The Na\(^+\)/Ca\(^{2+}\) exchanger in cardiac ischemia/reperfusion injury

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Source of support: This paper was supported by the National Natural Science Foundation of China (no. 30971065), Liaoning Province Department of Education Fund (no. 2009A194) and Science and technology project of Dalian (no. 2012E12SF074)

Summary

The Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) is an important electrogenic transporter in maintaining Na\(^+\) and Ca\(^{2+}\) homeostasis in a variety of mammalian organs, and is involved in the physiological and pathophysiological regulation of Ca\(^{2+}\) concentration in the myocardium. It can affect cardiac structure, electrophysiology and contractile properties. The role of the NCX in heart cells following ischemia/reperfusion (IR) has been investigated using a number of in vitro and in vivo models. During ischemia, ionic disturbances favor Ca\(^{2+}\)-influx mode activity as excess Na\(^+\) is extruded in exchange for Ca\(^{2+}\), giving rise to increased intracellular Ca\(^{2+}\) levels (Ca\(_i\)). This rise in Ca\(_i\) contributes to reversible cellular dysfunction upon reperfusion, such as myocardial necrosis, arrhythmia, systolic dysfunction and heart failure. We have reviewed the major in vivo and in vitro cardiac IR-related NCX studies in an attempt to clarify the functions of NCX in IR and conclude that recent studies suggest blockage of NCX has potential therapeutic applications. Although the use of different IR models, application of NCX stimulators and inhibitors, and development of NCX transgenic animals do help elucidate the role of this ion exchanger in heart cells, related mechanisms are not completely understood and clinically effective specific NCX inhibitors need further research.

key words: Na\(^+\)/Ca\(^{2+}\) exchanger • ischemia • reperfusion • KB-R7943
**Background**

The Na⁺/Ca²⁺ exchanger (NCX) is involved in the physiological and pathophysiological regulation of Ca²⁺ concentration in the myocardium. It is considered to function both in the forward (Ca²⁺ extrusion) and reverse (Ca²⁺ influx) modes [1]. Ischemia/reperfusion (IR) injury is the tissue damage caused when blood supply returns to the tissue after a period of ischemia. The absence of oxygen and nutrients from blood during the ischemic period creates a condition in which the restoration of circulation results in inflammatory and oxidative damage through the induction of oxidative stress rather than restoration of normal function. That means the progressive and irreversible damage incurred during myocardial ischemia can only be stopped by an immediate reperfusion. Otherwise, severe and irreversible myocardium damage during the ischemia phase could be caused by reperfusion injury [2,3]. NCX has a key role in the Ca²⁺ flux balance of cardiac myocytes, as it is the major Ca²⁺ efflux pathway to remove Ca²⁺ entry occurring during excitation-contraction coupling [4,5]; whereas during IR, NCX functions reverse ly to induce Ca²⁺ influx, which strengthens Ca²⁺ overload. Ca²⁺ overload is mainly involved in cell structural damage, arrhythmia and systolic dysfunction. Thus inhibition of NCX has become a new way to prevent IR injury.

**NCX and Its Regulation**

NCX is an antiporter membrane protein, which helps to maintain Ca²⁺ homeostasis in a wide variety of cell types. They are found in both the plasma membrane and intracellular organelle membranes. NCX consists of a transmembrane part and a large intracellular loop. The activation of the NCX transport function requires the binding of Ca²⁺ to 2 tandem C2 domains – Ca²⁺ binding domain 1 (CBD1) and CBD2 – which are an integral part of the exchanger’s intracellular loop [6]. Three mammalian isoforms have been cloned to date (NCX1-3), which consist of 920–970 amino acid residues that are predicted to possess 11 or 12 transmembrane (TM) domains. Interestingly, they possess a short motif (about 30 residues) that is similar to the Na⁺/K⁺-ATPase, although its function is unknown [7]. And residues 248–252 and 300–304 of the cardiac NCX are involved in its regulation by phosphorylation [10]. NCX1 has been found to be predominantly expressed in the heart, where it plays an important role in excitation-contraction coupling, but it is also abundant in a variety of other tissues. Recently, Song J et al. [11] have suggested that NCX1 may possess a more essential role in protecting the heart. They found that constitutive overexpression of phosphorylman S 68 E mutant, which inhibits NCX1 but not Na⁺/Ca²⁺ ATPase, resulted in arrhythmias, early mortality and heart failure, possibly because of involvement of NCX1. NCX2 and NCX3 transcripts have been detected in the brain and skeletal muscle. In addition, NCLX turned out to be the long-sought mitochondrial NCX in a recent study [12].

