Intracellular Sodium Accumulation During Ischemia as the Substrate for Reperfusion Injury

Kenichi Imahashi, Hideo Kusuoka, Katsuji Hashimoto, Jun Yoshioka, Hitoshi Yamaguchi, Tsunehiko Nishimura

Abstract—To elucidate the role of intracellular Na\(^+\) kinetics during ischemia and reperfusion in postischemic contractile dysfunction, intracellular Na\(^+\) concentration ([Na\(^+\)]\(i\)) was measured in isolated perfused rat hearts using \(^{23}\)Na nuclear magnetic resonance spectroscopy. The extension of the ischemic period from 9 minutes to 15, 21, and 27 minutes (at 37°C) increased [Na\(^+\)], at the end of ischemia from 270.0±10.4% of preischemic level (mean±SE, n=5) to 348.4±12.0% (n=5), 491.0±34.0% (n=7), and 505.3±12.1% (n=5), respectively, whereas the recovery of developed pressure worsened with the prolongation of the ischemic period (95.1±4.2%, 84.3±1.2%, 52.8±13.7%, and 16.9±6.4% of preischemic level). The kinetics of [Na\(^+\)]\(i\) recovery during reperfusion was analyzed by the fitting of a monoexponential function. When the hearts were reperfused with low-[Ca\(^+\)]\(o\) (0.15 mmol/L) solution, the time constants of the recovery (\(\tau\)) after 15-minute (8.07±0.85 minutes, n=5) and 21-minute ischemia (6.44±0.90, n=5) were significantly extended, with better functional recovery (98.5±1.4% for 15-minute [P<0.05]; 98.0±1.0% for 21-minute [P<0.05]) compared with standard reperfusion ([Ca\(^+\)]\(o\)=2.0 mmol/L, \(\tau\)=3.58±0.28 minutes for 15-minute [P<0.0001]; \(\tau\)=3.02±0.20 for 21-minute [P<0.0001]). A selective inhibitor of Na\(^+\)/Ca\(^+\)\(^+\) exchanger also decelerated the [Na\(^+\)]\(i\) recovery, which suggests that the recovery reflects the Na\(^+\)/Ca\(^+\)\(^+\) exchange activity. In contrast, high-[Ca\(^+\)]\(o\) reperfusion (5 mmol/L) accelerated the [Na\(^+\)]\(i\), recovery after 9-minute ischemia (\(\tau\)=2.48±0.11 minute, n=5 [P<0.0001]) and 15-minute ischemia (\(\tau\)=2.10±0.07, n=6 [P<0.05]), but functional recovery deteriorated only in the hearts with 15-minute ischemia (29.8±9.4% [P<0.05]). [Na\(^+\)]\(i\) recovery after 27-minute ischemia was incomplete and decelerated by low-[Ca\(^+\)]\(o\), reperfusion, with limited improvement of functional recovery (42.5±7.9%, n=5 [P<0.05]). These results indicate that intracellular Na\(^+\) accumulation during ischemia is the substrate for reperfusion injury and that the [Na\(^+\)]\(i\) kinetics during reperfusion, which is coupled with Ca\(^+\)\(^+\) influx, also determines the degree of injury. (Circ Res. 1999;84:1401-1406.)

Key Words: [Na\(^+\)]\(i\), \(^{23}\)Na nuclear magnetic resonance spectroscopy, functional recovery, time constant, low-/high-[Ca\(^+\)]\(o\), reperfusion

Na\(^+\) accumulates in the myocardium during ischemia and recovers rapidly to preischemic level after reperfusion.\(^1\) We have previously hypothesized that Na\(^+\) accumulation during ischemia induces Ca\(^+\)\(^+\) overload during the initial phase of reperfusion mediated by Na\(^+\)/Ca\(^+\)\(^+\) exchanger, resulting in myocardial stunning.\(^2,3\) Many studies have demonstrated that the inhibition of Na\(^+\) influx pathways reduced Na\(^+\) accumulation during ischemia and contributed to better functional recovery after reperfusion.\(^4-6\) Na\(^+\) accumulation during ischemia has also been considered to have deleterious effects on energy metabolism and to induce contractile dysfunction and/or ventricular fibrillation during reperfusion.\(^7,9\) Recently, it was reported that Na\(^+\) accumulation during ischemia or hypoxia causes net K\(^+\) loss, resulting in lethal arrhythmias.\(^10\) Thus, it has been considered that Na\(^+\) accumulation during ischemia is one of the major determinants of ischemia/reperfusion injury.

