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Reduced sarcolemmal expression and function of the NBCe1 isoform of the Na^{+}–HCO_3^{-} cotransporter in hypertrophied cardiomyocytes of spontaneously hypertensive rats: role of the renin–angiotensin system

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Aims Electroneutral (NBCn1) and electrogenic (NBCe1) isoforms of the Na^{+}–HCO_3^{-} cotransporter (NBC) coexist in the heart. We studied the expression and function of these isoforms in hearts of Wistar and spontaneously hypertensive rats (SHR), elucidating the direct implication of the renin–angiotensin system in the NBC regulation.

Methods and results We used myocytes from Wistar, SHR, losartan-treated SHR (Los-SHR), and Angiotensin II (Ang II)-induced cardiac hypertrophy. We found an overexpression of NBCe1 and NBCn1 proteins in SHR that was prevented in Los-SHR. Hyperkalaemic-induced pH_i alkalization was used to study selective activation of NBCe1. Despite the increase in NBCe1 expression, its activity was lower in SHR than in Wistar or Los-SHR. Similar results were found in Ang II-induced hypertrophy. A specific inhibitory antibody against NBCe1 allowed the discrimination between NBCe1 and NBCn1 activity. Whereas in SHR most of the pH_i recovery was due to NBCn1 stimulation, in Wistar and Los-SHR the activity of both isoforms was equitable, suggesting that the deteriorated cardiac NBCe1 function observed in SHR is compensated by an enhanced activity of NBCn1. Using the biotin method, we observed greater level of internalized NBCe1 protein in SHR than in the non-hypertophic groups, while with immunofluorescence we localized the protein in endosomes near the nucleus only in SHR.

Conclusions We conclude that Ang II is responsible for the impairment of the NBCe1 in hypertrophied hearts. This is due to retained transporter protein units in early endosomes. Moreover, NBCn1 activity seems to be increased in the hypertrophic myocardium of SHR, compensating impaired function of NBCe1.

Keywords Na^{+}–HCO_3^{-} cotransporter • Cardiac myocytes • Cardiac hypertrophy

1. Introduction

In the heart the main alkalinizing mechanisms which neutralize acid in the cell are the Na^{+}–H^{+} antiporter (NHE) and the Na^{+}–HCO_3^{-} cotransporter (NBC), which exports H^{+} and imports HCO_3^{-} into the cell, respectively. NBC is responsible for 40–50% of total acid extrusion in cardiac myocytes.[1–3] At present, it is known that in ventricular myocytes exist at least two functional isoforms of NBC: the electroneutral NBCn1, which introduces one molecule of Na^{+} per molecule of HCO_3^{-} and the electrogenic NBCe1, which promotes the co-influx of one molecule of Na^{+} per two molecules of HCO_3^{-}.[4,5] Both isoforms regulate intracellular pH (pH_i) and intracellular Na^{+} concentration ($[Na^{+}]_i$).[6] In addition, NBCe1 generates an anionic current that contributes to the action potential (AP) repolarization.[7–9]

In a recent study, using a selective inhibitory antibody against the extracellular loop 3 of NBCe1 (a-L3), we were able to discriminate

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the relative contribution of NBCe1 and NBCn1 to total NBC activity in feline ventricular cardiomyocytes during the recovery from acidosis.[4] In this work, we have shown that the activity of both isoforms is equivalent. Moreover, in the presence of the a-L3 the cardiac action potential duration (APD) was longer than in its absence, indicating the relevance of NBCe1 in cardiac electrical properties.[4]

It was demonstrated that Angiotensin II (Ang II) stimulates total NBC activity during the recovery from acidosis both in rat and in cat adult ventricular myocytes.[3,10] Moreover, we have recently shown a differential effect of Ang II on NBC isoforms, demonstrating that Ang II inhibits NBCe1 via p38-kinase activation, whereas activates NBCn1 in an ERK 1/2 and reactive oxygen species (ROS)-dependent mechanism. The net effect is an up-regulation of NBC activity since the Ang II-induced stimulation of NBCn1 is only partially masked by the inhibition of NBCe1.[9]

The spontaneously hypertensive rats (SHRs) are a well-established genetic model of hypertension and heart hypertrophy. It was demonstrated that the cardiac APD of SHR is prolonged in comparison with that of normotensive animals.[11] In addition, the $[Na^+]_i$ and $[Ca^{2+}]_i$ are increased in hypertrophied hearts.[12–14] It is also known that the renin–angiotensin system is involved in the development of hypertension, cardiac hypertrophy, and subsequent transition to heart failure. In concordance, the levels of Ang II[15–18] and the Ang II receptors AT_1[19] are increased in hypertrophied hearts.

It was proposed that at least part of the maladaptive cardiac mass growth is caused by an autocrine/paracrine event triggered by myocardial stretch that begins with the activation of AT_1 receptors,[20,21] involves the production of ROS[22] and the activation of several kinase pathways.[20] One clear target of these intracellular pathways is the NHE, which it has been shown to be up-regulated in several models of cardiac hypertrophy.[23–26] In addition, the NBC was shown to be overexpressed and hyperactive in aortic constriction-induced cardiac hypertrophy.[27] However, the precise implication of NBC in cardiac hypertrophy has not been well established yet. The aim of the present research was to investigate the pathophysiological role of the cardiac NBC isoforms in the SHR in order to bring new insights into their potential implication in the hypertensive cardiac hypertrophy.

2. Materials

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 the experimental protocol was approved by the Animal Welfare Committee of La Plata School of Medicine. Male rats (body weight 300–400 g) were anaesthetized by i.p. injection of sodium pentobarbital (35 mg/kg body weight) and hearts rapidly excised when plane three of phase III of anaesthesia was reached.

A group of SHR male rats was treated during 1 month with the Ang II type 1 receptor blocker losartan (40 mg/kg/day; Los-SHR).

2.1 Cell isolation

Ventricular myocytes were isolated according to the technique previously described,[28] using 6-month-old Wistar, SHR, and Los-SHR.

