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Original Article

The electrogenic cardiac sodium bicarbonate co-transporter (NBCe1) contributes to the reperfusion injury

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ABSTRACT

Background: Although the participation of the electrogenic sodium/bicarbonate cotransporter (NBCe1) in the recovery from an intracellular acid load is recognized, its role in ischemia–reperfusion is still unclear.
Methods and results: Our objective was to assess the role of NBCe1 in reperfusion injury. We use selective functional antibodies against extracellular loop 3 (a-L3) and loop 4 (a-L4) of NBCe1. a-L3 inhibits and a-L4 stimulates NBCe1 activity. Isolated rat hearts were submitted to 40 min of coronary occlusion and 1 h of reperfusion. a-L3, a-L4 or S0859 — selective Na^+-HCO_3^- co-transport inhibitor — were administered during the initial 10 min of reperfusion. The infarct size (IS) was measured by triphenyltetrazolium chloride staining technique. Postischemic systolic and diastolic functions were also assessed. a-L3 and S0859 treatments decreased significantly ($P<.05$) the IS (16±3% for a-L3 vs. 32±5% in hearts treated with control nonimmune serum and 19±3% for S0859 vs. 39±2% in untreated hearts). Myocardial function during reperfusion improved after a-L3 treatment, but it was not modified by S0859. The infusion of a-L4 did not modify neither the IS nor myocardial function.
Conclusions: The NBCe1 hyperactivity during reperfusion leads to Na^+ and Ca^{2+} loading, conducing to Ca^{2+} overload and myocardial damage. Consistently, we have shown herein that the selective NBCe1 blockade with a-L3 exerted cardioprotection. This beneficial action strongly suggests that NBCe1 could be a potential target for the treatment of coronary disease.

1. Introduction

The regulation of the intracellular H^+ homeostasis is dependent on the activity of Na^+/H^+ exchanger (NHE) and two HCO_3^--dependent systems, Na^+-HCO_3^- co-transport (NBC), and Na^+-independent Cl^-/HCO_3^- exchange (AE) [1–6]. The major transporters responsible for acid extrusion are NHE and NBC, which transport H^+ out and HCO_3^- into the cell, respectively. In medium with bicarbonate, like the blood, both mechanisms, NHE and NBC, are equally operative at an intracellular pH (pH_i) close to basal [7,8].

The metabolic changes occurring during ischemia lead to an intracellular acidosis. This increased generation of H^+ activates NHE and NBC, resulting in an increase in intracellular Na^+. This rise in intracellular Na^+, coupled with the depolarized plasma membrane, results in a reversal of Na^+-Ca^{2+} exchanger (NCX) to bring Ca^{2+} into the myocyte. In addition to NCX, Ca^{2+} entry via the L-type Ca^{2+} channel can contribute to the rise in Ca^{2+} during ischemia. During ischemia, intra- and extracellular pH are acidic; during reperfusion, extracellular pH rapidly returns to normal. However, at the beginning of reperfusion pH_i is still acidic, and this pH gradient facilitates extrusion of H^+ via NHE and entry of HCO_3^- via NBC. Previous experiments performed in isolated hearts showed that HCO_3^--dependent processes are responsible for between 30 and 40% of pH recovery during reperfusion [9,10].

Calcium overload secondary to NHE and NBC activation is a factor of injury suggesting that both transporters are implicated in the damage produced by ischemia–reperfusion [11,12].

Reperfusion is associated with a burst of reactive oxygen species (ROS) production [13]. Accumulation of ROS leads to cardiac Ca^{2+} overload, and it has been suggested that redox modulation of ion channels, transporters, and pump activity is directly responsible for postischemic damage [14].

Overall, it is thought that the combined effects of ROS and Ca^{2+} overload play a critical role in the transition from reversible to irreversible reperfusion injury and that mitochondria are the major target of these agents. In particular, they lead to the opening of the mitochondrial permeability transition pore that is now widely accepted to play a critical role in reperfusion injury [15].