The reperfusion with acidic,\(^1,1\) low-calcium,\(^1,2\) or high-sodium perfusate\(^3\) and the inhibition of Na\(^+\)/H\(^+\) exchange\(^13\) prevent subsequent contractile dysfunction, which suggests that Na\(^+\) accumulation during ischemia is not the only causal factor for postischemic injury. Several lines of evidence suggest a critical role of Na\(^+\)/Ca\(^+\)\(^+\) exchange during reperfusion.\(^3,14\) However, few studies have addressed the ion kinetics during the early phase of reperfusion. The mechanism of Na\(^+\) kinetics during reperfusion is still unclear.

The present study was performed to clarify whether the amount of Na\(^+\) accumulated during ischemia is a determinant...
of function after reperfusion. The effect of Na\(^+\) accumulation during ischemia on functional recovery was measured by changing the duration of the ischemic period. The effect of the [Na\(^+\)], kinetics during the early phase of reperfusion was measured by changing [Ca\(^{2+}\)] in the reperfusion solution or by adding a selective inhibitor of Na\(^+/\)Ca\(^{2+}\) exchanger.

**Materials and Methods**

The experimental model was described previously.\(^{18}\) Briefly, male Sprague-Dawley rats (400 to 450 g) were anesthetized with sodium pentobarbital (50 mg/kg IP; Abbott Laboratories) and heparinized. The heart was excised and perfused with modified HEPES buffer containing (in mmol/L) NaCl 108, KCl 5, MgCl\(_2\) 1, HEPES 5, CaCl\(_2\) 2, sodium acetate 20, and glucose 10. The pH was adjusted to 7.40 at 37°C, and the solution was bubbled continuously with 100% O\(_2\). Heart rate was maintained at 300 bpm by right ventricular pacing. A latex balloon tied to the end of a polyethylene tube was passed into the left ventricle through the mitral valve and connected to a pressure transducer (SPB-101, San-ei Electric Co, Ltd). Coronary flow rate was controlled by a peristaltic pump and was initially adjusted so that the coronary pressure equaled 75 to 80 mm Hg, after which the flow rate was kept constant throughout the experiment except during global ischemia. Coronary effluent was accumulated to measure the coronary flow. The experimental protocol was approved by the Animal Care and Use Committee of Osaka University Medical School.

**\(^{23}\)Na Nuclear Magnetic Resonance Spectroscopy (\(^{23}\)Na MRS) Measurements**

To measure intracellular Na\(^+\) concentration ([Na\(^+\)],), we applied \(^{23}\)Na MRS as described previously.\(^{16}\) Briefly, \(^{23}\)Na MRS spectra were obtained on a Bruker AMX-400wb spectrometer, the resonance frequency for \(^{23}\)Na of which was 105.843 MHz. Two hundred fifty-six free induction decays were collected into 1 spectrum; it took 90 seconds to obtain 1 spectrum. To distinguish intracellular and extracellular \(^{23}\)Na NMR signals, a perfusate of the following composition (in mmol/L) was used for \(^{23}\)Na MRS measurement: NaCl 18, KCl 5, MgCl\(_2\) 1, CaCl\(_2\) 2, HEPES 5, glucose 10, sodium acetate 20, and Tris(hydroxymethyl)aminomethane (Tris) 15, pH 7.4. The balloon was filled with a complex of dysprosium triply phosphate (PPP), Na\(_3\)PPP \(\cdot\) 3 NaCl solution as a reference. The baseline for the peak of intracellular Na\(^+\) on the \(^{23}\)Na MRS spectrum was obtained by extrapolating the slope of the peak for extracellular Na\(^+\).\(^{16}\) The area of the peak was measured by planimetry using a digitizer. The area was normalized by the reference peak in the left ventricular balloon. The calculated amount was divided by the measured weight of each heart to yield the intracellular concentration in units of micromoles per gram wet weight. This value can be converted to millimoles per liter by multiplying by 1.74.\(^{18}\)