2.2 pH_i measurements

pH_i was measured in single myocytes with an epi-fluorescence system (Ion Optix, Milton, MA, USA) using the previously described BCECF technique.[9] The intrinsic buffering capacity (βi; see Supplementary material online) was significantly higher in cardiac myocytes from Wistar than in cardiomyocytes from SHR (at pH_i of 6.9: 56.02 ± 0.10 mM/pH units, $n = 14$ vs. 34.12 ± 0.09 mM/pH units, $n = 12$; $P < 0.05$). This depressed βi of the SHR cardiomyocytes was not reversed by the treatment with losartan (at pH_i of 6.9: 33.91 ± 0.06 mM/pH units, $n = 6$).

2.3 Immunodetection

NBCe1 and NBCn1 expression were determined by western blots (W-B) with specific antibodies as previously described.[4] In addition, the biotin method was employed to determine the fraction of NBCe1 expressed in the sarcolemma.[29]

2.4 Immunostaining of cardiac myocytes and analysis by confocal microscopy

Fresh single dissociated rat ventricular myocytes were plated onto 22 × 22-mm laminin (30 μg/mL)-coated glass coverslips and incubated with specific antibodies, as previously described.[4]

2.5 Rats treated with Ang II

Male Wistar rats, weighing 300–400 g, were anaesthetized by inhalation of 2% sevoflurane, 98% oxygen, and an osmotic minipump (Alzet 2004) for s.c. of Ang II (200 μg/kg/day; Sigma) was implanted under the skin. The control animals were also anaesthetized in order to simulate the surgery. Blood pressure (BP) monitored by tail cuff plethysmography and echocardiographic parameters was recorded before and after treatment. On Day 30 after implantation of minipumps, animals were sacrificed and the hearts were isolated to perform the functional and molecular experiments.

2.6 Statistics

Data were expressed as means ± SEM and were compared with Student's *t*-test or one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test. A value of $P < 0.05$ was considered statistically significant (two-tailed test).

An expanded Methods section is available in Supplementary material online.

3. Results

3.1 Cardiac hypertrophy parameters

The systolic BP measured by the plethysmographic method and the hypertrophy indexes determined by echocardiography in Wistar, SHR, and Los-SHR are shown in *Table 1*. As expected, the hypertension and cardiac hypertrophy of SHR were partially reversed by the treatment with losartan.

3.2 Potassium pulses: the activity of NBCe1 is impaired in SHR

Wistar and SHR myocytes were exposed to a high extracellular K^+ solution. This hyperkalaemic solution induced a depolarization of the membrane potential which selectively stimulated NBCe1 and therefore resulted in cellular alkalization. The HCO_3^- influx ($J_{HCO_3^-}$) obtained from the initial velocity of increase in pH_i and the total intracellular buffering capacity was compared (see Supplementary material online). *Figure 1A* shows representative traces of continuous pH_i recordings in Wistar, SHR, and Los-SHR. As observed in the average results (*Figure 1B*), the $J_{HCO_3^-}$ was significantly smaller in SHR myocytes in comparison with

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Table 1 General characteristics of Wistar, SHR, and Los-SHR rats

	Wistar	SHR	Los-SHR
BP	118.4 ± 2.3 (n = 15)	198.5 ± 4.7 (n = 15)*	140.7 ± 1.6 (n = 11)#
BW	325.4 ± 9.2 (n = 15)	341.8 ± 9.5 (n = 15)	328.8 ± 7.3 (n = 15)
LVM	456 ± 28.9 (n = 15)	695.5 ± 19.01 (n = 15)*	516.5 ± 13.32 (n = 15)
LVMI	1.41 ± 0.09 (n = 15)	2.04 ± 0.06 (n = 15)*	1.57 ± 0.03 (n = 15)

BP, blood pressure (mmHg); BW, body weight (g); LVM, left ventricular mass (mg); LVMI, left ventricular mass index, calculated as the ratio LVM/BW (mg/g).
*$P < 0.05$ vs. Wistar and Los-SHR.
#$P < 0.05$ vs. Wistar.