The involvement of NHE on the postischemic alterations of myocardium has been previously shown by us in isolated heart model [16,17] and by other laboratories using cardiomyocytes, fibroblasts, in vivo infarct model, and humans [18–20]. However, there is scarce information about the role played by NBC in ischemia and reperfusion, a

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fact that can be due to the lack of specific NBC blockers. The availability of a specific inhibitor of NBC, named S0859 [21] and polyclonal antibodies opened new valuable paths to dissect the participation of NBC in different pathological situations. In this regard, a previous work [12] showed an improvement of cardiac function when rat hearts were pretreated with an antibody directed against the human cardiac NBC isoform NBCe1. Recently, in our laboratory, functional antibodies against extracellular loops 3 (a-L3) and 4 (a-L4) of NBCe1 were synthesized [22]. In this study, pH_i was measured in single myocytes by an *epi*-fluorescence system. To investigate the NBCe1 activity, a potassium pulse (increase in extracellular potassium concentration) was performed, and the pH_i changes were registered. Interestingly, we detected that the increase of pH_i produced by the potassium pulse was abolished by a-L3 and was higher when a-L4 was added. Thus, we showed that these antibodies exerted opposite actions on NBCe1 activity: a-L3 inhibited and a-L4 stimulated NBCe1 function.

Therefore, the aim of the present study was to employ these selective tools to assess the contribution of NBCe1 to alterations subsequent to coronary occlusion and reperfusion in isolated rat hearts.

2. Methods

All procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and to the guidelines laid down by the Animal Welfare Committee of La Plata School of Medicine.

2.1. Isolated heart preparation

Wistar rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60-mg/kg body wt). The heart was rapidly excised and perfused by the nonrecirculating Langendorff technique with Ringer's solution containing (in mmol/L): 118 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.35 $CaCl_2$, 20 $NaCO_3H$, and 11.1 dextrose. The buffer was saturated with a mixture of 95% O_2–5% CO_2, had a pH 7.4, and maintained at 37°C. The conductive tissue in the atrial septum was damaged with a fine needle to achieve atrioventricular block, and the right ventricle was paced at 280±10 beats/min. A latex balloon tied to the end of a polyethylene tube was passed into the left ventricle through the mitral valve; the opposite end of the tube was then connected to a Statham P23XL pressure transducer. The balloon was filled with water to provide an end-diastolic pressure [left ventricular end diastolic pressure (LVEDP)] of 8–12 mmHg, and this volume was unchanged for the rest of the experiment. Coronary perfusion pressure was monitored at the point of cannulation of the aorta and adjusted to approximately 60–70 mmHg. Coronary flow, controlled with a peristaltic pump, was 11±2 ml/min. Left ventricular pressure (LVP) was acquired by using an analog-to-digital converter and acquisition software (Chart V4.2.3 ADInstruments).

2.2. Experimental protocols

Myocardial infarction was induced after 20 min of stabilization by occluding the left anterior descending (LAD) coronary artery during 40 min followed by a 60-min reperfusion. The coronary artery, 3–4 mm from its origin, was encircled by a 6–0 polypropylene suture attached to a small curved needle, and the two ends of the suture were threaded through a length of plastic tubing, forming a snare, which could be tightened.

Four experimental protocols were performed:

(1) : Nonischemic control (n=4): Hearts were perfused for 120 min without any treatment
(2) : Ischemic control (IC, n=10): Hearts were reperfused with the preischemic solution.
(3) : Nonimmune serum (S, n=4): To examine the effects of the serum, this solution in a dose of 10 μM was administered during the first 10 min of reperfusion.
(4) : a-L3 (n=6): To examine the effects of the extracellular loop domain 3 (L3) of NBCe1, 10 μM of a specific antibody (a-L3) was administered during the first 10 min of reperfusion.
(5) : a-L4 (n=6): To examine the effects of the extracellular loop domain 4 (L4) of NBCe1, 10 μM of a specific antibody (a-L4) was administered during the first 10 min of reperfusion.
(6) : S0859 (n=5): To examine the effects of both NBC isoforms (electrogenic NBCe1, and electroneutral NBCn1), a specific NBC blocker S0859 — an *N*-cyanosulphonamide compound — 10 μM was administered during the first 10 min of reperfusion.

The effect of S0859, a-L3, and a-L4 on contractility was assessed in others hearts (n=4 for each one) in which 10 μM of the drug was added and the perfusion was extended for 120 min.

As previously reported [22], antibodies were raised against fusion proteins corresponding to putative third (EC3) and fourth (EC4) extracellular loops of human NBCe1 and used to detect NBCe1. The alignments of EC3 and EC4, although showing clusters of conserved human sequence, present enough nonconserved aminoacids to constitute dissimilar antigen determinant regions, minimizing the risk of inducing the generation of antibodies that could produce cross-reaction between the different bicarbonate transporters.