The time constant (\(\tau\)) for the recovery of [Na\(^+\)], during the initial 3 minutes of reperfusion was determined by the following equation:

\[
\%[\text{Na}^+] = 100 \exp(-t/\tau)
\]

where \%Na\(^+\) is the value of [Na\(^+\)], normalized by that at the end of ischemia, and \(t\) is the duration after the start of reperfusion.

**Experimental Protocol**

After the stabilization of isolated perfused hearts, the perfusate was switched from the standard one to that containing a shift reagent, and bathing was started for \(^{23}\)Na MRS measurement. Then the hearts were subjected to 0-flow global ischemia at 37°C. After the predetermined ischemic period, the hearts were reperfused with the solution containing shift reagent for 6 minutes and without shift reagent for 24 minutes. Pacing was discontinued after 5 minutes of ischemia and restarted at the beginning of reperfusion. The duration of the ischemic period was 9, 15, 21, or 27 minutes. \(^{23}\)Na MRS spectra were acquired 3 minutes before ischemia, during ischemia, and during the initial 6 minutes of reperfusion. Functional recovery after ischemia was assessed by the developed pressure during reperfusion normalized by preischemic level perfused with standard solution. In hearts reperfused with low-[Ca\(^{2+}\)] solution, hearts were reperfused with the solution containing 0.15 mmol/L [Ca\(^{2+}\)], for the initial 6 minutes of reperfusion. After 6 minutes, [Ca\(^{2+}\)], in perfusate was increased to 0.5 and 1.0 mmol/L every 3 minutes. Finally, the perfusate was switched to the standard one to measure the recovery of contractile function. In the high-[Ca\(^{2+}\)] reperfusion protocol, the hearts were reperfused with the solution containing 5.0 mmol/L [Ca\(^{2+}\)], for 6 minutes, and then the perfusate was changed to the standard one.

To confirm the role of Na\(^+/\)Ca\(^{2+}\) exchanger in [Na\(^+\)\] recovery during reperfusion, some hearts were treated with a selective inhibitor of the exchanger, KB-R7943 (Kanebo Ltd),\(^{19}\) for 5 minutes before 21-minute ischemia and 10 minutes of reperfusion. KB-R7943 was dissolved in DMSO at 100 mmol/L and diluted further in buffer to a final concentration (3 or 10 \(\mu\)mol/L).

**Statistical Methods**

Data are presented as mean\(\pm\)SE. Statistical analysis was performed using the unpaired \(t\) test or one-way ANOVA where appropriate. A value of \(P<0.05\) was considered significant.

**Results**

**Relation Between Na\(^+\) Accumulation During Ischemia and Functional Recovery**

Intracellular Na\(^+\) was accumulated during ischemia and decreased rapidly during reperfusion. Na\(^+\) accumulation at the end of ischemia was increased with prolongation of the ischemic period (Figure 1). [Na\(^+\)], was 1.98\(\pm\)0.04 \(\mu\)mol/g wet weight at the preischemic, control period (\(n=48\), all groups) and increased to 5.37\(\pm\)0.19 (\(n=5\)), 7.57\(\pm\)0.09 (\(n=5\)), 8.81\(\pm\)0.22 (\(n=7\)), and 10.35\(\pm\)0.19 (\(n=5\)) at the end of 9-, 15-, 21-, and 27-minute ischemia, respectively. The recovery of developed pressure after reperfusion was better in the 9-minute ischemia group (95.1\(\pm\)4.2% of preischemic level) and moderate after 15-minute ischemia (84.3\(\pm\)1.2%). However, the recovery after 21 minutes of ischemia was extremely delayed and depressed in 3 hearts (14.4\(\pm\)1.3%), but in the remaining hearts the recovery was 81.6\(\pm\)3.8% (52.8\(\pm\)13.7% in total hearts). When the ischemic period was...
more extended to 27 minutes (n=5), the functional recovery was also extremely depressed (16.9±6.4%). The relation between Na\(^+\) accumulation during ischemia and functional recovery is depicted in Figure 2. There was a significant correlation between Na\(^+\) accumulation and functional recovery when the hearts were reperfused with standard reperfusion (Figure 2, open symbols; r=-0.76, P<0.0001). This result suggests that Na\(^+\) accumulation during ischemia determines the functional recovery when the reperfusion was not modified.

