+}$ uptake is expressed as μmol/g dry weight of tissue [20].

2.7. Sensitivity of the reverse mode of Na$^{+}$/Ca$^{2+}$ exchange to KB-R7943 in isolated myocytes

Isolated myocytes of young SD (n=3) and young Fischer 344 rat (n=3) were obtained from hearts perfused by the Langendorff technique. Hearts were perfused at 37°C with HEPES buffered solution containing (in mmol/l): NaCl 118, NaHCO$_3$ 25, KCl 4.7, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, CaCl$_2$ 0.5, glucose 11, pyruvate 5, pH 7.4. After 5 min of wash to eliminate the remaining blood, the hearts were then perfused with fresh buffer mixed with 1 mg/ml collagenase type 2 (Worthington Biochemical Corporation) and 40 μM CaCl$_2$ for 15 to 20 min. Both atria were removed and the ventricles were then cut into several pieces and myocytes were gently dissociated in the following solution A (in mmol/l): l-glutamic acid potassium 70, KCl 30, KH$_2$PO$_4$ 10, MgCl$_2$(6H$_2$O) 0.5, HEPES 5, taurin 15, EGTA 0.5, glucose 60, pH 7.4 and centrifuged at 300×g for 3 min. Supernatant was discarded and isolated myocytes were resuspended in the solution A containing 0, 1 nM or 10 μM of KB-R7943 and 3 μM of nifedipine. After the stabilization for 10 min, myocyte suspension was mixed quickly with the solution A containing 1 mM CaCl$_2$ and a trace level of $^{45}$Ca$^{2+}$. An aliquot of myocyte suspension was filtered using 0.45 μM millipore filter (Millipore Corporation, Bedford, MA) after 2 min incubation and the extracellular $^{45}$Ca$^{2+}$ on the filter was washed.
2.8. Analysis of ventricular tachycardia (VT) and fibrillation (VF)

An epicardial electrocardiogram (ECG) was recorded throughout the experiment by using three platinum electrodes attached directly to the left atrium, the right ventricle, and the apex of the left ventricle. The epicardial ECGs were analyzed to determine the incidence and duration of VT and/or VF according to the criteria of the Lambeth Conventions (1988) [22]. The duration of ventricular tachyarrhythmias in the reperfusion period was calculated. The incidence of ventricular tachyarrhythmias was also calculated by dividing number of hearts with VT and/or VF by total number of hearts in each group.

2.9. Immunoblot protocols

Young (n = 12) and aged (n = 12) Fischer 344 and young SD (n = 12) rat hearts were minced, homogenized (Physcotron, Nition), and centrifuged for 10 min at 700 × g. The supernatant was centrifuged again for 30 min at 70,000 × g. The pellet was washed once with a solution containing 20 mM HEPES–Tris (pH 7.4) and 150 mM NaCl and centrifuged for 30 min at 70,000 × g. Isolated crude membranes were subjected to SDS–PAGE on 8.0% acrylamide gels and then electrophoretically transferred to nitrocellulose membranes (Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. The blots were blocked in PBS containing 5% nonfat dry milk and then washed several times, and incubated for 1 h with PBS–milk containing alkaline phosphatase-rabbit antimouse IgG (1:1000 dilution). The immunoreaction was visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Quantification was achieved by densitometric analysis of the results of at least five separate experiments.

2.10. Materials

Chemicals were purchased from Sigma or Wako Pure Chemical Co. KB-R7943 was a generous gift from Nippon Organon K.K. (Osaka, Japan).

2.11. Statistical analysis

Data are expressed as the mean ± S.E.M. of 9–24 hearts. For differences between groups or for different concentrations of KB-R7943 in one group, we used two-way analysis of variance with Tukey’s or Dunnett’s test, respectively. For the analysis of the duration of VT and/or VF, the Mann–Whitney test was used. A value of P < 0.05 was considered statistically significant.

