Role of Intracellular Na⁺ Kinetics in Preconditioned Rat Heart

Kenichi Imahashi, Tsunehiko Nishimura, Jun Yoshioka, Hideo Kusuoka

Abstract—To elucidate the role of intracellular Na⁺ kinetics in the mechanism for ischemic preconditioning (IPC), we measured intracellular Na⁺ concentration ([Na⁺]) using 23Na–magnetic resonance spectroscopy in isolated rat hearts. IPC significantly delayed the initial [Na⁺], increase (d[Na⁺]/dt) compared with non-IPC control, resulting in attenuation of Na⁺ accumulation (Δ[Na⁺]) during 27 minutes of ischemia with better functional recovery. [Na⁺], in IPC, but not in control, recovered to preischemic level during a 6-minute reperfusion. The Na⁺-H⁺ exchange inhibitor further suppressed d[Na⁺]/dt in both control and IPC hearts with concomitant improvement of functional recovery, suggesting little contribution to the mechanism of IPC. The mitochondrial ATP-sensitive K⁺ (mito K<sub>ATP</sub>) channel activator diazoxide (30 μmol/L) completely mimicked both [Na⁺], kinetics and functional recovery in IPC without any additive effects to IPC. The mito K<sub>ATP</sub> channel blocker 5-hydroxydecanoic acid (100 μmol/L) lost protective effect as well as the attenuation of d[Na⁺]/dt and [Na⁺], recovery induced by diazoxide. However, 5-hydroxydecanoic acid also lost IPC-induced protection, but incompletely abolished the alteration of d[Na⁺]/dt and the [Na⁺], recovery. The Na⁺/K⁺-ATPase inhibitor ouabain (200 μmol/L) did not change d[Na⁺]/dt in non-IPC hearts, but it abolished the IPC- or diazoxide-induced reduction of d[Na⁺]/dt and the [Na⁺], recovery, whereas IPC followed by ouabain treatment showed partial functional recovery with smaller Δ[Na⁺], than other ouabain groups. In conclusion, alteration of Na⁺ kinetics by preserving Na⁺ efflux via Na⁺/K⁺-ATPase mediated by mito K<sub>ATP</sub> channel activation contributes to functional protection in IPC hearts. The contribution of mito K<sub>ATP</sub> channel–independent pathway relating to Na⁺ kinetics including reduced Na⁺ influx is limited in functional protection of IPC. (Circ Res. 2001;88:1176-1182.)

Key Words: ion transport ■ ischemia ■ mitochondria ■ nuclear magnetic resonance ■ reperfusion

We have previously shown that intracellular Na⁺ accumulation during ischemia is the substrate for reperfusion injury, and the recovery kinetics during reperfusion, which is coupled with Ca²⁺ influx, also determines the degree of injury. A phenomenon in which brief episodes of ischemia and reperfusion before a prolonged ischemia reduces postischemic injury has been well recognized as ischemic preconditioning (IP). It has been demonstrated that IPC attenuates Na⁺ accumulation and Ca²⁺ overload during ischemia and reperfusion. The reduced Na⁺-H⁺ exchange (NHE) activity was suggested as a contributor to less Ca²⁺ influx during reperfusion in IPC hearts. However, the role of NHE in the mechanism of IPC has been recently questioned. In contrast, the mitochondrial ATP-sensitive K⁺ (mito K<sub>ATP</sub>) channel has been focused on as a critical mediator of the mechanism; the activation of this channel exerts cardioprotection, and the protective effect of IPC was abolished by the treatment with the mito K<sub>ATP</sub> channel blocker. However, the downstream pathway after the channel activation has not been elucidated. Furthermore, it has been demonstrated that the inhibition of Na⁺/K⁺-ATPase also abolished the infarct size–limiting effect induced by IPC. Although these candidates are considered to modify intracellular Na⁺ kinetics that is critically coupled with the subsequent Ca²⁺ overload, the details of the change in Na⁺ kinetics are not clear.

To elucidate the mechanism for the alteration in intracellular Na⁺ kinetics induced by IPC, we measured intracellular Na⁺ concentration during ischemia and reperfusion in IPC rat hearts. In particular, we characterized the role of NHE and Na⁺/K⁺-ATPase in the Na⁺ kinetics of IPC hearts and the contribution of the mito K<sub>ATP</sub> channel to the mechanism for IPC.