NCX mediates the counter transport of 3 or 4 Na⁺ ions for 1 Ca²⁺ ion across the membrane. It is a bidirectional transport process, capable of moving Ca²⁺ in either direction across the sarcolemma, depending on the membrane potential and the transmembrane gradients of Na⁺ and Ca²⁺, as described by the following equation:

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\text{Equilibrium potential} = \left(\frac{RT}{F}\right) \ln \left(\frac{[Ca]_i}{[Ca]_o}\right) \cdot \frac{Na}{Na_0}^{3} \]

where the equilibrium potential (or reversal potential) is the membrane potential at which NCX is at equilibrium.

Using the intracellular concentrations of Na⁺ and Ca²⁺ found in murine myocytes (Na⁺=15 mmol/L; Ca²⁺=0.13 mmol/L) and the extracellular concentrations of Na⁺ and Ca²⁺ used in a type of buffer solution (Na⁺=145 mmol/L; Ca²⁺=1.75 mmol/L), the equilibrium potential was calculated to be ~73 mV. At membrane potentials more negative than the equilibrium potential, NCX will operate in the direction of Ca²⁺ efflux. Conversely, at membrane potentials more positive than the equilibrium potential, at resting Ca⁺ concentrations, NCX will operate in the direction of Ca²⁺ influx. As the resting potential of the cell is ~80 mV, NCX will operate normally in the Ca²⁺ efflux mode, and it has been demonstrated that Na⁺/Ca²⁺ exchange is the major Ca²⁺ efflux mechanism in the heart [13].

The expression and activities of NCX can be regulated by a variety of factors, among which, membrane potential, as well as Na⁺ and Ca²⁺ gradients, are crucial. It can be activated by Li⁺, K⁺, and inhibited by Ni²⁺, Cd²⁺, Mn²⁺, Mg²⁺. In addition, phorbol ester and endothelin-1 alter functional expression of NCX in myocytes [15]. 2-[4-{(2,5-difluorophenyl) methoxy} phenyl]-5-ethoxyaniline(SEA0400) and 2-[2-{4-(4-nitrobenzoyl)phenyl}ethyl]sulfthioiuca (KB-R7943) are at present commonly utilized to inhibit NCX experimentally. In fact, the exchange inhibitory peptide (XIP; RRLLFYKYKRYRAGKQRG) is the most strongly selective inhibitor of NCX, but it does not appear to permeate through the cell membranes. KB-R7943 is synthesized as a potent inhibitor of NCX; however, the results of previous studies do not support the selectivity of KB-R7943 for NCX [16]. SEA0400 is a highly potent and selective inhibitor of NCX. SEA0400, in the concentration range that inhibits NCX, exhibits negligible affinities for the Ca²⁺ channels, Na⁺ channels, K⁺ channels, noradrenaline transporter and 14 receptors; and it does not affect the activities of the store-operated Ca²⁺ channel, Na⁺/H⁺ exchanger (NHE), and several enzymes including Na⁺/K⁺-ATPase and Ca²⁺-ATPase [17]. Since NCX plays a key role in IR injury and no inhibitor of NCX is clinically used, SEA0400 warrants further study.

It has long been postulated that NCX was phosphorylated by the cAMP-dependent protein kinase A (PKA) in vitro, which increased its activity, but this finding has always been controversial and no phosphorylation site has so far been identified. Wanichawan et al. [18] recently confirmed that NCX1 was not a direct target for PKA phosphorylation by using bioinformatic analysis and peptide arrays. Since NCX1 is widely expressed in the heart, studies of this subject are in fully underway.