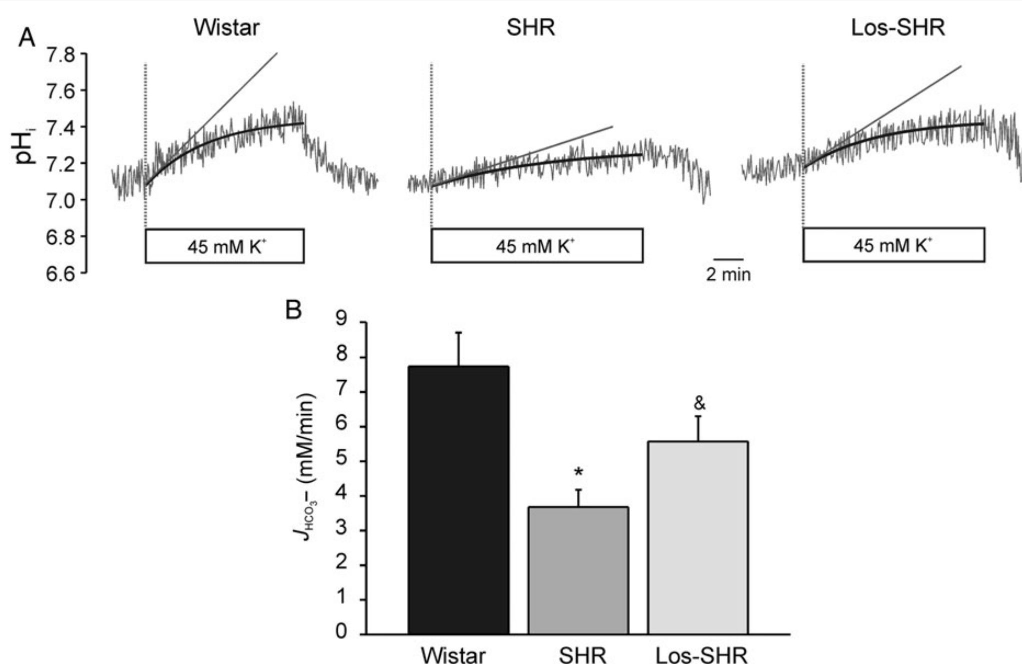

Figure 1 NBCe1 activity determined by the potassium pulse: (*A*) Representative traces of pH_i recorded from ventricular myocytes isolated from Wistar, SHR, and Los-SHR during exposure to the potassium pulse. The slope (grey line), corresponding to the initial velocity during the alkalinization produced by the potassium pulse, was used to determined HCO_3^- influx. The vertical dotted lines indicate the points on the fit curves at which these slopes were determined. (*B*) Average data of the $J_{HCO_3^-}$ obtained from the initial velocity of the pH_i alkalinization induced by the hyperkalaemic-induced depolarization of membrane potential in myocytes from Wistar (n = 23 myocytes from 11 rats), SHR (n = 25 myocytes from 9 rats), and Los-SHR (n = 23 myocytes from 7 rats). *$P < 0.05$ vs. Wistar and Los-SHR. &$P < 0.05$ vs. Wistar.

their Wistar controls. In addition, the myocytes derived from Los-SHR presented an NBCe1 activity intermediate between that of the Wistar and SHR. These results indicate that the NBCe1 activity is impaired in SHR, and that this behaviour is partially prevented when the AT_1 receptors were blocked with losartan.

3.3 Ammonium pulses: NBCn1 overexpression compensates NBCe1-impaired activity in SHR

The total NBC (NBCe1 + NBCn1) activity was assessed by evaluating the pH_i recovery from an ammonium pre-pulse-induced acute acid load in a medium containing bicarbonate and in the continuous presence of HOE 642 10 μM (NHE blocker). Serum containing the inhibitory antibody against the extracellular loop 3 of the NBCe1 (a-L3)[4] was used to differentiate the relative participation of both isoforms during the recovery from the acidosis; NBCn1 activity was estimated as the pH_i recovery in the presence of a-L3, whereas NBCe1 activity was calculated as the control pH_i recovery (total NBC) minus the NBCn1 activity. Non-immune serum was used as a control.[4] The selective inhibitory effect of a-L3 on the function of the NBCe1 expressed in the rat cardiomyocytes is shown in Supplementary material online, *Figure S1*. *Figure 2A* illustrates superimposed traces of Wistar, SHR, and Los-SHR myocytes in the presence and absence of a-L3 (dilution 1/500). *Figure 2B* compares the $J_{HCO_3^-}$ calculated at pH_i of 6.9 in the three different conditions. Total $J_{HCO_3^-}$ (NBCe1 + NBCn1) was smaller in SHR than in Wistar or Los-SHR, although this difference did not attain a statistical significance. More interestingly, whereas the Wistar myocytes showed an equivalent NBCn1 and NBCe1 activity, the SHR myocytes exhibited almost exclusive NBCn1 activity. Importantly, the Los-SHR recovered the equitable

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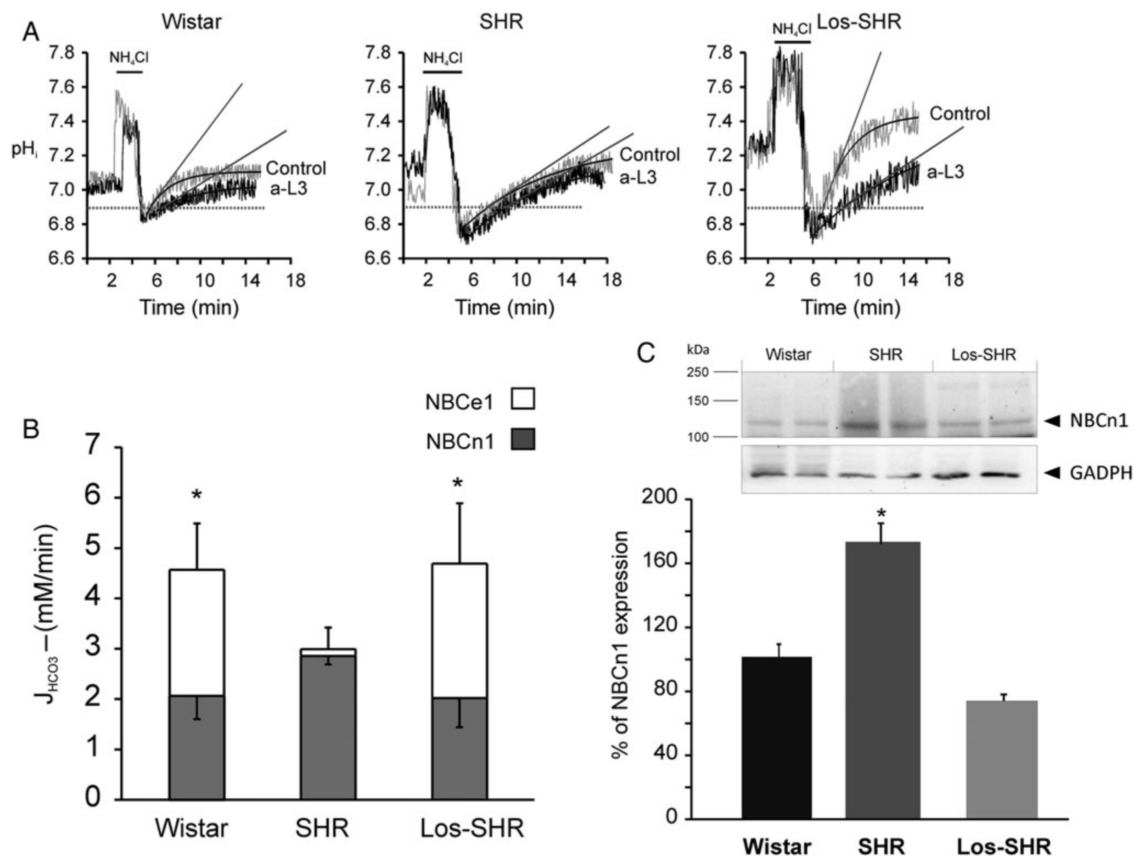