2.3. Infarct size determination

Infarct size (IS) was assessed by the widely validated triphenyltetrazolium chloride (TTC) staining technique [23]. At the end of reperfusion, the LAD was occluded again, and the myocardium was perfused during 1 min with a 0.1% solution of blue dye. This procedure delineated the nonischemic myocardium as dark blue. The freezed heart was cut into six transverse slices, which were incubated for 5 min at 37°C in a 1% solution of TTC. All atrial and right ventricular tissues were excised. To measure myocardial infarction, the slices were weighed and scanned. The infarcted (pale), viable ischemic/reperfused (red), and nonischemic (blue) areas were measured by computed planimetry (Scion Image 1.62; Scion Corp., Frederick, MD, USA). Noninfarcted viable myocardium was stained brick red with TTC, whereas the infarcted tissue remained unstained. The area at risk (AAR), the portion of the left ventricle supplied by the previously occluded coronary artery, was identified by the absence of blue dye. Infarct weights were calculated as $(A1\times W1)+(A2\times W2)+(A3\times W3)+(A4\times W4)+(A5\times W5)+(A6\times W6)$, where A is the area of infarct for the slice and W is the weight of the respective section. The weight of the AAR was calculated in similar fashion. IS was expressed as a percentage of AAR [24].

2.4. Systolic and diastolic function

Myocardial contractility was assessed by the left ventricular developed pressure (LVDP), obtained on subtracting LVEDP to LVP peak values and maximal rise velocity of LVP ($+dP/dt_{max}$). Data were expressed as percentage of their respective preischemic values. The diastolic function was evaluated by isovolumic LVEDP.

2.5. Immunostaining of rat cardiac myocytes

Isolated adult rat cardiomyocytes were fixed to laminin-coated coverslips and permeabilized as previously described [22]. Myocytes were incubated with rabbit anti-NBCe1 antibody (ab3212, Millipore, Temecula, CA, USA; 1:100 dilutions). Secondary chicken antirabbit conjugated to Alexa fluor 488 was used at 1:200 dilutions. Coverslips were washed three times in phosphate buffered saline (PBS) containing 0.2% gelatin and mounted and viewed with a confocal microscope.

Immunostained cells were mounted in ProLong Antifade solution (Molecular Probes, Eugene, OR, USA) and imaged with an Olympus Bx61 laser-scanning confocal microscope imaging system. Images were collected with an oil immersion X60 1.4 objective (numerical aperture 0.2, plan Apochromat, Zoom 1.5X). Images were captured in a sequential manner and analyzed with the Fluoview 3.3 Software.

2.6. Immunodetection of NBCe1

Samples of rat heart lysates or isolated ventricular myocytes lysates were resolved by SDS-PAGE on 7.5% acrylamide gels. Proteins were transferred to PVDF membranes and then incubated with rabbit anti-NBCe1 antibody (Millipore, Temecula, CA, USA; 1:1000 dilutions). Membranes were blocked with 5% nonfat milk. Immunoblots were incubated with donkey antirabbit IgG conjugated to horseradish peroxidase and visualized using the enhanced chemiluminiscence (ECL) reagent and a Chemidoc Image Station (Bio-Rad, Hercules, CA, USA).

3. Results

3.1. NBCe1 blockade and IS

Forty minutes of regional ischemia followed by 1 h of reperfusion in rat hearts without any treatment caused an IS of ~40% of risk area. The administration of antibody against extracellular loop 4 of NBCe1 (a-L4) and S did not modify the IS detected in IC hearts. However, a similar significant reduction was obtained when the antibody against extracellular loop 3 of NBCe1 (a-L3) or the NBC specific blocker S0859 (which simultaneously blocks NBCe1 and NBCn1) were added to the perfusate at the beginning of reperfusion (Fig. 1). Since a-L3 is a functional inhibitory antibody, these results indicate that NBCe1 participates in the generation of infarct produced by ischemia and reperfusion.