Materials and Methods

Isolated Rat Heart Preparation

The whole-heart preparation was described previously. Briefly, hearts were excised from male Sprague-Dawley rats (body weight, 400 to 450 g; Nihon-Dobutsu, Osaka, Japan) anesthetized with...
pentobarbital sodium (50 mg/kg IP; Abbott Laboratories), and the hearts were heparinized. After excision, the aorta was cannulated for Langendorff perfusion with modified HEPES buffer (in mmol/L) NaCl 108, KCl 5, MgCl 2 1, HEPES 5, CaCl 2 2, sodium acetate 20, 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 it was connected to pressure transducer (SPB-101, San-ei Electric). Coronary flow rate was controlled by a peristaltic pump and was initially adjusted so that coronary pressure equaled 75 to 85 mm Hg, after which the flow rate was kept constant throughout the experiment except during global ischemia. All experiments were performed with the approval of the Animal Care and Use Committee of Osaka University Medical School.

Nuclear Magnetic Resonance Spectroscopy (MRS) Measurements
To measure intracellular Na⁺ concentration ([Na⁺]), we acquired 23Na-MRS spectra obtained on a Bruker AMX-400wb spectrometer; the resonance frequency for 23Na was 105.843 MHz, as described previously.1,15–17 Two hundred fifty-six free induction decays were collected into 1 23Na-spectrum; it took 90 seconds to obtain 1 spectrum. To distinguish intra- and extracellular 23Na-nuclear magnetic resonance signals, the perfusate with the following composition (in mmol/L) was used for 23Na-MRS measurement: NaCl 18, KCl 5, MgCl 2 1, CaCl 2 2, HEPES 5, glucose 10, sodium acetate 20, and dysprosium triethylenetetraminehexaacetic acid [Na 3 Dy(TTHA) · 3NaCl] 15 as a shift reagent (the solution was supplemented with CaCl 2 1.5 to compensate for the binding to Dy(TTHA) 3). The bathing solution contained mannitol 150, HEPES 5, KCl 5, MgCl 2 1, CaCl 2 2, tris(hydroxymethyl)aminomethane 15, (Tris)Dy(TTHA) · 3Tris, at a pH of 7.4. The reference filled in the left ventricular balloon was prepared from dysprosium triopolyphosphate (Na 7 Dy(PPP) 2 · 3NaCl). The areas of intracellular Na⁺ peak in the 23Na-MRS spectra were measured by using planimetry, normalized by the peak for the reference, and corrected with the measured weight of each heart, resulting in the intracellular concentration in units of micromoles per gram wet weight ([Na⁺]).

Experimental Protocols
IPC protocol consisted of 4 cycles of 5 minutes of ischemia separated by 10 minutes of reflow. For 23Na-MRS measurements, the perfusate was switched from a standard one to one containing a shift reagent during global ischemia. Furthermore, 5HD was also administered during the IPC protocol consisting of 4 cycles of 5 minutes of ischemia separated by 10 minutes of reflow. For 23Na-MRS measurements, the perfusate was switched from a standard one to one containing a shift reagent (SPB-101, San-ei Electric). Coronary flow rate was controlled by a peristaltic pump and was initially adjusted so that coronary pressure equaled 75 to 85 mm Hg, after which the flow rate was kept constant throughout the experiment except during global ischemia. All experiments were performed with the approval of the Animal Care and Use Committee of Osaka University Medical School.

Statistical Analysis
Data were presented as mean±SEM. Statistical analysis was performed using ANOVA except comparison between control and IPC hearts, which was performed with unpaired t test. P<0.05 was considered significant.

