Forum Minireview

Forefront of $\text{Na}^+/\text{Ca}^{2+}$ Exchanger Studies: Role of $\text{Na}^+/\text{Ca}^{2+}$ Exchanger – Lessons From Knockout Mice

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Abstract. We used $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) knockout mice to evaluate the effects of NCX in cardiac function and the infarct size after ischemia/reperfusion injury. The contractile function in NCX KO mice hearts was significantly better than that in wild type (WT) mouse hearts after ischemia/reperfusion and the infarcted size was significantly smaller in NCX KO mice hearts compared with that in WT mice hearts. NCX is critically involved in the development of ischemia/reperfusion-induced myocardial injury, and therefore the inhibition of NCX function may contribute to cardioprotection against ischemia/reperfusion injury.

Keywords: $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), knockout mouse, heart, ischemia/reperfusion injury

Introduction

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) is an important electrogenic transporter in maintaining calcium homeostasis in a variety of mammalian organs (1). NCX catalyzes electrogenic exchange of Na^+ and Ca^{2+} across the plasma membrane in either the Ca^{2+} -efflux (the forward mode) or Ca^{2+} -influx (the reverse mode), depending on the electrochemical gradients of the substrate ions. In the heart, NCX plays an important role in excitation-contraction coupling as the dominant myocardial Ca^{2+} -efflux system (2). On the other hand, the reverse mode of NCX is associated with in cytoplasmic Ca^{2+} levels in cardiomyocytes during digitalis treatment or ischemia/reperfusion (3). It has been reported that NCX inhibitors and NCX antisense oligonucleotides protect the heart from ischemia/reperfusion injury (4, 5). However, two putative NCX inhibitors, KB-R7943 and SEA0400, have been reported to be not specific for NCX (6). Therefore, it remains unclear whether NCX indeed plays a crucial role in mediating Ca^{2+} influx that leads to Ca^{2+} overload and cellular injury after myocardial ischemia, reperfusion injury.

We generated *Ncx1*-deficient mice by gene targeting to determine the in vivo function of the exchanger (7). Homozygous *Ncx1*-deficient mice died between embry-

onic days 9 and 10. Their hearts did not beat, and cardiac myocytes showed apoptosis. No forward mode or reverse mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchange activity was detected in null mutant hearts. The Na^+ -dependent Ca^{2+} exchange activity as well as protein content of NCX1 were decreased by approximately 50% in the heart, kidney, aorta, and smooth muscle cells of the heterozygous mice, and tension development of the aortic ring in Na^+ -free solution was markedly impaired in heterozygous mice. These findings suggest that NCX1 is required for heartbeats and survival of cardiac myocytes in embryos and plays critical roles in Na^+ -dependent Ca^{2+} handling in the heart and aorta.

The functional activity as well as the protein content of NCX in the myocardium of NCX KO mice is approximately half of those of WT mice

Twelve-week-old male heterozygous knockout (KO) mice and wild type (WT) littermates were used. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). Ventricular cells were prepared from adult mouse hearts by standard enzymatic digestion (8). Whole-cell membrane currents were recorded by the patch-clamp method and the current-voltage relationship was obtained by voltage clamp ramp pulses as described previously (9). Under

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these conditions, the Ni^{2+} -sensitive current represents NCX current (10). All data were acquired and analyzed by the pCLAMP (version 5.5; Axon Instrument) software.

Expression levels of dihydropyridine (DHP) receptor (L-type Ca^{2+} channel) and SR Ca^{2+} -ATPase 2 (SERCA2) were analyzed by Western blot as described previously (11). Briefly, tissue was homogenized in lysis buffer containing 25 mM Tris-HCl (pH 7.4), 25 mM NaCl, 0.5 mM EGTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM NaF, 10 nM okadaic acid, 1 mM PMSF, 20 $\mu\text{g}/\text{ml}$ aprotinin, and 20 $\mu\text{g}/\text{ml}$ leupeptin. Protein concentration was determined using a protein assay kit (BioRad, Hercules, CA, USA) and equal amounts of total protein (40 $\mu\text{g}/\text{lane}$) were separated on 8% SDS-polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane (Amersham Life Science, Arlington Heights, IL, USA). Membranes were incubated with anti-mouse dihydropyridine L-type Ca^{2+} channel α -2 subunit monoclonal antibody (Affinity Bioreagents, Inc., Golden, CO, USA) or anti-mouse SERCA2 monoclonal antibody (Affinity Bioreagents) at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody for 1 h. Immunoreactive protein was visualized using an enhanced chemiluminescence detection kit (ECL, Amersham).