3.2. NBCe1 blockade during reperfusion and myocardial function

At the end of 120 min, nonischemic hearts exhibited a decrease in contractility of approximately 25%. After 40 min, ischemia and a 1-h reperfusion LVDP decreased to approximately 45% of the preischemic value. Postischemic recovery was not modified by S but was significantly improved by a-L3 treatment, reaching LVDP values of ~80% (Fig. 2, upper panel). The infusion of a-L4 or S0859 did not attenuate the postischemic systolic dysfunction detected in non-treated hearts. A similar pattern was observed when $+dP/dt_{max}$ was analyzed (Fig. 2, lower panel).

The LVEDP (an index of diastolic stiffness) was approximately 12 mmHg at the end of the stabilization period in the different experimental groups (Fig. 3). This parameter significantly increased reaching a value of approximately 37 mmHg after 1 h of reperfusion. The hearts treated with S, S0859, or a-L4 did not show significant changes of LVEDP compared to nontreated ischemic hearts. However, the treatment with a-L3 abolished the increase of LVEDP. In other words, the increase of myocardial diastolic stiffness detected in hearts after ischemia and reperfusion was avoided by selective inhibition of NBCe1 with a-L3.

In parallel experiments, the administration of a-L3 and a-L4 did not modify the decay of contractility detected in nontreated nonischemic hearts (75±5% for a-L3 and 72±4% for a-L4 vs. 75±5% for nontreated nonischemic hearts) while a significant decay was obtained after S0859 treatment (59±6%). The infusion of S0859 also produced an increase of LVEDP reaching a value of 26±6 mmHg at the end of perfusion. This value was significantly different to that observed in nontreated hearts (15±5 mmHg). The treatment with a-L3 and a-L4 produced similar changes than that observed in nontreated hearts. Fig. 4 shows a typical experiment of a heart treated with S0859 in which it can be observed the negative inotropic response and the impairment of diastolic stiffness produced by the drug.

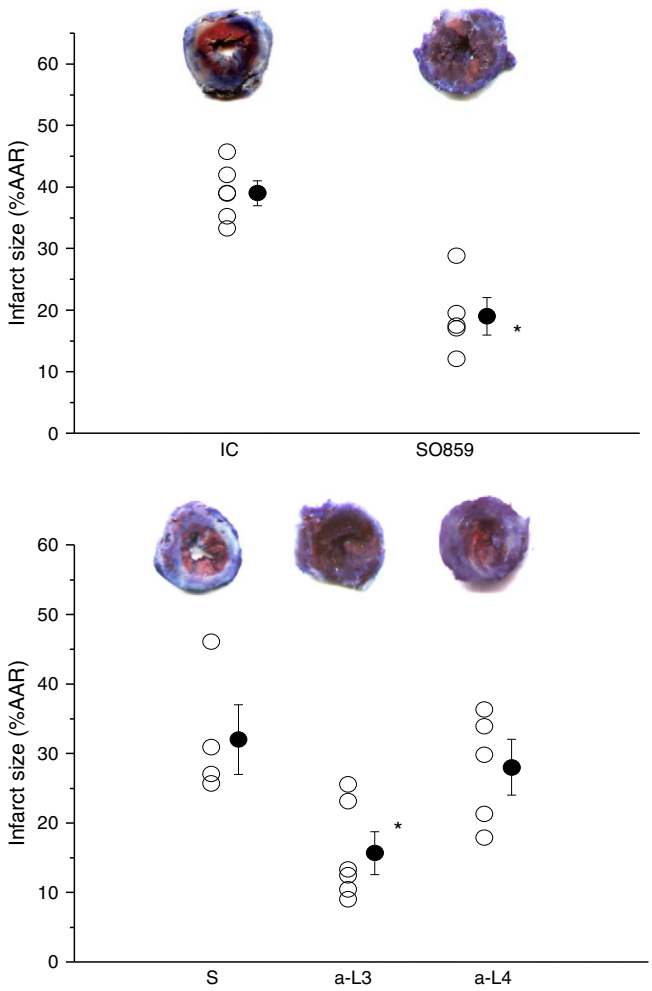

Fig. 1. IS, expressed as a percentage of risk area, in IC and in hearts treated with S, NBCe1 loop 3 and loop 4 antibodies (a-L3 and a-L4) and NBC blocker S0859. Observe that the treatment with a-L3 and S0859 decreased the IS obtained in IC and S hearts. *P<.05 vs. IC or S.