Results
Alteration of Na⁺ Kinetics in IPC Hearts
Functional recovery was significantly better in IPC hearts (protocol 2 in Figure 1, 55.9±4.2%, n=7) than in control hearts (protocol 1, 15.6±5.4%, n=6, P<0.05). During 27 minutes of ischemia at 37°C, [Na⁺], in control hearts increased to 508.0±10.2% of preischemic level, whereas IPC slightly but significantly attenuated Na⁺ accumulation as shown in Figure 2A (359.3±14.9%, P<0.0001). The degree of Na⁺ accumulation and its relation to functional recovery were equivalent to those in the hearts subjected to 21 minutes of ischemia without IPC (Figure 2 in Reference 1). Figure 2 suggests that the Na⁺ kinetics in IPC hearts is characterized by the delay of Na⁺ accumulation in the early phase of ischemia. Thus, we calculated the initial [Na⁺] increase rate (d[Na⁺]/dt) as the index of the Na⁺ kinetics during early ischemia. As shown in Figure 2B, d[Na⁺]/dt (0.27±0.02
μmol/g wet weight/min), which was calculated from the data during the initial 15 minutes of ischemia, in IPC hearts was significantly less than that in control (0.35±0.01 μmol/g wet weight/min, P<0.05). In contrast, the increment rate of [Na⁺]i during the late phase of ischemia (18 to 27 minutes) was not significantly different between IPC and control (0.21±0.02 versus 0.22±0.01, P=0.62). After 6 minutes of reperfusion, the recovery of [Na⁺]i was not completed in the control hearts, whereas [Na⁺]i in the IPC hearts rapidly recovered to preischemic levels (Figure 2A).

**Contribution of NHE to the Mechanism of IPC**

EIPA (1 μmol/L) significantly decreased d[Na⁺]/dt both in non-IPC and IPC hearts (protocols 3 and 4, Figure 2A), resulting in the amelioration of accumulated Na⁺ during ischemia (non-IPC, 289.0±18.4%, n=5, P<0.01 versus control; IPC, 276.6±22.7%, n=5, P<0.05). EIPA had no additional effect on IPC on the Na⁺ recovery during reperfusion. [Na⁺]i at the end of ischemia was not significantly different between EIPA-treated non-IPC and EIPA-treated IPC hearts (P>0.05). Functional recovery was significantly better in the EIPA-treated hearts than non-treated hearts (P<0.05, Figure 3) even after IPC. These results suggest that the contribution of NHE to the mechanism of IPC is limited.

**Contribution of Mito K_ATP Channel to the Mechanism of IPC**

A potent activator of mito K_ATP channel, diazoxide (30 μmol/L), was administered for 10 minutes before the 27 minutes of ischemia both in non-IPC (protocol 5 in Figure 1) and IPC hearts (protocol 6). Diazoxide delayed the initial increase in [Na⁺]i (0.25±0.02 μmol/g wet weight/min, n=5, P<0.05 versus control; P>0.05 versus IPC in Figure 2B) and attenuated Na⁺ accumulation during ischemia in non-IPC hearts (Figure 2A), as IPC did. After 6 minutes of reperfusion, Na⁺ recovery completely preischemic levels in diazoxide-treated hearts as in IPC hearts. Diazoxide improved functional recovery, which was almost equivalent with that in IPC hearts (P<0.05 versus control, P>0.05 versus IPC, Figure 3). In addition, the administration of diazoxide followed by 10 minutes of washout before ischemia (protocol 7 in Figure 1), which mimicked protocol 2, also induced the protection [DIAZO(E) in Figure 3] with altering [Na⁺]i kinetics (Figure 2B). Thus, mito K_ATP channel activation completely mimicked [Na⁺]i kinetics obtained by IPC. Furthermore, administration of diazoxide had no additional effects to IPC on [Na⁺]i kinetics and functional recovery (P>0.05 versus IPC and diazoxide-treated, Figures 2 and 3).

Next, we confirmed whether the alteration of [Na⁺]i kinetics and protection mentioned above were caused by activation of the mito K_ATP channel. When the mito K_ATP channel was blocked by 5HD (100 μmol/L) in control hearts (protocol 8), it did not change d[Na⁺]/dt (0.39±0.01 μmol/g wet weight/min, n=4, P>0.05 versus control), and Na⁺ remained elevated after 6 minutes of reperfusion (Figure 4). 5HD abolished the protective effect induced by IPC (protocol 9) or diazoxide (protocol 10); functional recovery in IPC or diazoxide-treated hearts simultaneously exposed to 5HD was not significantly different compared with control (P>0.05, Figure 4C). 5HD reversed the delayed increase in [Na⁺]i (n=5, P>0.05 versus control, Figure 4B) obtained by diazoxide treatment, and [Na⁺]i was elevated after reperfusion (Figure 4A). This complement effect was also observed in the early diazoxide-treatment group (protocol 11, Figures 4B and 4C). In contrast, when 5HD was applied through IPC (pro-
The delayed increase in $[\text{Na}^+]_i$ during ischemia (d$[\text{Na}^+]_i$/dt), whereas factors other than $\text{Na}^+/\text{K}^+$-ATPase activity are also required for the functional protection induced by IPC.