### Kinetics of [Na\(^+\)]\(_i\) Recovery During Reperfusion

Upon reperfusion, [Na\(^+\)]\(_i\) returned to the preischemic level exponentially (Figure 1), and the time constants of the recovery (\(\tau\)) were identical among the hearts with 9-, 15-, and 21-minute ischemia (9-minute, 3.44±0.14 minutes [n=5]; 15-minute, 3.58±0.28 [n=5]; and 21-minute, 3.02±0.20 [n=7]; Figure 3). In 3 hearts showing extremely depressed functional recovery after 21-minute ischemia, [Na\(^+\)]\(_i\) recovery was identical to that of the other hearts during the early period but was not completed after 3 minutes of reperfusion, resulting in [Na\(^+\)]\(_i\) elevation. When the ischemic period was extended to 27 minutes, the [Na\(^+\)]\(_i\) recovery was not completed and remained at an elevated level even after 3 minutes of reperfusion (Figure 1). The time constant (4.36±0.10 minutes, n=5) was prolonged compared with that of the 9- and 21-minute ischemia groups (Figure 3; P<0.05).

The effect of [Ca\(^+\)]\(_i\), in reperfusate on functional recovery was measured to clarify whether Na\(^+\) accumulation during ischemia is the only causal factor. The recovery of [Na\(^+\)]\(_i\) after 15-minute ischemia in the hearts reperfused with low-[Ca\(^+\)]\(_i\) solution (\(\tau\)=8.07±0.85 minutes, n=5) was significantly slower compared with standard reperfusion (Figure 4; P<0.0001). Functional recovery was significantly better with low-[Ca\(^+\)]\(_i\) reperfusion (98.5±1.4%) than with standard reperfusion (84.3±1.2%; P<0.05; Figure 3). In contrast, when the hearts were reperfused with high-[Ca\(^+\)]\(_i\), solution, the [Na\(^+\)]\(_i\) recovery (\(\tau\)=2.10±0.07, n=6) was faster (Figure 4; P<0.05), and functional recovery deteriorated (29.8±9.4%; P<0.01; Figure 3). Similar results were observed in the experiments with 9-minute ischemia (high-[Ca\(^+\)]\(_i\) reperfusion, \(\tau\)=2.48±0.11, n=5; P<0.0001; Figure 3), 21-minute ischemia (low-[Ca\(^+\)]\(_i\) reperfusion, \(\tau\)=6.44±0.90, n=5; P<0.0001; Figure 3), and 27-minute ischemia (low-[Ca\(^+\)]\(_i\) reperfusion, \(\tau\)=6.52±0.32, n=5; P<0.0001; Figure 3). These results suggest that [Na\(^+\)]\(_i\) recovery in this protocol reflects a mirror image of Ca\(^+\)\(_i\) influx and is partially mediated by Na\(^+\)/Ca\(^+\)\(_i\) exchanger during the initial 3 minutes of reperfusion. To confirm the effect of Na\(^+\)/Ca\(^+\)\(_i\) exchanger in [Na\(^+\)]\(_i\) recovery, a selective inhibitor of the exchanger, KB-R7943, was applied; KB-R7943 decelerated the recovery of [Na\(^+\)]\(_i\) during 3 minutes of reperfusion dose dependently (Figure 5; P<0.001), as was observed in low-[Ca\(^+\)]\(_i\) reperfusion. The functional recovery of the hearts reperfused with low-[Ca\(^+\)]\(_i\) or high-[Ca\(^+\)]\(_i\) solution were outside the 95% confidence range of the regression that was based on the Na\(^+\) accumulation during ischemia (Figure 2). These results indicate that the degree of functional recovery is also influenced by the means of