3.3. NBCe1 is expressed in rat cardiac myocytes

To confirm the presence of NBCe1 in Wistar rat cardiomyocytes, immunohistochemistry and immunoblot experiments were performed. Antibodies against NBCe1 revealed labeling surrounding the isolated rat cardiomyocytes, consistent with sarcolemmal staining (Fig. 5A) and intracellular localization, with invaginations that run toward the center of the myocytes. As previously reported [22,25], such distribution is consistent with the presence of NBCe1 in the sarcolemma and along the transverse tubular system (t-tubules), as suggested by the longitudinal bands. This distribution can be associated to a possible role for NBCe1 in cardiomyocytes excitation–contraction coupling. On the other hand, immunoblots of samples of rat heart and isolated ventricular myocyte demonstrated a band with a molecular mass of ~130 kDa, corresponding to NBCe1 (Fig. 5B).

4. Discussion

In this study we demonstrated for the first time that the selective blockade of NBCe1 at the beginning of reperfusion decreases the IS and improves the myocardial postischemic function in a regional ischemia model.

In the myocardium, the maintenance of pH_i is critical to sustain contractility and to prevent damage. During ischemia, pH_i falls due to an increased anaerobic metabolism combined with the lack of acids

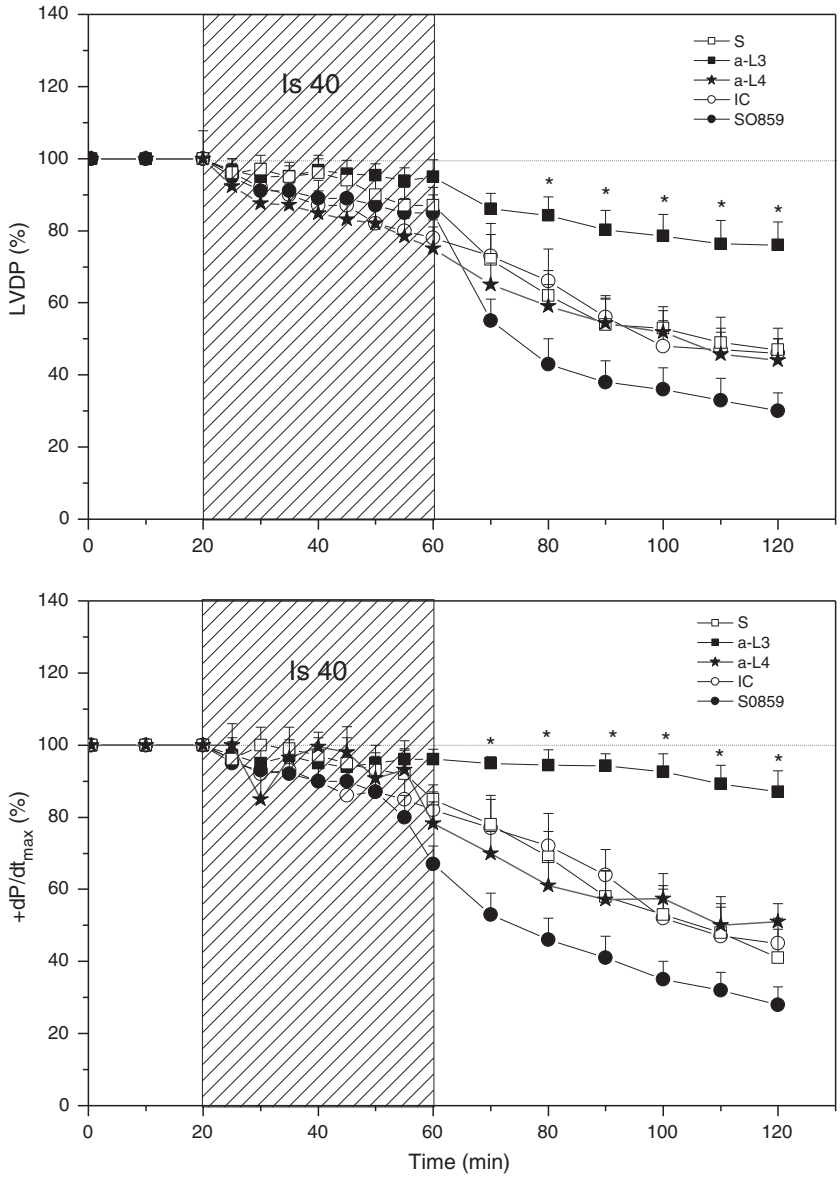

Fig. 2. Changes of LVDP and $+dP/dt_{max}$, expressed as percentage of preischemic values, in IC and in hearts treated with S, NBCe1 loop 3 and loop 4 antibodies (a-L3 and a-L4) and NBC blocker S0859. Note that only the treatment with a-L3 significantly improved the postischemic recovery of systolic function. *P<.05 vs. S.