Figure 2 Total NBC activity determined by the ammonium pulse. (*A*) Superimposed representative recordings of pH_i obtained from myocytes isolated from Wistar, SHR, and Los-SHR exposed to an ammonium pulse in the absence (N-I serum representing control) or presence of the serum containing the functional inhibitory antibody against NBCe1 (a-L3; 1:500). The experiments were performed in the continuous presence of HOE642 (10 μM) in order to block NHE-1 activity. During these experiments, cells are exposed to external solution containing NH_4Cl. External NH_3 enters the cell far more rapidly than external NH_4^+ thereby alkalinizing the cell. This alkalinization will continue until the internal and external concentration of NH_3 are the same. Upon removal of external NH_4Cl, all the original NH_4^+ formed upon exposure will dissociate and regenerate NH_3 (which diffuses from the cell) and H^+. In this point the cell will reach the most acid pH_i and will begin the recovering due to the activation of NBC. The slope (grey line), corresponding to the velocity corresponding to pH_i 6.9 during the recovery from acidosis, was used to determined HCO_3^- influx. The horizontal dotted lines indicate pH_i 6.9, which are the points on the fit curves at which these slopes were determined. (*B*) $J_{HCO_3^-}$ calculated at pH_i 6.9 in the absence or presence of a-L3 in Wistar ($n = 10$ myocytes from 5 rats), SHR ($n = 10$ myocytes from 6 rats), or Los-SHR ($n = 6$ myocytes from 4 rats). NBCn1 activity was estimated as the pH_i recovery in the presence of a-L3, whereas NBCe1 activity was calculated as the control pH_i recovery (total NBC) minus the NBCn1 activity. Non-immune serum was used as a control. $^*P < 0.05$ vs. NBCn1. (*C*) Percentage average expression of NBCn1 (normalized by GAPDH expression) obtained from lysates of ventricles from Wistar ($n = 11$), SHR ($n = 5$), or Los-SHR ($n = 4$). The expression of NBCn1 in Wistar was taken as 100%. $^*P < 0.05$ vs. Wistar and Los-SHR. A representative blot is shown at the top.

participation of NBC isoforms, similar to the one exhibited by the Wistar myocytes (*Figure 2B*). In concordance with these functional results, W-B analysis demonstrated that, compared with Wistar hearts, NBCn1 expression in SHR was significantly higher (*Figure 2C*). Furthermore, the treatment with losartan abrogated the up-regulation of NBCn1 in SHR hearts (*Figure 2C*). These data indicate that in SHR, the NBCe1-impaired activity might be compensated by an Ang II-induced NBCn1 up-regulation.

3.4 Distribution of NBCe1 protein in SHR

Parallel experiments were performed to investigate the NBCe1 expression. Opposite to it was expected, the NBCe1 expression in SHR hearts was higher than in Wistar. In addition, the treatment with losartan prevented this increase (*Figure 3*).

Previous studies had shown that Ang II induces internalization of NBCe1 from the plasma membrane in *Xenopus laevis* oocytes.[30] To investigate whether this phenomenon is taking place in SHR myocytes, confocal images were taken in both Wistar and SHR myocytes and the biotinylation technique followed by W-B analysis was used to determine the level of NBCe1 surface expression. We found that in both strains NBCe1 was expressed along the sarcolemmal membrane and T tubules, but interestingly, in SHR myocytes an important fraction of NBCe1 was retained near the nucleus, possibly in the endoplasmic reticulum, suggesting a disturbance in NBCe1 trafficking (*Figure 4A*). Consistently with an involvement of Ang II in this phenomenon, the treatment of SHR with losartan reversed this localization pattern (*Figure 4A*). The overlay of the NBCe1 signal with an endosome marker (transferrin receptor; Tfr) was evaluated to quantify the presence of NBCe1 in endosomes near the nucleus. The signal for NBCe1

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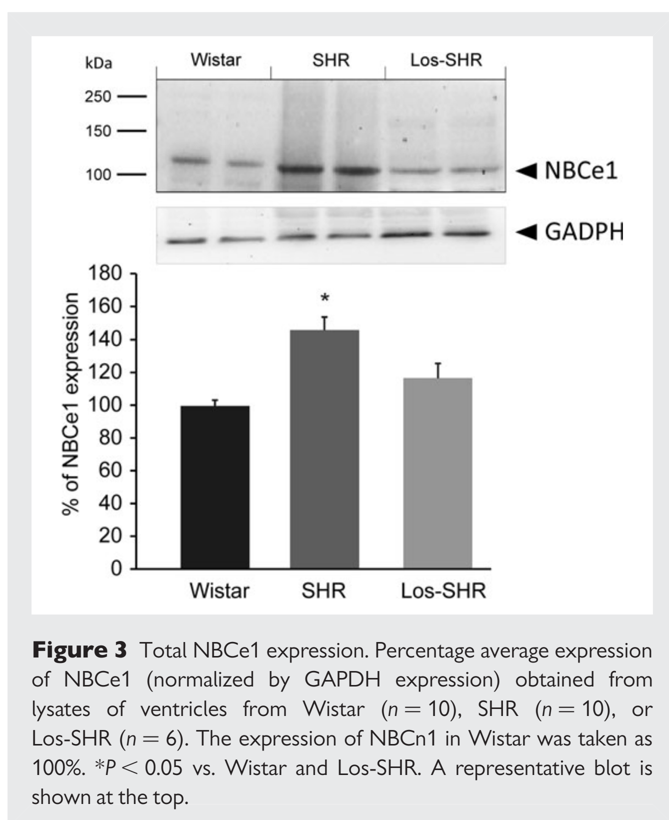

Figure 3 Total NBCe1 expression. Percentage average expression of NBCe1 (normalized by GAPDH expression) obtained from lysates of ventricles from Wistar ($n = 10$), SHR ($n = 10$), or Los-SHR ($n = 6$). The expression of NBCn1 in Wistar was taken as 100%. *$P < 0.05$ vs. Wistar and Los-SHR. A representative blot is shown at the top.

and Tfr was line-scanned on the same area of the nucleus in each cell examined. *Figure 4B* shows the representative traces for such line-scannings of the cells corresponding to *Figure 4A*. As the figure shows, the nucleus exhibited no signal, whereas signals for NBCe1 and Tfr can be observed in the adjacent areas. In myocytes from Wistar and Los-SHR, the signals for NBCe1 are low and do not merge with the signal for Tfr. In contrast, in the myocytes from SHR, the signals for NBCe1 and Tfr clearly overlay, indicating the presence of NBCe1 in early endosomes. *Figure 4C* shows average fluorescence detected in the nucleus adjacent area for the NBCe1 and Tfr signals. The average fluorescence values of the NBCe1 and Tfr signals were significantly different in the myocytes from Wistar and Los-SHR and not different in those from SHR, supporting the existence of an alteration of NBCe1 trafficking in the hypertensive rats.