**Discussion**

**Intracellular $\text{Na}^+$ Kinetics During Ischemia and Reperfusion in IPC Hearts**

The present results demonstrate that IPC slightly but significantly attenuates the initial $\text{Na}^+$ accumulation during ischemia and completed the $[\text{Na}^+]_i$ recovery after 6 minutes of reperfusion compared with that in non-IPC hearts. In particular, when the recovery process was assessed by the time constant in the regression with $%\Delta[\text{Na}^+]_i=(100-\alpha)\exp(-t/\tau)+\alpha$, the time constants ($\tau$) were not significantly different between control (1.09±0.20 minutes) and IPC hearts (1.30±0.17 minutes, $P>0.05$), but the irreversible accumulation ($\alpha$) was significantly smaller in IPC hearts (1.03±0.80 versus 3.73±3.88 minutes in control, $P<0.05$). This indicates that the number of the irreversibly injured myocytes is significantly reduced in IPC hearts.

It was reported that IPC stimulates $\text{Na}^+$ accumulation during ischemia. Although this was detected by $^{23}\text{Na-MRS}$, the appropriate methods to improve the resolution between intra- and extracellular $\text{Na}^+$ peaks were not applied. In contrast, we carefully measured to assess $[\text{Na}^+]_i$ by $^{23}\text{Na-MRS}$ with a shift reagent. In the present study, the reference, which was adjacent to the heart and simultaneously measured, and the bathing solution to wash out $\text{Na}^+$-containing perfusate.
were applied to compensate weak resolution between intracellular and extracellular signals. These methods improved resolution for reliable quantification of \([Na^+]\). Finally, our results indicate that \(Na^+\) accumulation during ischemia is attenuated in IPC hearts, and it is consistent with the report by Steenbergen et al\(^a\) that applied another shift reagent.

The degree of \(Na^+\) accumulation during the 27 minutes of ischemia and the functional recovery after reperfusion in IPC hearts was equivalent to that in the 21-minute ischemia group (see Figure 2 in Reference 1). This reduction in IPC hearts was characterized by the delay of \(Na^+\) increase during the early phase of ischemia. Thus, we applied the initial \([Na^+]\), increase rate \((d[Na^+]/dt)\) as an index of \(Na^+\) kinetics during ischemia. However, it has been reported that \(Na^+\) efflux activity via \(Na^+/K^+\)-ATPase is decreased by the duration of ischemia,\(^{19,20}\) and \(Na^+\) recovery kinetics during reperfusion after prolonged ischemia (ie, 27 minutes) is not completed.\(^{21}\)

Furthermore, as we have previously shown, the \(Na^+\) kinetics during reperfusion as well as “substrate” \(Na^+\) accumulation during ischemia is an important factor when determining the degree of reperfusion injury.\(^1\) It is reasonable that the complete \([Na^+]\), recovery in IPC hearts also contributes to better functional recovery. Thus, we focused on both \(d[Na^+]/dt\) during the early ischemia and the \([Na^+]\), recovery kinetics after reperfusion as the main indexes of major determinants for functional recovery. \([Na^+]\), kinetics during ischemia and reperfusion is regulated by a balance between \(Na^+\) influx and efflux across the cell membrane. We focused on NHE activity as the \(Na^+\) influx pathway and \(Na^+/K^+\)-ATPase activity as the efflux pathway during ischemia and reperfusion in IPC hearts.

### Contribution of NHE to \(Na^+\) Kinetics in IPC Hearts

NHE activity is critically involved in the mechanism of ischemia/reperfusion injury. NHE activation induces \(Na^+\) accumulation during ischemia by compensating to extrude H\(^+\), resulting in subsequent Ca\(^{2+}\) influx via \(Na^+/Ca^{2+}\) exchange after reperfusion.\(^{1,2,15,22}\) The inhibition of NHE improves functional recovery and prevents the incidence of arrhythmias.\(^{22-24}\) The attenuation of Ca\(^{2+}\) overload has been demonstrated in IPC hearts.\(^6\) Thus, the reduced NHE activity has been proposed as a main mechanism of IPC.\(^6\) The present study demonstrated that the administration of the NHE inhibitor attenuated \(Na^+\) accumulation during ischemia both in non-IPC and in IPC hearts and exerted additive protection on functional protection. Although EIPA reduced \(Na^+\) accumulation during ischemia at almost the same level in non-IPC and IPC hearts, the functional recovery in EIPA-treated IPC hearts was significantly higher than that in EIPA-treated non-IPC hearts. This implies that the contribution of the NHE activity to the mechanism of IPC is very limited, and it strongly supports that the mechanism of NHE inhibitor–induced protection is different from that of IPC.\(^{10,24,25}\) Furthermore, the reduced \(Na^+\) accumulation during ischemia per se is not the sole determinant of functional recovery in IPC hearts.