removal [10]. This intracellular acidosis leads to activation of NHE and NBC as alkalinizing mechanisms [7,18]. The activity of both transporters leads to a rapid drop in extracellular pH, which in turn reduces the efficiency of the extrusion of acids through the sarcolemma [26]. Reperfusion washes out the extracellular acidosis, releasing the inhibition of the alkalinizing transporters and allowing the extrusion of H^+.

Secondarily to the intracellular Na^+ loading, the NHE and NBC activation carries to Ca^{2+} overload which promotes myocardial cell damage. This reveals that both transporters are contributing to the reperfusion injury. Consistently, a previous investigation [11] shows that the simultaneous inhibition of NHE and NBC protects the myocytes against reoxygenation hypercontracture. However, it has also been shown that the exclusive pharmacological inhibition of NHE cardiac isoform (NHE-1), either prior to ischemia [27,28] or during reperfusion [16,20] is able to prevent Ca^{2+} overload and alterations derived from ischemia–reperfusion.

In relation to NBC participation on reperfusion injury the evidences are less abundant. A previous work [12], using a polyclonal antibody during global ischemia and reperfusion, described the protective action of NBCe1 blockade on postischemic myocardial function. Our results also highlight the cardioprotective role of NBCe1 inhibition against ischemia and reperfusion injury. However, our study has two important differences compared to that work: we used a regional ischemia model instead of global ischemia — closer to the situation of human coronary disease — and the NBC blockers were administered at the beginning of reperfusion instead of prior ischemia, which allows the evaluation of the employment of this compound in the clinical scenario. Thus, the NBCe1 inhibition might represent a pharmacological tool for clinical therapy.

We have recently demonstrated that a-L3 and a-L4 recognize NBCe1 in cat myocardium [19]. Surprisingly, these antibodies have opposite effects on this transporter function: the a-L3 is inhibitory, and the a-L4 is excitatory. Thus, the recovery of acidosis applied to cat or rat [28–30] ventricular myocytes decreased in the presence of a-L3. In contrast, a-L4 accelerated the recovery from acidosis in cat ventricular myocytes [22]. Extrapolating these data to our present results we can hypothesize that the cardioprotection detected after a-L3 treatment could be due to the delay in the recovery from acidosis at the onset of reperfusion, whereas opposite changes could be occurring with a-L4. This hypothesis makes

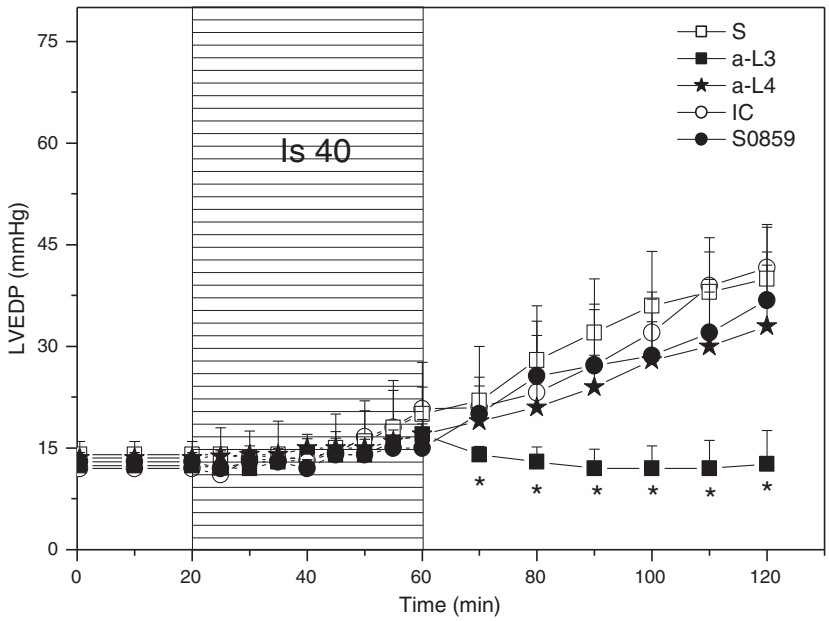

Fig. 3. Changes of LVEDP, expressed in mmHg, in IC and in hearts treated with S, NBCe1 loop 3 and loop 4 antibodies (a-L3 and a-L4) and NBC blocker S0859. Observe that only the a-L3 treatment significantly attenuated the increase of diastolic stiffness. *P<.05 vs. S.