In concordance with this, the percentage of NBCe1 internalization was significantly higher in SHR myocytes in comparison with Wistar (*Figure 4D*). In addition, the treatment with losartan prevented this phenomenon (*Figure 4D*). These results suggest that the reduction in NBCe1 activity was secondary to a decrease in its sarcolemmal expression, likely due to Ang II-induced alteration of NBCe1 trafficking to the plasma membrane.

A relevant functional consequence of the altered NBCe1 sarcolemmal expression in the cardiomyocytes of SHR would be a reduced impact of this electrogenic transporter on the AP configuration. To evaluate this hypothesis, we recorded APs with the patch-clamp technique. As previously reported,[11] the APD was significantly longer in the SHR cardiomyocytes than in those from the normotensive animals (*Figure 5A* and *B*). *Figure 5B* and *C* shows the effect of a-L3 on the APD determined at 90% of repolarization time (APD_{90}). The inhibitory antibody was able to significantly prolong the APD in the myocytes of the Wistar rats (~14%, *Figure 5C*), but not in those from the SHR, supporting the depressed membrane expression of NBCe1 in the latter, as suggested above.

We finally attempted to further evaluate the involvement of Ang II on the structural and functional remodelling of NBCe1 by treating Wistar rats with this hormone (200 μg/kg/day) during 1 month. Ang II induced a significant percentage increase in BP (23.61 ± 0.07%, $n = 7$) and left ventricular mass index (LVMI; 17.64 ± 0.02%, $n = 7$). These parameters were not affected in the control rats (BP; 0.99 ± 0.02%, $n = 6$ and LVMI; 0.94 ± 0.07%, $n = 6$). *Figure 6A* shows representative traces of pH_i recorded during the potassium pulse in myocytes isolated from control- and Ang II-treated rats. As shown in *Figure 6B*, the average $J_{HCO_3^-}$ obtained from the initial velocity of increase in pH_i during the potassium pulse was significantly lower in myocytes from the Ang II-treated rats than in those from control. These results further support the idea that the cardiac NBCe1 dysfunction detected in the SHR is induced by Ang II.

Figure 6C and *D* depicts the average expression of NBCe1 and NBCn1 in the myocardium from control- and Ang II-treated rats. Similarly to the observations made for the SHR hearts, the NBCe1 and NBCn1 expression were increased in the hearts from the Ang II-treated rats. The depressed cardiac NBCe1 function, despite its enhanced expression, might reflect the trafficking disturbance of the transporter protein demonstrated for the SHR myocytes.

4. Discussion

The present work shows that in the SHR exists a remodelling in cardiac NBC isoforms, which results in a decrease of NBCe1 in the cell surface and in a compensatory up-regulation of NBCn1. These alterations were reversed by treatment of the hypertrophic rats with the AT_1 antagonist losartan, suggesting that Ang II is involved in such remodelling. Furthermore, the infusion of Wistar rats with Ang II for 30 days induced cardiac hypertrophy and mimicked the results on NBC isoforms expression and NBCe1 function obtained in SHR. These findings allow us to assert that Ang II is directly involved in the remodelling of NBC isoforms during the cardiac hypertrophy of SHR rats.

Previous investigations have demonstrated that Ang II stimulates NBC activity in rat and cat cardiomyocytes.[3,10] Furthermore, we have reported that Ang II regulates NBC isoforms activity in an opposite way, activating NBCn1 via ROS and ERK 1/2 kinase stimulation and inhibiting NBCe1 in a p38-kinase-dependent manner.[9] We found that the NBCn1 stimulation overrules the NBCe1 inhibition and therefore resulted in a total NBC stimulation.[9] The Ang II-induced NBCe1 inhibition was also demonstrated in renal tubules in a pathway that involves arachidonic acid.[31,32]

It is well known that Ang II is involved in the pathophysiology of cardiac hypertrophy secondary to diverse stimuli. We have previously suggested that Ang II and endothelin-1 can trigger an intracellular pathway that stimulates NHE-1 in an ROS and ERK1/2-kinase-dependent manner. This effect increases $[Na^+]_i$, activates the reverse mode of the Na^+/Ca^{2+} (rNCX), enhances the intracellular calcium concentration ($[Ca^{2+}]_i$), and finally leads to cell growth.[23,24,33] Furthermore, it was demonstrated that chronic inhibition of NHE-1 prevents or reverses cardiac hypertrophy.[34,35] On the other hand, the overexpression of the NHE-1 was shown to induce cardiac hypertrophy.[36] Based on these results, it is possible to speculate that a similar intracellular pathway to the one described above for the NHE-1 might be involved in the Ang II-induced up-regulation of NBCn1 observed herein in the hypertrophic hearts. In other words, since it was reported that NBC is