3. Results

3.1. Pilot study

In previous studies, inhibitory effects of KB-R on the reverse mode of Na⁺/Ca²⁺ exchange were reported at a concentration of 5 to 10 μmol/l in SD rats [13,14]. Therefore, we initially performed experiments using 10 μmol/l KB-R in Fischer 344 rats. However, the recovery of LV function after reperfusion did not improve in this strain, although we confirmed the beneficial effects of this concentration of KB-R in SD rats (Table 1). We hypothesized that treatment with 10 μmol/l KB-R in Fischer 344 rats may be toxic due to its non-specific effects at higher concentrations on other ion transport systems. Finally, we found that 1 nmol/l KB-R had beneficial effects on the recovery of function in Fischer 344 rat hearts. In the following experiments, we used 1 nmol/l KB-R.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Young Sprague–Dawley rats (n=9)</th>
<th>Young Fischer 344 rats (n=12)</th>
<th>Aged Fischer 344 rats (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control KB-R 1 μM KB-R 10 μM</td>
<td>Control KB-R 1 nM KB-R 10 μM</td>
<td>Control KB-R 1 μM KB-R 10 μM</td>
</tr>
<tr>
<td>Preischemic LVSP (mmHg)</td>
<td>112.2±13.5         100.4±7.4                        103.6±2.3            105.4±7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%LVSP (%)</td>
<td>17.2±5.1               55.0±5.1                     28.5±6.3                27.5±5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemic (LVDP) (mmHg)</td>
<td>109.0±12.9          90.2±7.5                      100.5±5.3               95.7±7.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%LVDP (%)</td>
<td>8.5±2.8               43.4±6.6                   18.8±4.3                14.3±3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP (μmol/g dry weight)</td>
<td>6.5±0.3               10.7±0.5                   7.9±0.8                 6.6±0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (μmol/g dry weight)</td>
<td>9.5±0.6               22.1±1.3                   15.6±2.3                10.5±2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate (μmol/g dry weight)</td>
<td>28.3±2.9           8.7±1.0                     27.1±5.4                21.4±2.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The percentage of preischemic values was calculated for each heart. Results are expressed as the mean ± S.E.M. KB-R, KB-R7943; LVSP, left ventricular systolic pressure; %LVSP, percent recovery of LVSP; LVDP, left ventricular developed pressure=LVSP–LVEDP (left ventricular end-diastolic pressure); %LVDP, percent recovery of LVDP; ATP, adenosine triphosphate; CP, creatine phosphate. *, **, ***P < 0.05 vs. control for each strain.
3.2. Studies in aged Fischer 344 rat hearts

3.2.1. Recovery of LV function

There were no significant differences in the preischemic indices of LV function between the control and KB-R groups of aged Fischer 344 rats (Table 2). Hearts stopped beating within 3 min after the onset of ischemia and the intraventricular pressure increased 10 to 20 min after the onset of ischemia. When the LVEDP was readjusted to 10 mmHg (9 to 11 mmHg) after 30 min of reperfusion, the recovery of LVSP, LVEDP, peak positive \( dP/dt \), and peak negative \( dP/dt \) was better in the KB-R group than in the control group (Table 2). The beneficial effects of KB-R on the recovery of function in aged Fischer 344 rat hearts were comparable to those in young SD or young Fischer 344 rat hearts (Table 1).

3.2.2. Recovery of myocardial energy metabolites

The level of ATP and creatine phosphate contents after reperfusion was significantly higher (Fig. 2A and B) and the accumulation of lactate at the end of reperfusion was significantly less in the KB-R group of aged Fischer 344 rat hearts than in the control group (Fig. 2C). These beneficial effects were similar to those in young SD and young Fischer 344 rat hearts (Table 1).

3.2.3. CK release in the coronary effluent

CK release in aged Fischer 344 rat hearts was reduced when treated with KB-R as compared with the control hearts without KB-R (Fig. 3).

3.2.4. Intracellular \( Ca^{2+} \) content after reperfusion

Myocardial \( 45Ca^{2+} \) uptake in aged Fischer 344 hearts without ischemic damage was 0.67±0.05 \( \mu \text{mol} / \text{g dry weight of tissue} \) and significantly less in the KB-R group of aged Fischer 344 rat hearts than in the control group (Fig. 2C). These beneficial effects were similar to those in young SD and young Fischer 344 rat hearts (Table 1).