We previously reported that the protein content of NCX in NCX KO mouse hearts was approximately 50% of that in WT mouse hearts (12). To elucidate the functional activity, we examined NCX current densities from -40 mV to 40 mV in WT ($n=9$) and NCX KO ventricular cells ($n=6$) (Fig. 1). The densities of the reverse mode of NCX at 40 mV in ventricular cells of KO mice (0.57 ± 0.07 pA/pF) were approximately half (55.4%) compared with those of WT mice (1.04 ± 0.14 pA/pF). These results suggest that the functional

activity as well as the protein content of NCX in the myocardium of NCX KO mice is approximately half of those of WT mice.

Western blot analysis revealed that there was no difference in the protein levels of L-type Ca^{2+} channel and SERCA2 between the two groups (data not shown).

The infarct size was significantly smaller in KO hearts than in WT hearts

Hearts were excised from mice and connected to the perfusion canula via the aorta as described previously (8). Retrograde perfusion was maintained with Krebs-Henseleit solution. To evaluate the contractile function, a polyethylene film balloon was inserted into the cavity of the left ventricle through the left atrium. The balloon was filled with saline to adjust the baseline end-diastolic pressure to 5–10 mmHg. Hearts were subjected to no-flow, global ischemia by clamping the perfusion line. After 30 min of ischemia, the clamp was released and the hearts were reperfused for 120 min. Left ventricular developed pressure (LVDP) was designated as difference between systolic and diastolic pressures of the left ventricle. After 120 min, the heart was incubated for 5 min at 37°C in a 1% solution of triphenyl-tetrazolium chloride (TTC). The sizes of the infarcted area and viable ischemic-reperfused area were measured by computed planimetry (Scion Image 1.62; Scion Corporation, Frederick, MD, USA). Infarct size was calculated as described previously (13).

There were no significant differences in the basal hemodynamic parameters, including heart rate, left ventricular pressure, end-diastolic pressure, and positive and negative dP/dt , between WT and KO mice (Table 1). After ischemia, there was no significant difference between the two groups in several parameters such as time to no beating, time to contracture, and left

Fig. 1. NCX current densities. The densities of the reverse mode of NCX at 40 mV in ventricular myocytes isolated from WT ($n=9$) and NCX KO mice hearts ($n=6$). Values are expressed as the mean $\pm$ S.E.M. * $P<0.05$ vs WT mice.

Table 1. Hemodynamic parameters of NCX KO mice

	WT (n = 6)	NCX KO (n = 7)
HR (bpm)	356 ± 40	378 ± 77
LVP (mmHg)	142.8 ± 40	146.3 ± 34.5
EDP (mmHg)	4.4 ± 1.5	4.3 ± 1.3
dP/dt (mmHg/s)	7368 ± 630	7845 ± 2582
$-\text{dP}/\text{dt}$ (mmHg/s)	5204 ± 782	5539 ± 1157
Time to no beating (min)	2.2 ± 0.9	2.2 ± 1.6
Time to contracture (min)	6.2 ± 1.7	6.3 ± 2.0
EDP at 25 min (mmHg)	67.3 ± 9.2	63.8 ± 10.8

HR, heart rate; LVP, left ventricular pressure; EDP, LV end-diastolic pressure; dP/dt and $-\text{dP}/\text{dt}$, positive and negative first derivatives for maximal rates of LV pressure development, respectively.

Fig. 2. Ex vivo studies. Changes in LVP during ischemia/reperfusion. Representative LVP records of WT and NCX KO mouse hearts are shown. Note that KO mouse hearts started to contract earlier than WT mouse hearts after reperfusion.