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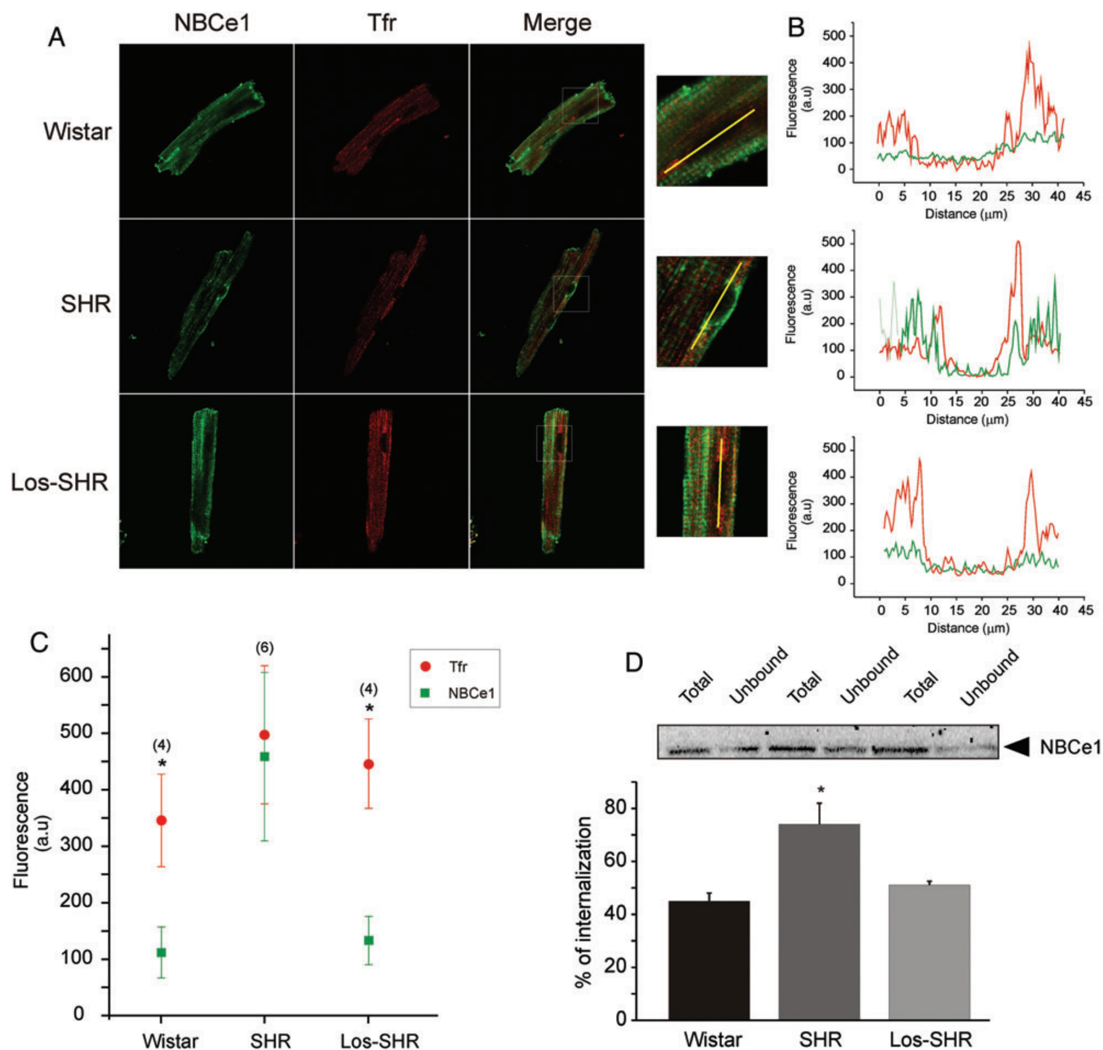

Figure 4 Localization of NBCe1 in rat ventricular myocytes. (*A*) Confocal immunofluorescence analysis of the distribution of NBCe1 in rat myocytes isolated from Wistar, SHR, and Los-SHR. Freshly isolated cardiomyocytes were stained with a combination of rabbit anti-NBCe1 antibody and goat polyclonal anti-transferrin receptor antibody (Tfr; marker for endosomal vesicles). Combined primary antibodies were used at 1:100 dilutions. Secondary chicken anti-rabbit conjugated to Alexa fluor 488 (green) and chicken anti-goat conjugated to Alexa fluor 594 (red) was used at 1:200 dilutions. Squares represent the area amplified shown in the left. (*B*) Representative line-scan fluorescence corresponding to Wistar, SHR, and Los-SHR rats, plotted as the signals for NBCe1 (green) and Trf (red), which were taken in the nucleus and adjacent areas (*A*; inset, yellow line). (*C*) Average of NBCe1 and Trf fluorescence in the nucleus adjacent area (*n* values are shown between brackets). $^{*}P < 0.05$ vs. Trf (a.u.). (*D*) Percentage average expression of internalized cardiac NBCe1 of Wistar ($n = 4$), SHR ($n = 4$), and Los-SHR ($n = 4$) obtained from the subtraction of the intracellular expression of NBCe1 (unbound to the sarcolemmal marker biotin) to the total expression of NBCe1 (unbound plus bound to biotin). $^{*}P < 0.05$ vs. Wistar and Los-SHR. A representative blot is shown at the top.

responsible for 30% of Na^+ influx into the cells during the recovery from acidosis,[6] the overexpression and up-regulation of NBCn1 in SHR might be part of the cause of the $[Na^+]_i$ overload observed in cardiac hypertrophy.[12–14] Moreover, taking into account the stoichiometry of both NBC isoforms, which could lead to the consideration of NBCe1 as a 'Na^+-sparing' bicarbonate transporter, it is feasible to anticipate that not only the overexpression of NBCn1, but also the reduction in NBCe1 function in the hypertrophied hearts would further contribute to the deleterious effects on $[Na^+]_i$ and $[Ca^{2+}]_i$ overload.

We have suggested that Ang II-induced NBCe1[9] inhibition might be the responsible, at least in part, for the characteristic APD prolongation induced by this hormone. Herein, we present new data which show that in SHR myocytes the inhibitory antibody against NBCe1 (a-L3) exerts no effect on AP configuration, suggesting that NBCe1 might be already inhibited due to the up-regulation of the renin–angiotensin system in SHR.