3.2.5. Reperfusion-induced VT and/or VF

We evaluated the effect of KB-R on the duration of VT and/or VF during reperfusion (Fig. 5). The duration of VT and/or VF in the control group without KB-R treatment represented 70% of the total reperfusion period. The duration of VT and/or VF was significantly reduced when hearts were treated with KB-R. Although the incidence of reperfusion-induced VT and/or VF during post-ischemic reperfusion in each group did not achieve statistical significance (control: 100% (24/24), KB-R: 83.3% (20/24)), the ventricular tachyarrhythmias in the KB-R group appeared only in a very short time in the initial phase of reperfusion.

3.3. Sensitivity of the reverse mode of \( Na^+ /Ca^{2+} \) exchange to KB-R7943 in SD and Fischer 344 rat myocytes

In isolated myocytes of Fischer 344 rats, \( 45Ca^{2+} \) uptake via the reverse mode of \( Na^+ /Ca^{2+} \) exchange was significantly inhibited with lower concentration of KB-R7943 (% of control without KB-R; 10 \( \mu \text{M} \) 51.6±1.9%, 1 nM 49.6±2.3%, mean±S.E.M., \( *P<0.05 \) vs. control without KB-R). However, \( 45Ca^{2+} \) uptake was not inhibited at 1 nM of KB-R7943 in SD rat myocytes (% of control without KB-R; 10 \( \mu \text{M} \) 61.9±9.3%, 1 nM 92.0±8.7%, mean±S.E.M., \( *P<0.05 \) vs. control without KB-R).

3.4. Amount of NCX1 in young SD, and young and aged Fischer 344 rat hearts

Immunoblot analysis with anti-\( Na^+ /Ca^{2+} \) antibody revealed a single broad band with a molecular weight of 130 to 150 kDa in both young SD and young and aged Fischer 344 rat myocytes. The amount of NCX1 protein was similar in these three groups (Fig. 6).

4. Discussion

The present study is the first to show that the aged myocardium can be protected against ischemia-reperfusion injury when treated with the selective reverse-mode of \( Na^+ /Ca^{2+} \) exchange inhibitor, KB-R7943, during the initial reperfusion period.

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Table 2
Effects of 1 nM KB-R7943 on left ventricular function before ischemia and after ischemia-reperfusion in aged Fischer 344 rats

<table>
<thead>
<tr>
<th></th>
<th>C (n=24) Before ischemia</th>
<th>After reperfusion</th>
<th>K (n=24) Before ischemia</th>
<th>After reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVSP (mmHg)</td>
<td>118.8±14.0</td>
<td>15.6±10.5</td>
<td>112.9±11.5</td>
<td>63±10.0*</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>109.3±13.5</td>
<td>12.8±8.6</td>
<td>98.8±11.9</td>
<td>43±9.5*</td>
</tr>
<tr>
<td>( +dP/dt ) (mmHg/s)</td>
<td>3050±456</td>
<td>369±247</td>
<td>2800±247</td>
<td>1616±323*</td>
</tr>
<tr>
<td>( -dP/dt ) (mmHg/s)</td>
<td>988±243</td>
<td>163±110</td>
<td>1074±160</td>
<td>826±165*</td>
</tr>
</tbody>
</table>

Data represent the mean±S.E.M. C, 78-week-old rat hearts without KB-R7943; K, 78-week-old rat hearts treated with KB-R7943; \( +dP/dt \), left ventricular peak positive \( dP/dt \); \( -dP/dt \), left ventricular peak negative \( dP/dt \), \( *P<0.05 \) vs. C.
4.1. Ischemic tolerance with aging

Several investigators including our laboratory have reported that the hearts of senescent animals are less tolerant to ischemia-reperfusion injury than are the hearts of young adult animals [4–8]. However, these previous reports did not determine the reasons why aged hearts become less tolerant. Several mechanisms have been proposed to explain this difference. Firstly, a decrease in the activity of superoxide dismutase has been reported for...
the myocardium of aged Fischer 344 rats [24]. This diminished oxygen-free radical scavenging capacity could lead to a reduction in ischemic tolerance, although the levels of catalase and glutathione peroxidase do not change with aging. Secondly, the myocardial contents of ATP and creatine phosphate during normoxic perfusion are lower in senescent animals than in young animals [25]. Therefore, the level of ATP should also be lower at the onset of ischemia. This lower tolerance in aged hearts may also be due to the depletion of ATP that occurs during ischemia, which possibly accelerates cross bridge formation or perturbs energy-dependent processes. Rigor will be caused by conformation of myosin interdomain interaction induction when glycolytic ATP in myofibrils is depleted by myosin ATPase to fairly low levels (10–30%) [26,27]. However, local accumulation of MgADP in myofibrils due to a fall in cellular phosphocreatine and inability of myofibrillar creatine kinase to rephosphorylate ADP produced by myosin ATPase could be an important mechanism of diastolic pressure rise in ischemic conditions [28]. In the present study, the intraventricular pressure tended to be higher at the end of ischemia in aged hearts compared with young hearts. However, at the end of reperfusion, we did not find any differences in the myocardial ATP contents between the values for the aged and young groups without KB-R treatment (Table 1 and Fig. 2).