### Mito \(K\text{ATP}\) Channel Activation Mimics \(Na^+\) Kinetics in IPC Hearts

The contribution of mito \(K\text{ATP}\) channel to the mechanism of IPC has been strongly suggested from the results using \(K\text{ATP}\) channel openers or inhibitors.\(^{11,26}\) Furthermore, in the present results, diazoxide completely mimicked \([Na^+]\), kinetics observed in IPC hearts. Both this similarity and no additive effect in diazoxide-treated IPC hearts strongly support that the mito \(K\text{ATP}\) channel is the central mediator of IPC.\(^{27,28}\)

Mito \(K\text{ATP}\) channel blockade by 5HD abolished the protection induced by either IPC or diazoxide treatment as demonstrated previously.\(^{13}\) 5HD reversed the delay of the \(Na^+\) increase during ischemia induced by diazoxide, and \([Na^+]\), remained elevated after reperfusion. This reversibility by 5HD was also observed in the early treatment of diazoxide (protocols 7 and 11), suggesting that the mito \(K\text{ATP}\) channel is not only a trigger but also an inducer of diazoxide-mediated protection. In contrast to diazoxide, 5HD abolished IPC-induced alteration of \([Na^+]\), kinetics during ischemia and reperfusion as well as functional protection. However, this reversibility was partial for \(Na^+\) recovery after reperfusion. Furthermore, when 5HD was administered after the IPC cycle (protocol 12), no reversibility was observed although functional recovery was abolished. These results indicate that there was the dissociation between \([Na^+]\), kinetics and functional recovery only in the IPC hearts treated with 5HD (see Figure in online data supplement, available at http://www.circresaha.org), suggesting the existence of mito \(K\text{ATP}\) channel-independent pathways to alter \([Na^+]\), kinetics in IPC hearts. Because this mito \(K\text{ATP}\) channel-independent pathway is not the major contributor to IPC-mediated protection as we have indicated in the first part of this study, functional protection was not achieved in 5HD-treated IPC hearts even when \([Na^+]\), kinetics was almost consistent with non-treated IPC hearts.

Mitochondrial dysfunction has been considered one of the mechanisms for reperfusion injury.\(^{29}\) The mito \(K\text{ATP}\) channel modulates mitochondrial function,\(^{30}\) and the activation of the mito \(K\text{ATP}\) channel protects the myocardium against ischemia through maintaining intramitochondrial Ca\(^{2+}\) homeostasis, ie, enhancement of Ca\(^{2+}\) release from and reduction of Ca\(^{2+}\) uptake into mitochondria.\(^{31}\) Thus, preserved mitochondrial function by mito \(K\text{ATP}\) channel activation\(^{28}\) may be necessary to achieve better functional recovery after reperfusion. However, the downstreams after the mito \(K\text{ATP}\) channel activation is still unclear. Especially, it has not been elucidated how the mito \(K\text{ATP}\) channel contributes to the alteration of \([Na^+]\), kinetics.

### Contribution of \(Na^+\)/\(K^+\)-ATPase to \(Na^+\) Kinetics in IPC Hearts

The \(Na^+\)/\(K^+\)-ATPase activity is important for extruding \(Na^+\) accumulated during the initial phase of ischemia and after reperfusion.\(^{32,33}\) Inhibition of \(Na^+\)/\(K^+\)-ATPase reduced the infarct size–limiting effect by IPC,\(^{14}\) suggesting the important role of the ATPase. Functional interaction between the sarcolemmal \(K\text{ATP}\) channel and \(Na^+\)/\(K^+\)-ATPase has been reported.\(^{34,35}\) There is the possibility that the mito \(K\text{ATP}\) channel interacts with \(Na^+\)/\(K^+\)-ATPase because the interac-
tion between actin microfilament and the sarcolemmal K
ATP channel and or mitochondrial K
ATP channel has been demonstrated. Because free radicals can alter the activity of Na
K-ATPase, the alterations activated by the mitochondrial K
ATP channel may enhance the enzyme activity leading to [Na
atom], kinetics in IPC hearts.