**NCX Participates in IR**

Basically, during ischemia, anaerobic glycolysis and ATP degradation produce H⁺ that activate the NHE, causing Na⁺ influx. Na⁺ efflux is attenuated because the Na⁺/K⁺-ATPase is inhibited during ischemia; therefore, Na⁺/H⁺ exchange activity leads to increased Nai. The increase in Na⁺ during ischemia is accompanied by an increase in Cai through reverse-mode NCX, and this is high Ca²⁺ that is thought to
cause myocardial injury [19]. Recent studies conducted by Gershome et al. [20] proposed that it was the overlapping expression and distribution of voltage-gated Na⁺(NaV1) channel isoforms and the NCX that allowed a Na⁺ current to cause an elevation in the Na⁺ concentration sufficiently large to bring Ca²⁺ into the myocyte through reverse-mode NCX.

As mentioned before, the function of NCX is strongly influenced by Cai, Nai, and membrane potential. Actually, it is local submembrane Cai (Casm) rather than average Cai that is sensed by NCX. Thus submembrane space here is not anatomically defined, but is functionally defined by average NCX behavior [19]. This may suggest that when IR is applied, Casm determines how NCX functions more accurately than Cai.

Recently, Salas et al. [21] suggested that NCX mediated the increase in Ca²⁺-calmodulin-dependent protein kinase II (CaMKII) activity at the onset of reperfusion. The onset of reperfusion increased the phosphorylation of Thr²⁷ site of phospholamban (PLN), without changes in total protein, consistent with an increase in CaMKII activity. They showed that inhibition of NCX by KB-R7943 produced a significant decrease in the phosphorylation of Thr²⁷ of PLN. In contrast, inhibition of L-type Ca²⁺ channels with nifedipine failed to affect this phosphorylation. Moreover, NCX inhibition also decreased infarct size, lactate dehydrogenase (LDH) release, and apoptosis produced by IR injury. Taken together, these experiments indicate that the reverse NCX mode is a major pathway of Ca²⁺ influx upon reperfusion able to activate CaMKII.

Although ischemia and reperfusion are often referred to simultaneously, NCX functions differently during ischemia and reperfusion. Nai rises during ischemia, as shown in several previously published studies [22,23]. Because of the marked decrease in pH during ischemia, it has generally been assumed that NCX becomes inhibited. Although NCX activity is reduced at low pH, it is not totally inhibited, particularly under conditions of elevated Na⁺. The increase in Ca²⁺ during ischemia has been shown to be modulated by Na⁺, suggesting that NCX operates during ischemia. During ischemia, Kenichi Imahashi et al. [24] observed a slower decline in ATP, a slower rate of ischemic contracture, and less of a rise in Nai in cardiac-specific ablation of NCX-KO hearts. In conclusion, these data suggest increased Ca²⁺ entry via NCX during ischemia in wild-type (WT) hearts. The lack of Ca²⁺ entry via the reverse-mode NCX would decrease the detrimental effects of Ca²⁺ overload and better preserve ATP, which would prolong the activity of the Na⁺/K-ATPase [25], leading to less Na⁺ accumulation during ischemia in NCX-KO hearts. On reperfusion, the better-preserved ATP in the NCX-KO hearts facilitates Na⁺ extrusion by Na⁺/K-ATPase.

**NCX and IR-induced Myocardial Necrosis, Arrhythmia, Contractile Dysfunction and Heart Failure**

During ischemia and reperfusion, with an increase in Nai and a depolarized membrane potential, Ca²⁺ enters the myocyte in exchange for intracellular Na⁺ via reverse-mode NCX. Kenichi Imahashi et al. [24] studied mice with cardiac-specific ablation of NCX-KO and demonstrated that reverse-mode Ca²⁺ influx was absent in the NCX-KO myocytes and that IR injuries were diminished. They found that hearts lacking NCX exhibited less of a decline in ATP during ischemia, delayed ischemic contracture, and reduced maximum contracture. Furthermore, on reperfusion following ischemia, NCX-KO hearts had much less necrosis, better recovery of left-ventricular developed pressure, improved phosphocreatine recovery, and reduced Na⁺ overload. The improved recovery of function following ischemia in NCX-KO hearts was not attributable to the reduced preischemic contractility in NCX-KO hearts, because when the preischemic workload was matched by treatment with isoproterenol, NCX-KO hearts still exhibited improved postischemic function compared with WT hearts. All these results proved that NCX was associated with myocardial necrosis and contractile dysfunction during IR. Matsumot et al. [26] also demonstrated that NCX was responsible for myocardial necrosis. They induced ischemia in isolated rabbit hearts and determined injury severity through infarct size. KB-R7943 infusion similarly reduced infarct size both when infused before ischemia and when infused for only 5 min after reperfusion. Protein levels of NCX after 30-min ischemia and 30-min ischemia/30-min reperfusion were similar to baseline values in both untreated controls and hearts treated with 0.5 microM KB-R7943 upon reperfusion.