In the present study, we observed that despite that βi depends on the amount of protein in the cell, the larger cardiomyocytes from the SHR exhibited a 40% lower βi value than that obtained from Wistar rats. It has been suggested that the increased protein synthesis in hypertrophy improves intracellular H^+ buffering capacity, which might improve the ability to resist acute acid–base changes.[37] However, most of the studies performed in the myocardium from hypertrophied hearts did not report a significant increase in the value of βi in comparison with

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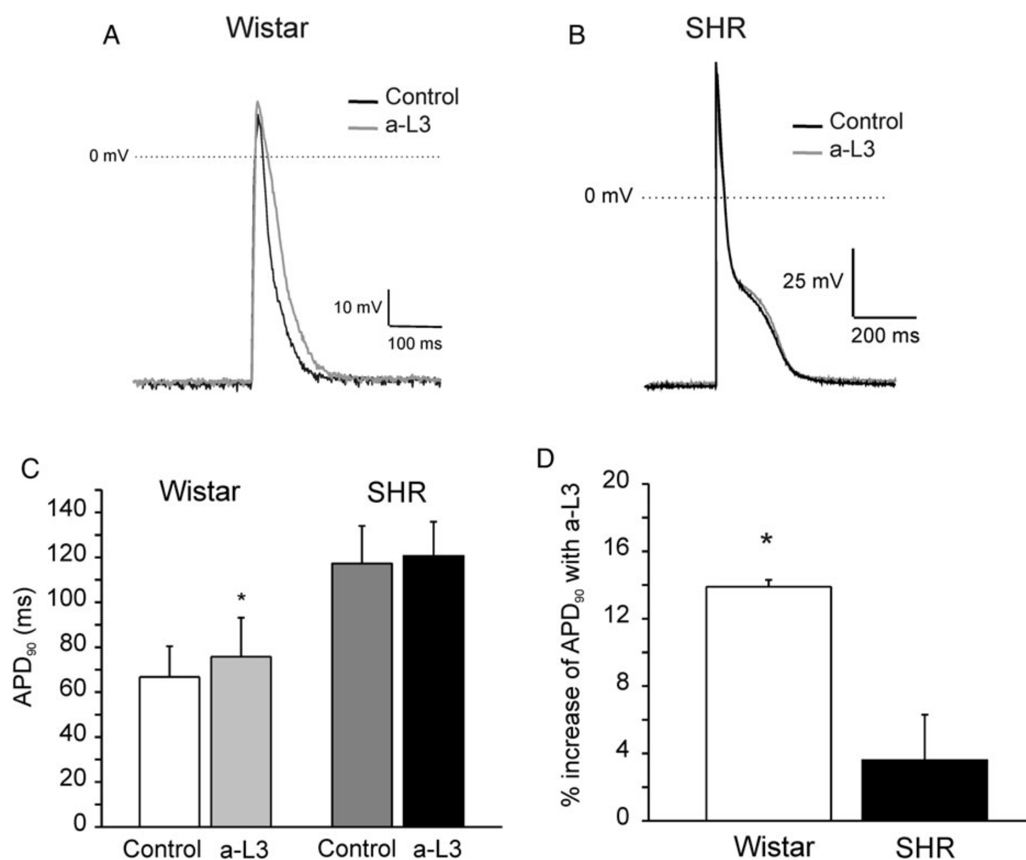

Figure 5 Impact of NBCe1 current in Wistar and SHR cardiac action potential (AP) configuration. (*A*) Representative AP recordings performed with current-clamp in perforated-patch configuration obtained from ventricular myocytes isolated from Wistar and SHR rats, successively exposed to bicarbonate solution without a-L3 (control) and bicarbonate solution with a-L3 (a-L3; dilution 1/500). (*B*) Average values of APD_{90} of Wistar myocytes in the absence and presence of a-L3 ($n = 5$) and of SHR myocytes in the absence and presence of a-L3 ($n = 6$). $^{*}P < 0.05$ vs. control. (*C*) Average of the percentage increase of APD_{90} after exposing the cell to a-L3 in Wistar and SHR rats. Asterisk indicates that the Difference is statistically significant.

control.[38–41] Moreover, we showed herein that the treatment with losartan did not normalize the decreased intrinsic buffer capacity observed in the hearts of SHR. Thus, it is possible that the cardiac hypertrophy is not responsible for the altered intrinsic buffering capacity, but rather an endogenous property of the strain.

In the last years, the implication of NBC in cardiac pathophysiology started to be studied. Several studies have demonstrated that cardiac NBC is activated during ischaemia-reperfusion.[42–44] Moreover, Khandoudi *et al.*[45] have demonstrated that selective inhibition of NBCe1 during reperfusion after ischaemia significantly improved contractile recovery, indicating that this transporter contributes to the characteristic intracellular Na^{+} and Ca^{2+} overload produced by this pathology. These authors also showed that NBCe1 is overexpressed in human heart failure.[45] It was also demonstrated that local myocardial infarction leads to an increase in both mRNA level and NBC protein expression and, as a consequence, NBC activity was enhanced.[46] Chronic treatment with blockers of the Ang II signalling, either with ACE inhibitor or with AT_1 receptor antagonists, effectively reduced mRNA[27] and protein NBC up-regulation and transport activity,[46] demonstrating a close relationship among NBC, Ang II, and myocardial infarction.

Yamamoto *et al.*[27] have demonstrated that NBCe1 and NBCn1 were overexpressed in ventricular myocytes isolated from hypertrophied rat hearts subjected to non-ischaemic pressure overload. Although the authors suggested that the function of both isoforms was also enhanced, they could not clearly demonstrate the functional up-regulation of NBCe1 in the hypertrophied hearts.[27] Nevertheless, our results show that, as in Yamamoto's work, the expression of both isoforms is increased, but in contrast with their study, the activity of NBCe1 was significantly decreased, probably as a consequence of a reduced NBCe1 protein in the cell surface. The disagreement between both investigations is not clear to us, but should be explained by the different stimulus that generates the hypertrophy and/or the time that they expended in this condition. Interestingly, in both studies the changes in the expression and function of NBC isoforms were prevented using an AT_1 receptor blocker,[27] suggesting that this hormone might be involved in the changes in NBC function and expression during the development of the maladaptive cardiac hypertrophy. In the present work, we confirmed that Ang II is directly involved in NBC isoforms remodelling during cardiac hypertrophy. However, at the moment we cannot state if the changes in NBC isoforms expression and activity are a cause or a consequence of cell growth. Nevertheless, the possibility that the remodelling of the NBC isoforms may not be a cause or a consequence of the pathology but simply a different phenotype of a different rat strain cannot be discarded.