The delayed increase in Na
atom during the early ischemia and the complete recovery during reperfusion observed in IPC and diazoxide-treated hearts can be attributed to the prevention of ischemia-induced dysfunction of Na
K-ATPase. Inhibition of Na
K-ATPase abolished the beneficial change in [Na
atom], kinetics induced by IPC and diazoxide-treated hearts. However, the amount of Na
atom accumulation during ischemia and at 6 minutes of reperfusion was significantly smaller in ouabain-treated IPC hearts than in other ouabain-treated hearts. Furthermore, function was not recovered in the ouabain-treated hearts except IPC hearts. This indicates that the prevention of ischemia-induced reduction of Na
atom efflux via Na
K-ATPase resulted from the activation of the mitochondrial K
ATP channel. Preserved Na
K-ATPase activity reflects the delayed [Na
atom], increase during early ischemia and the complete [Na
atom], recovery during reperfusion and contributes to functional protection in IPC hearts. However, the mitochondrial K
ATP channel-independent pathway, including the reduced Na
atom influx, although the contribution may be limited, is required for both the change in Na
atom kinetics and functional protection in IPC hearts.

Mechanism for the Change in Na
atom Kinetics in IPC Hearts

The experiments using 5HD and ouabain suggest the mechanism for the dissociation between Na
atom kinetics and functional protection in IPC hearts followed by 5HD (see Figure in online data supplement, available at http://www.circresaha.org). Although the intracellular Na
atom was almost identical in non-treated IPC hearts and IPC hearts followed by 5HD, these underlying mechanisms may be different. When the prevention of ischemia-induced reduction of Na
atom efflux by 5HD, the reduced Na
atom influx and the other mitochondrial K
ATP channel-independent pathway contribute to the alteration of Na
atom kinetics in IPC hearts followed by 5HD. But the inhibition of Na
K-ATPase suggests that the Na
atom extrusion during reperfusion is mainly mediated in turn by Na
atom-Ca
2+-exchange, leading to Ca
2+ overload and resulting in the lack of functional protection in these groups with 5HD. Thus, even when the Na
atom kinetics is consistent, the different underlying mechanisms for the alteration of Na
atom kinetics lead to the dissociation observed in 5HD-treated hearts.

In conclusion, the alteration of Na
atom kinetics by preserving Na
atom influx via Na
K-ATPase mediated by mitochondrial K
ATP channel activation maintains functional protection in IPC hearts. The contribution of the mitochondrial K
ATP channel-independent pathway relating to Na
atom kinetics, including reduced Na
atom influx, is limited in functional protection of IPC.

Acknowledgments

This work is partly supported by grants from the Ministry of Health and Welfare of Japan (to H.K.) and from the Japan Society for the Promotion of Science (JSPS, to K.I.). K.I. is a research fellow of JSPS. We thank Yasuo Katsuki for the support of nuclear magnetic resonance facilities and Katsuji Hashimoto (Osaka National Hospital) and Shinni Hasegawa (Osaka University) for their suggestions. We also thank Yuka Tamai for laboratory assistance.

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Circ Res. 2001;88:1176-1182; originally published online May 24, 2001;
doi: 10.1161/hh1101.092139

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Figure

A

Initial Increase of $[\text{Na}^+]_{i}$ During Ischemia
($\mu\text{mol/g wet weight/min}$)

B

Remained $[\text{Na}^+]_{i}$ level After Reperfusion
($\mu\text{mol/g wet weight}$)
**Figure.** (A) The relation between the initial increase rate of [Na\(^+\)]\(_i\) during ischemia (d[Na\(^+\)]/dt) and functional recovery after reperfusion. (B) The level of remained [Na\(^+\)]\(_i\) after reperfusion and functional recovery after reperfusion. The remained level of [Na\(^+\)]\(_i\) was determined as the increment of [Na\(^+\)]\(_i\) at 6 min of reperfusion from the pre-ischemic level. Abbreviations indicated were previously defined in print version of the manuscript.