sense considering that it has been described that the infusion of an acid solution at the beginning of reperfusion is cardioprotective [31,32]. Indeed, the action of a-L3 validated part of our hypothesis. On the other hand, we initially hypothesized that a-L4 would exacerbate the reperfusion injury, as expected if it would activate NBCe1. However, a-L4 did not worsen the reperfusion injury. We did not elucidate herein the reason for this discrepancy, but we could speculate that maximal activation of NBCe1 is achieved during reperfusion and thus, a-L4 is not able to further stimulate it and increase cell damage.

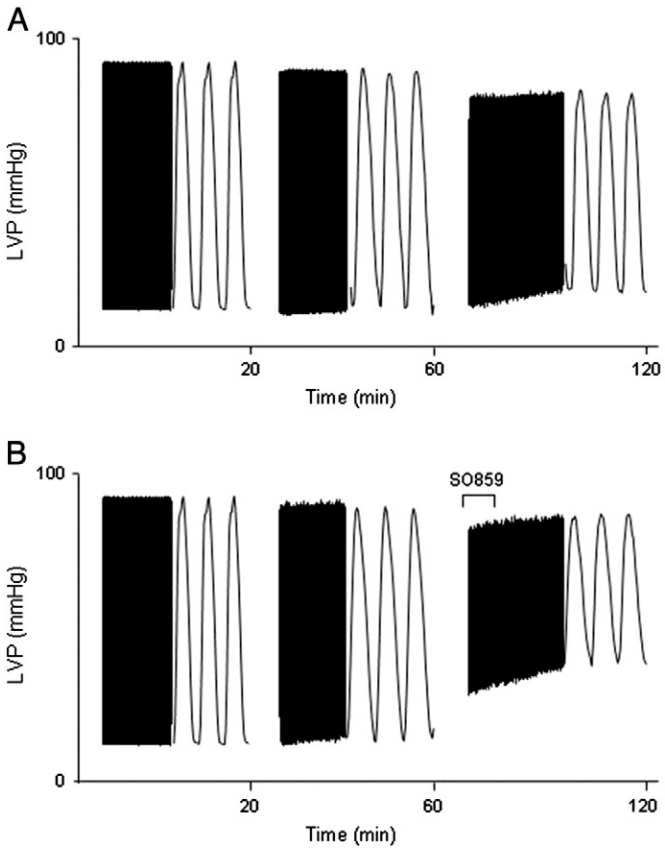

Fig. 4. Typical traces of LVP during the perfusion of a nontreated and nonischemic heart (A) and in a heart treated with S0859 (B). At the end of perfusion the S0859 treatment significantly increased the LVEDP and decreased the LVDP in comparison to the values obtained in nontreated heart.

Our finding that the treatment with S0859 limits IS suggests that both NBC isoforms (NBCe1 and NBCn1) are responsible for the reperfusion damage. However, S0859 produced a similar effect on IS than a-L3 (which exclusively inhibit NBCe1), suggesting that this NBCe1 is the only NBC isoform that contributes to the generation of IS after ischemia and reperfusion. On the other hand, a-L3 but not S0859 was able to improve the postischemic recovery of contractile function. The reason for this discrepancy is not apparent to us at the present moment. However, the decrease in basal contractility observed with S0859 (Fig. 4) might explain the lack of mechanical protection. Nevertheless, a negative nonselective off-target effect of this compound cannot be rule out.

Expression of NBCe1 has been previously observed in heart tissue [33–35]. In this study, using antibody raised against fusion protein corresponding to putative third extracellular loop of human NBCe1 we detected NBCe1 protein in the rat myocardium. Data from immunostaining of rat cardiac myocytes revealed the presence of NBCe1 in the sarcolemma, along the transverse tubular system and co-localized with vinculin. This localization is similar to that recently published by us in feline cardiomyocyte [22].