Ataka et al. [7] reported that the increase in the cytosolic calcium concentration after ischemia is greater in aged rabbit hearts (28 to 38 months old) compared with young hearts (18 to 25 weeks old). Furthermore, the magnitude of L-type Ca$^{2+}$ current increases in parallel with the enlargement of cardiac myocytes during aging [29]. This augmentation of Ca$^{2+}$ influx may allow the myocytes of older hearts to maintain normal function in the face of other age-related cellular alterations (e.g. decreases in the SR Ca$^{2+}$ pump rate [30] and expression of NCX1 [31]). This augmented Ca$^{2+}$ influx, under certain circumstances, may be responsible for the lower threshold of aged cardiac myocytes at which Ca$^{2+}$ overload and Ca$^{2+}$-dependent arrhythmias develop. However, the age-related changes in L-type Ca$^{2+}$ channel current cannot be explained on the single channel level. Tani et al. [4] showed that the increase in intracellular Na$^+$ at the end of ischemia was greater in aged Fischer 344 rat hearts (50 and 100 weeks old) than in young animals (12 weeks old), but the decrease in the intracellular K$^+$ content was similar in both groups, emphasizing the importance of Ca$^{2+}$ influx via the Na$^+$/Ca$^{2+}$ exchanger. The present study showed that a selective inhibitor of the reverse mode of Na$^+$/Ca$^{2+}$ exchange, KB-R7943, can block Ca$^{2+}$ overload following an increase in the intracellular Na$^+$ concentration in the aged rat hearts. This study also revealed the importance of modification of NCX1 in improving ischemic tolerance to aged rat hearts.

4.2. Effect of KB-R7943 on the recovery of LV function and energy metabolites in young SD, and young and aged Fischer 344 rat hearts

Iwamoto et al. [13] reported that KB-R at 0.3 to 10 μM (IC$_{50}$=1.2–2.4 μM), blocked NCX1-mediated Ca$^{2+}$ influx into cells, but did not significantly affect the activities of other ion transporters, such as the Na$^+$/H$^+$ exchanger, dihydropyridine-sensitive Ca$^{2+}$ channels, sarcolemmal and SR Ca$^{2+}$-ATPases, and Na$^+$, K$^+$-ATPase. In addition, it did not affect passive Na$^+$ permeability. Previous reports have shown that pretreatment with KB-R at micromolar concentrations protects ischemia-reperfused myocardium in SD rats [32]. In the present pilot study, we analyzed the
effects of 1 nM and 10 μM KB-R in both young SD, and young and aged Fischer 344 rat hearts. The 10 μM KB-R improved recovery of LV function and myocardial energy metabolites in young SD rat hearts (Table 1). However, in young and aged Fischer 344 rat hearts, KB-R at a concentration of 1 nM but not 0.1 or 10 μM improved the recovery of LV function and metabolites after ischemia-reperfusion (Tables 1 and 2, and Fig. 2). These differences may be attributable to strain differences. We confirmed the differential sensitivity of NCX1 to KB-R7943 by 45Ca2+ uptake between these strains. This result was in beautiful agreement with the data of the pilot study. Differences in drug effectiveness may be due to changes in the expression or physiologic activity of NCX1. KB-R at very low concentrations may have different effects on other ion transport systems in different strains of rats.