Fig. 3. LVDP of hearts of NCX KO mice ($n=7$) and WT mice ($n=6$) hearts after reperfusion. Values are expressed as the mean $\pm$ S.E.M. * $P<0.05$ vs WT mice.

ventricular end-diastolic pressure (Fig. 2). After reperfusion, however, hearts of KO mice started to beat earlier than those of WT mice (Fig. 2). At 120 min after reperfusion, contractile function (left ventricular developed pressure) of KO mouse hearts was significantly better ($51.7 \pm 12.7\%$ of preischemic value) than that of WT mouse hearts ($26.3 \pm 6.9\%$, $P<0.05$) (Fig. 3). After ischemia/reperfusion, there was much more viable myocardium in KO hearts than WT hearts (red lesion (printed in black) in Fig. 4A). The infarcted size was significantly smaller in KO hearts ($32 \pm 9\%$) than in WT hearts ($68 \pm 10\%$, $P<0.05$) (white lesion in Fig. 4A and Fig. 4B).

Concluding remarks

Myocardial cell injury is induced by a combination of mechanical and chemical stresses during ischemia

Fig. 4. Infarct size. A: Representative TTC staining photographs of WT and NCX KO mice hearts after ischemia/reperfusion are shown. Infarct area is expressed as a white lesion and viable myocardium is expressed as a red lesion (printed in black). Bar = 2 mm. B: Myocardial infarct size is expressed as percentage for the total heart of WT mice ($n=6$) and NCX KO mice ($n=7$). Values are expressed as the mean $\pm$ S.E.M. * $P<0.05$ vs WT mice.

(14). Reoxygenation after extended periods of ischemia rapidly induce hypercontracture of cardiomyocytes (15) and aggravate the preexisting injury (16). The hypercontracture represents a major cause of acute lethal cell injury in the reperfused myocardium (17, 18). It has been hypothesized that an increase in intracellular Ca^{2+} levels of cardiomyocytes through NCX induces the hypercontracture state after reperfusion but not during ischemia by the mechanism described below (5). During myocardial ischemia, anaerobic metabolism induces acidosis both inside and outside of cardiomyocytes. The Na^+/H^+ exchanger does not operate at this moment because of no difference in H^+ concentration across the plasma membrane of cardiomyocytes. Reperfusion restores extracellular acidosis, leading to a disparity in H^+ concentration between the inside and outside of cardiomyocytes. The increase in intracellular H^+ concentration activates the Na^+/H^+ exchanger, and the elevated intracellular Na^+ concentration triggers a rise in intracellular Ca^{2+} by the reverse mode of NCX (5). The excessive Ca^{2+} overload induces the catastrophic hypercontracture of cardiomyocytes. In fact, it has been reported that reduction of Ca^{2+} concentration protects cardiomyocytes against hypercontracture evoked by reoxygenation (19). In contrast, overexpression of NCX

increased ischemia/reperfusion injury in mice (20). Pharmacological inhibition of reverse mode of NCX protected reperfusion injury in cardiomyocytes (19). These results suggest that NCX is critically involved in the myocardial ischemia/reperfusion injury; however, NCX inhibitors have been recently reported to be not specific to NCX (6). Two putative NCX inhibitors, KB-R7943 and SEA0400, depressed the Ca^{2+} transients even in cardiomyocytes of NCX null mice (7). Although these NCX inhibitors have been reported to suppress the reverse mode but not the forward mode of NCX, the administration of high dose of these inhibitors increased infarct size possibly by inhibiting the forward mode of NCX (21). We here demonstrated an important role of NCX in myocardial ischemia/reperfusion injury by using NCX KO mice. The reverse mode of NCX current in KO mice was decreased to half that of WT mice. Loss of function of NCX is assumed to result in alleviation of Ca^{2+} overload, hypercontracture, and cell death after reperfusion. Our present study clearly indicates that the inhibition of NCX contributes to cardioprotection against myocardial ischemia/reperfusion injury and suggests that specific inhibitors of the reverse mode of NCX may be useful to prevent the myocardial ischemia/reperfusion injury.

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