![Figure 2](image2.png)

**Figure 2.** Relation between Na\(^+\) accumulation during ischemia and the percentage recovery of developed pressure. Na\(^+\) accumulation was defined as the difference between [Na\(^+\)]\(_i\) at the end of ischemia and that before ischemia. ■, □, △, and ○ represent the hearts reperfused after 9, 15, 21, and 27 minutes of ischemia, respectively. Open symbols represent the hearts reperfused with standard solution. Closed and dotted symbols represent the hearts reperfused with the low-[Ca\(^+\)]\(_i\), and high-[Ca\(^+\)]\(_i\), solution, respectively. Solid line indicates regression, with a 95% confidence interval (broken lines) obtained from the data with standard reperfusion.

![Figure 3](image3.png)

**Figure 3.** Relationship between the time constant of [Na\(^+\)]\(_i\), recovery during reperfusion (\(\tau\)) and the recovery of developed pressure. ■, □, △, and ○ represent the hearts reperfused with standard solution after 9, 15, 21, and 27 minutes of ischemia, respectively. Open symbols represent the hearts reperfused with standard solution. Closed and dotted symbols represent the hearts reperfused with the low-[Ca\(^+\)]\(_i\), and high-[Ca\(^+\)]\(_i\), solutions, respectively. The line indicates the regression obtained from the hearts subjected to 15-minute ischemia. Note that the time constant was plotted by logarithmic scale.

![Figure 4](image4.png)

**Figure 4.** Effects of [Ca\(^+\)]\(_i\) in reperfusate on the recovery of [Na\(^+\)]\(_i\) during reperfusion in hearts subjected to 15 minutes of ischemia. ○ represents hearts reperfused with standard solution, the same as that in Figure 1; ■ and △, hearts reperfused with low-[Ca\(^+\)]\(_i\) and high-[Ca\(^+\)]\(_i\), solutions, respectively.


Effects of KB-R7943 on the recovery of [Na+] during reperfusion in hearts subjected to 21-minute ischemia. A, ○ represents hearts reperfused with standard solution, the same as that in Figure 1; ●, hearts treated with KB-R7943 (10 μmol/L). B, Relation between KB-R7943 concentration and time constant of recovery during reperfusion (n=3 for 3 μmol/L; n=5 for 10 μmol/L).

The early phase of reperfusion has been indicated. In contrast, the detailed role of intracellular Na+ in reperfusion injury is not clarified yet, although it may play some key roles. This study has demonstrated that both Na+ accumulation during ischemia and the kinetics of [Na+] recovery mainly determine the functional recovery after reperfusion.

Role of Na+ Accumulation During Ischemia in Reperfusion Injury

We have previously hypothesized that Na+ accumulation during ischemia induces Ca2+ influx during reperfusion mediated by Na+/Ca2+ exchange and that Ca2+ entry during the initial phase of reperfusion is important to produce Ca2+ overload, causing stunning. Previous studies demonstrated that reduction of Na+ accumulation during ischemia by Na+/H+ exchange inhibitors, Na+ channel blocker, or the reduced activity of Na+/H+ exchange in diabetic rat hearts protects myocardium against stunning. However, few studies have examined the relationship between the augmentation of Na+ accumulation and the degree of functional recovery. In the present study, we titrated functional recovery and Na+ accumulation during ischemia by changing the ischemic period. Prolongation of ischemia consumes ATP and induces acidosis and Ca2+ overload as well as the accumulation of Na+. However, we previously demonstrated that the reduction of Na+ accumulation during ischemia by 5-(N-ethyl-N-isopropyl) amiloride (EIPA), a potent inhibitor of Na+/H+ exchanger, contributed to better functional recovery without affecting intracellular acidosis or high-energy phosphates. We also reported that functional recovery is independent of intramyocardial ATP content. When Na+ accumulation was matched by changing ischemic period or using EIPA, functional recovery was consistent, although the levels of ATP and intracellular acidosis were different. A significant correlation between Na+ accumulation and the degree of functional recovery was observed when the reperfusion was not modified. These results indicate that Na+ accumulation during ischemia is one of the determinants of functional recovery after reperfusion.