5. Conclusions

Our data demonstrate that NBCe1 blockade at the beginning of reperfusion decreases IS and myocardial postischemic dysfunction produced by 40 min of coronary occlusion and 1 h of reperfusion in isolated rat heart. This cardioprotective effect could be attributed to the diminution of Ca^{2+} overload.

6. Limitations

Although our data show that NBC blockers are useful pharmacological tools to decrease postischemic alterations in isolated heart, further

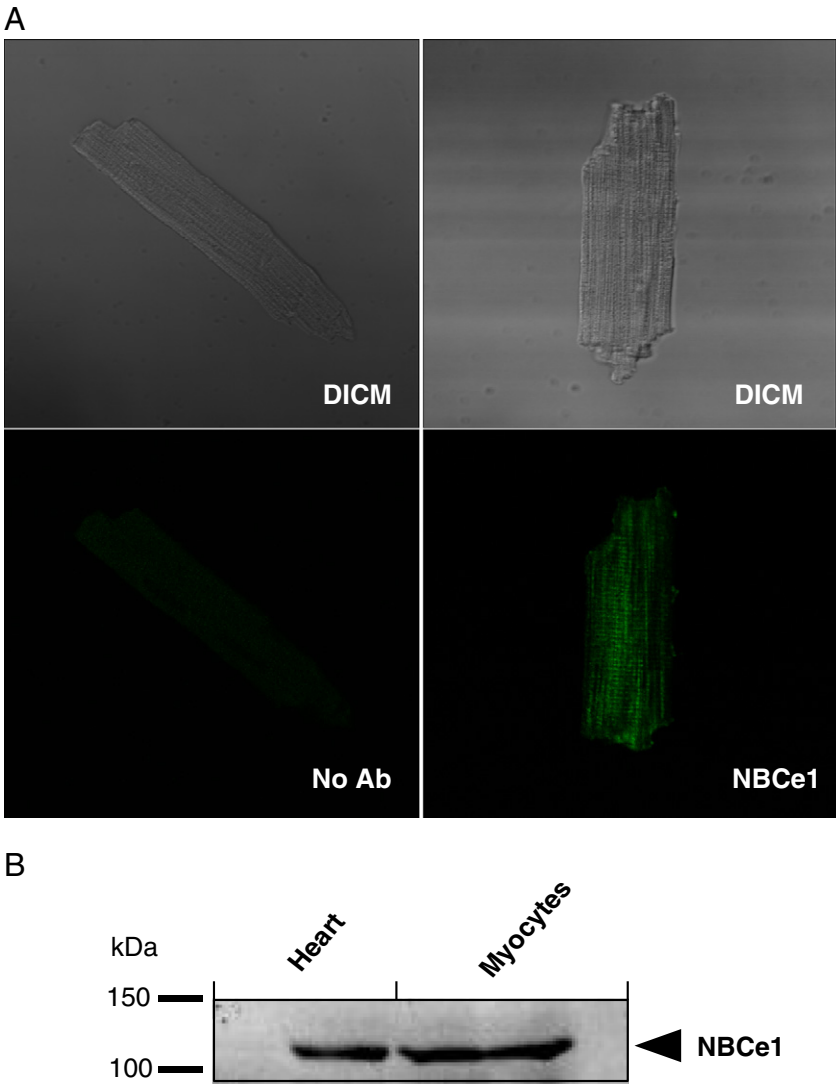

Fig. 5. (A) Confocal immunofluorescence analysis of the distribution of NBCe1 in rat myocytes. Freshly isolated rat ventricular myocytes were stained with rabbit anti-NBCe1 antibody or without antibody (No Ab). (B) Samples of rat heart or isolated ventricular myocytes were resolved by SDS-PAGE on 7.5% gels, blotted, and probed with an anti-NBCe1 antibody (arrow).

research using experimental models closer to clinical situation would be needed to ensure the treatment application for human coronary disease. Longer term effects "in vivo" (functional studies, histological analysis of AAR, IS, and fibrosis) need to be studied using a standard ischemia/reperfusion model to evaluate how these seemingly short-term benefit effects are clinically relevant to the vast remodeling process and could potentially affect patient morbidity and mortality.

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