It has been demonstrated that Ang II induces NBCe1 internalization in Xenopus oocytes.[30] Our data demonstrated that in SHR there is a

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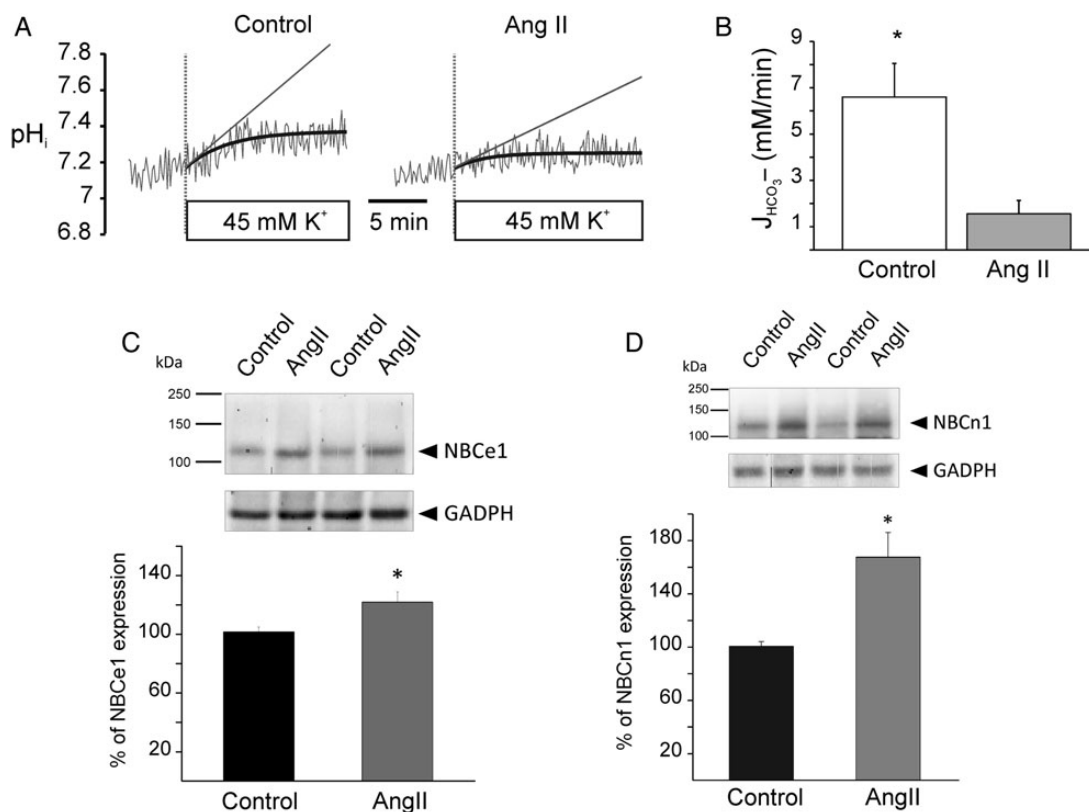

Figure 6 NBC activity and expression in rats infused with Ang II. (*A*) Representative traces of pH_i recorded from ventricular myocytes isolated from a control and from rats treated during 30 days with Ang II. The slope (grey line), corresponding to the initial velocity during the alkalinization produced by the potassium pulse, was used to determined HCO_3^- influx. The vertical dotted lines indicate the points on the fit curves at which these slopes were determined. (*B*) Average data of the $J_{HCO_3^-}$ obtained from the initial velocity of the pH_i alkalinization induced by the hyperkalaemic-induced depolarization of membrane potential in myocytes from a control ($n = 8$) and from Ang II-treated rats ($n = 9$). $*P < 0.05$ vs. Ang II. Percentage average expression of NBCe1 (*C*) or NBCn1 (*D*) (normalized by GAPDH expression) obtained from lysates of ventricles from a control ($n = 6$) or with Ang II ($n = 7$). The expression of NBCe1 or NBCn1 in a control was taken as 100%. $*P < 0.05$ vs. control. A representative blot is shown at the top.

greater amount of NBCe1 protein inside the myocyte in comparison with the cell surface. The qualitative confocal images suggest that NBCe1 is retained near the nucleus, probably in the endoplasmic reticulum. This altered NBCe1 distribution correlates with less activity of this NBC isoform measured with the potassium and ammonium pulses. Nevertheless, we cannot clearly state whether Ang II decreases sarcolemmal stabilization of the protein stimulating its internalization,[30] or there is an inhibition of the recycling of endosomes with NBCe1 during the development of cardiac hypertrophy, or there is a disturbance in the trafficking to the membrane that prevents the arrival of the protein to the surface. More experiments are needed to clarify this issue.

In the present work, we described for the first time the remodelling of the NBC isoforms in the hypertrophied hearts of the SHR. These changes in NBC isoform expression and function described herein appears to be deleterious rather than compensatory, which allows us to speculate that these alterations might be indeed involved in the development or progression of cardiac hypertrophy. The up-regulation of NBCn1, which carries more sodium to pull the same amount of bicarbonate than the NBCe1, could fairly contribute to $[Na^+]_i$ and $[Ca^{2+}]_i$ overload. On the other hand, the prolongation of the cardiac AP expected after NBCe1 inhibition[4,9] might be detrimental due to the increase in calcium influx through voltage-dependent L-type Ca^{2+} channel during the plateau. Indeed, the cardiac AP lengthening represents a hallmark of cardiac hypertrophy[47] and it was widely reported as one of the main causes that lead to this maladaptive growth of the heart.[48] Overall, the results of the present work open a new perspective regarding the investigation of the NBC isoforms as potential new therapeutic targets in the treatment of the pressure-overload induced hypertrophy.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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