Contribution of [Na+], Kinetics During Reperfusion to Reperfusion Injury

We have indicated the important role of Ca2+ influx via Na+/Ca2+ exchange during the early phase of reperfusion. This has been suggested by several lines of evidence. Interventions such as acidic, low-calcium, and high-sodium reperfusion improved the functional recovery. However, it has not been directly measured whether these interventions result in less Ca2+ overload. In the present study, we measured [Na+] instead of [Ca2+]. We measured the kinetics in [Na+] recovery by regulating Ca2+ influx into myocardium by changing Ca2+, in the perfusate, because, under our experimental conditions, [Na+] inversely reflects Ca2+ influx and is mediated by Na+/Ca2+ exchanger. This was also confirmed by the treatment with the selective inhibitor of Na+/Ca2+ exchanger. The present results indicate that the degree of functional recovery can also be modified by the [Na+] recovery during reperfusion and are consistent with the idea that the target of modified reperfusion is the...


<table>
<thead>
<tr>
<th>Duration of Ischemia (Minutes)</th>
<th>Time Constant (τ, Minutes)</th>
<th>Irreversible Accumulation (α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>1.29±0.08</td>
<td>0.32±0.09</td>
</tr>
<tr>
<td>15</td>
<td>1.79±0.16</td>
<td>0.34±0.43</td>
</tr>
<tr>
<td>21</td>
<td>1.55±0.11</td>
<td>8.54±3.33</td>
</tr>
<tr>
<td>27</td>
<td>1.15±0.23</td>
<td>38.65±4.46</td>
</tr>
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\( \Delta [\text{Na}^+] \) was the increment of [\text{Na}^+] from the control level and normalized by that at the end of ischemia (%Δ[Na^+]). The recovery kinetics during the initial 6 minutes of reperfusion was analyzed with Equation 2.

\[ \% \Delta [\text{Na}^+] = (100 - \alpha) \exp(\frac{-t}{\tau^*}) + \alpha \]

Instead of Equation 1, the time constants (τ*) were not significantly different among 9-, 15-, 21-, and 27-minute ischemic groups, whereas irreversible accumulation of Na⁺ (α) was significantly higher only in the 27-minute ischemic group (Table). These results indicate that incomplete recovery in [Na⁺], after 27-minute ischemia reflects 2 different populations of myocytes, one irreversibly injured with high [Na⁺], and the other normal. It is also not deniable that the translocation of Na⁺/K⁺-ATPase away from sarcolema may contribute to the slower and incomplete [Na⁺] recovery after prolonged ischemia.

Determinants of Functional Recovery

The present study indicates that the functional recovery was determined not only by Na⁺ accumulation during ischemia, but also by the [Na⁺] recovery kinetics during reperfusion, which may be coupled with Ca²⁺ influx. This means that Na⁺ accumulation during ischemia is the substrate for reperfusion injury. However, high-[Ca], reperfusion after 9 minutes of ischemia did not significantly aggravate the functional recovery. This suggests that there might be a threshold in the amount of Na⁺ accumulation during ischemia to induce the deleterious effect on reperfused myocardium. Our conclusion is clearly consistent with the beneficial effect of Na⁺/H⁺ exchange inhibitors, which reduces this substrate, whereas it is inconsistent with the hypothesis that Na⁺ entry via Na⁺/H⁺ exchange just after reperfusion is a critical trigger for reperfusion injury.

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