On the other hand, the problem whether NCX1 is damaged by ischemia is an important point for all the agents used during ischemia-reperfusion. Goldhaber reported that H2O2, an oxygen free radical generating compound, markedly stimulate Na+/Ca2+ exchange activity in guinea pig ventricular myocytes in an extracellular Na+-dependent manner. He also showed another free radical-generating system, xanthine oxidase, produced similar results and suggested that enhancement of Na+/Ca2+ exchange by oxygen free radicals during reperfusion, when intracellular Na+ was elevated, might promote intracellular Ca2+ overload and triggered arrhythmias [33]. Recently, Shigematsu and Arita reported that NCX1 in guinea pig ventricular myocyte was inhibited during simulated ischemia mainly due to secondary intracellular acidosis but that NCX1 activity was rapidly restored by subsequent reoxygenation [34]. These reports suggested that NCX1 activity might decrease during ischemia but would recover or exceed its preischemic function, which is not inconsistent with our data in the present study. KB-R is fairly specific for the reverse mode of Na+/Ca2+ exchange and has no effects on other ion transporters up to 20 μM [13]. KB-R at concentrations used in the present study was much lower and did not have any effects on left ventricular function or heart rate in non-ischemic control hearts of both strains. Therefore, it is very unlikely that KB-R at such low concentration in the present study could protect hearts and reduced Ca2+ influx by mechanism(s) other than inhibition of the reverse mode of Na+/Ca2+ exchange.

4.3. Effects of KB-R7943 on arrhythmias and Ca2+ overload during reperfusion in aged Fischer 344 rats

Intracellular Ca2+ overload causes oscillatory Ca2+ release from the SR with subsequent activation of the Ca2+-induced transient inward current [35]. The Ca2+ oscillation then induces Na+ influx through both the forward-mode of Na+/Ca2+ exchange and non-selective cation channels. This Na+ influx forms an ionic current component identified as TI [36,37]. TI induces oscillatory depolarization, and, when the depolarization reaches a threshold, voltage-gated Na+ channels are activated, resulting in the generation of an action potential. This action potential does not represent a regular beat, but is an extrasystole, and causes cardiac arrhythmias [38,39]. Therefore, activation of the reverse-mode of Na+/Ca2+ exchange could lead to reperfusion-induced ventricular tachyarrhythmias. This concept is supported by the present results, in that the duration of reperfusion-induced VT/VF decreased with the reduction in 45Ca2+ uptake during reperfusion when aged Fischer 344 rats were treated with KB-R.

4.4. Effects of KB-R7943 on CK release during early perfusion in aged Fischer 344 rats

Typically, CK is released mainly during early reperfusion. However, in more damaged hearts with relatively large areas of no-reflow, the CK release may be delayed and less prominent during early reperfusion because of inadequate washout. In the present study, the reduction of the release of CK during reperfusion by KB-R was statistically significant in aged Fischer 344 rat hearts.

4.5. Amount of NCX1 in young SD, and young and aged rat hearts

NCX1 is highly expressed in late fetal and neonatal Wistar rat hearts, decreases to adult levels 20 days after birth, and then reaches its lowest level of 24 and 72 weeks but again increases in the 96-week-old senescent rat [31]. Cardiac genes, such as α-MHC, β-MHC, and SERCA2, are known to have thyroid hormone response elements. The circulating thyroid hormone concentration is known to be highest at or around birth, and decreases with age. Because hypothyroidism increases NCX1 expression [40], it is possible that circulating thyroid hormone concentration may be involved in this age-related change in NCX1 expression. This augmentation of NCX1 expression at advanced ages may compensate for the augmentation of Ca2+ influx via L-type Ca2+ channels with aging [29]. However, we could not find any differences in the amount of NCX1 between young SD, young Fischer and aged Fischer, which cannot provide the reason for strain differences in the sensitivity to KB-R.

4.6. Limitation of the study

This study indicated that KB-R attenuated ischemic reperfusion injury, improved the recovery of contractile function, and reduced irreversible Ca2+ overload and ventricular tachyarrhythmias in aged Fischer 344 hearts. This finding raises the possibility that the post-ischemic inhibition of the Na+/Ca2+ exchanger represents a novel
method for antiarrhythmic therapy. However, the strain difference observed in the present study suggests the existence of significant species differences, which must be taken into consideration before these drugs are used in patients with ischemic heart disease.

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