Generally speaking, 25% of ischemic and 75% of reperfusion arrhythmia is induced by non-reentrant mechanisms such as delayed afterdepolarizations. The NCX current (INcx) is inward for Ca²⁺ removal (forward mode) and outward for Ca²⁺ influx (reverse mode). INcx can be arrhythmogenic because the inward current during Ca²⁺ release from the sarcoplasmic reticulum (SR) can give rise to delayed afterdepolarizations when SR Ca²⁺ release occurs outside the normal excitation-contraction coupling cycle [27]. Outward INcx can by itself contribute to Ca²⁺ loading when Nai is increased, leading to arrhythmia. Indeed, in conditions of increased Ca²⁺ influx via reverse mode, such as block of the Na⁺/K pump, KB-R7943 and SEA0400 reduced arrhythmias [28]. Milberg et al. [29] proved that acute inhibition of NCX reduced proarrhythmia in an experimental model of chronic heart failure. They concluded that inhibition of NCX: 1) reduced monophasic ventricular action potential duration, 2) decreased dispersion of repolarization, and 3) suppressed early afterdepolarizations and ventricular tachyarhythmias. This may also indicate that NCX is involved in IR-induced heart failure. Takahashi et al. [30] found that a high dose of SEA0400 could reduce the incidence of ventricular fibrillation to 30% and mortality to 20%.

Heart failure is a dangerous complication of IR. Myocardial hypertrophy, as well as left ventricular (LV) fibrosis and stiffening, play crucial roles in the development of heart failure with preserved ejection fraction (HFPEF). It has already been suggested that hypertrophied myocardial dysfunction may be attributed to changes in intracellular calcium cycling [31]. Studies of Kamimura et al. [32] suggested that digitalis-like factors and the subsequently activated NCX entry mode may play an important role in the development of hypertensive HFPEF. They created a heart failure model using Sprague-Dawley rat hearts. They found that SEA0400 suppressed the enhancement of H-proline incorporation in cardiac fibroblast and that improved the survival rate in association with the attenuation of LV fibrosis and stiffening in the HFPEF model.
CONDITIONS RELATED TO NCX DURING IR

Several conditions could enhance or mitigate IR injuries by having an effect on NCX. Chen et al. [33] found that intermittent hypoxia protected cardiomyocytes against IR injury-induced alterations in Ca\(^{2+}\) homeostasis and contraction via the SR and NCX mechanisms. Through experiments on Sprague-Dawley rats, they found that the decay rate of caffeine-induced Ca\(^{2+}\) transients, which mainly reflects NCX activity, increased significantly at 20-min reperfusion and 30-min reperfusion in myocytes from the normoxia group, and that intermittent high-altitude (IHA) hypoxia totally preserved these changes without affecting T (time constants), represents a decay of Ca\(^{2+}\) transients. Then they examined I\(_{\text{Na}}/\text{Ca}\) and found that ischemia at 20 min in normoxic myocytes significantly decreased both the inward- and outward-directed I\(_{\text{Na}}/\text{Ca}\) and shifted the apparent reversal potential to a positive direction. They also found that IHA hypoxia totally preserved these changes with similar apparent reversal potential. Moreover, NCX content was not altered by IR in both groups. These data indicate that IHA hypoxia relieves the ischemia-induced depression of NCX activity. Cross et al. [15] concluded that exacerbation of IR injury by over-expression of the NCX can be partially overcome by female-specific hormones such as estrogen. They used the hearts of male and female transgenic mice that over-express the NCX protein, and hearts of their WT littermates in order to test the hypothesis. Generally, posts ischemic function was lower in male transgenic than in male WT hearts, but there was no difference between female transgenic and female WT hearts. However, the functional recoveries of ovariectomized female transgenic hearts were lower than those of WT and sham-operated transgenic females. They presumed this specific female-protection could possibly be estrogen.

Recently, Barry et al. [34] demonstrated that non-anticoagulant heparin reduced myocyte Na\(^{+}\) and Ca\(^{2+}\) loading during simulated ischemia and decreased reperfusion injury, not only by anti-inflammatory activities as previously proposed, but also via NCX. They suggested that an agent such as heparin, desulfated at the 2-O and 3-O positions (ODSH), decreased myocyte Ca\(^{2+}\) influx via reverse-mode NCX early during reperfusion and increases Ca\(^{2+}\) extrusion via forward NCX later after reperfusion, and would be expected to provide substantial protection from reperfusion injury.

Nevertheless, several conditions contribute to IR injury, among which, reactive oxygen species (ROS) was recently proposed. Activity of NCX1 in calcium-influx mode contributes to the pathological intracellular calcium overload during cardiac IR injury. ROS also gives rise to myocardial dysfunction in IR and are reported to alter NCX1 activity. DiPolo et al. [35] have already demonstrated that oxidative stress inhibits the NCX by impairing the Ca\(^{2+}\)-regulatory site in dialyzed squid axons. Soliman et al. [36] used the patch-clamp technique to study the effects of the ROS, H\(_{2}\)O\(_2\) on recombiant NCX1 splice variants. H\(_{2}\)O\(_2\) irreversibly increased calcium-influx mode activity in the cardiac NCX1.1 splice variant, without affecting calcium-efflux mode activity. Since KB-R7943 was almost 7-fold less potent at inhibiting NCX1 activity after H\(_{2}\)O\(_2\) modification, they also emphasized that the potency of NCX1 inhibitors may be impaired under conditions of oxidative stress. Ions besides Na\(^{+}\) and Ca\(^{2+}\) participate in regulation of NCX as well. The studies of Feng Wu et al. [37] showed that low K\(^{+}\) increased Ca\(^{2+}\) oscillation and injury by activating the reverse-mode NCX and inhibiting the Na\(^{+}/K\(^{+}\)ATPase in rat cardiomyocytes. Compared to controls, myocytes reperfused with low K\(^{+}\) had greater number of calcium oscillations and reverse-mode NCX activity, which were accompanied with decreased cell length recovery and cell viability. Reperfusion with KB-R7943 attenuated the effects of low K\(^{+}\) on all the parameters.

Although NCX is responsible for IR injury, enhanced NCX expression does not necessarily increase myocardial vulnerability to IR injury, which was proposed through experiments on rabbit hearts by Tomoaki Matsumoto et al. [38]. Using separate groups of hearts, myocardial infarction was induced by 30-min global ischemia/2-h reperfusion with or without treatment with 0.3 µM KB-R7943. Heart weight-to-body weight ratio was larger and NCX protein level was much higher in the post-MI group than in the sham group. But there were no significant differences between severities of myocardial stunning after the repetitive IR and between infarct sizes after 30-min ischemia in both groups. This is interesting, since NCX increased in expression upon survival of infarction. Later, experiments conducted by Li et al. [39] clearly explained the mechanisms. They found that NCX activity significantly decreased during simulated reperfusion after severe metabolic inhibition (index ischemia) in myocytes subjected to metabolic inhibition preconditioning. This inhibitory effect on NCX activity correlated with the enhancing effect of metabolic inhibition preconditioning on cell viability following ischemic insult.

CONCLUSIONS

NCX plays an important role in regulating Ca\(^{2+}\) homeostasis, and it also contributes to Ca\(^{2+}\) overload, leading to a series of IR injuries. Blocking of NCX has become a new target to treat IR injury. The function of NCX has been well-explained in recent years, but its mechanism needs further elucidation. For instance, estrogen possesses a specific protection from IR injury, but how does it work? By itself directly or by activating other substances indirectly? Inhibition of NCX has therapeutic applications and that there is increasing demand by both researchers and clinicians for new NCX inhibitors with significantly enhanced selectivity and functionality. Unfortunately, to date there is no clinical evidence of an ideal drug for use in inhibiting NCX. Azimilide inhibits NCX at supratherapeutic concentrations [40]. Recently, Rapamycin was proposed to confer preconditioning-like protection against ischemic-reperfusion injury in isolated mouse heart cultures, probably by stimulating NCX to extrude Ca(2+) outside the cardiomyocytes, but its mechanism is not yet fully understood [41]. These are issues that need to be addressed in future research projects.

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