ORIGINAL RESEARCH

Regulation of Intracellular pH is Altered in Cardiac Myocytes of Ovariectomized Rats

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***Background*—**It is well known that after menopause women are exposed to a greater cardiovascular risk, but the intracellular modifications are not properly described. The sodium/proton exchanger (NHE) and the sodium/bicarbonate cotransporter (NBC) regulate the intracellular pH and, indirectly, the intracellular sodium concentration ($[Na^+]$). There are 2 isoforms of NBC in the heart: the electrogenic ($1Na^+/2HCO_3^-$; NBCe1) and the electroneutral ($1Na^+/1HCO_3^-$; NBCn1). Because NHE and NBCn1 hyperactivity as well as the NBCe1 decreased activity have been associated with several cardiovascular pathologies, the aim of this study was to investigate the potential alterations of the alkalinizing transporters during the postmenopausal period.

***Methods and Results*—**Three-month ovariectomized rats (OVX) were used. The NHE activity and protein expression are significantly increased in OVX. The NBCe1 activity is diminished, and the NBCn1 activity becomes predominant in OVX rats. p-Akt levels showed a significant diminution in OVX. Finally, NHE activity in platelets from OVX rats is also higher in comparison to sham rats, resulting in a potential biomarker of cardiovascular diseases.

***Conclusions*—**Our results demonstrated for the first time that in the cardiac ventricular myocytes of OVX rats NHE and NBC isoforms are altered, probably because of the decreased level of p-Akt, compromising the ionic intracellular homeostasis. **(*J Am Heart Assoc*. 2019;8:e011066. DOI: 10.1161/JAHA.118.011066.)**

Key Words: Alcalinizing transporters • cardiac myocyte • cardiac remodeling • cardiovascular pathophysiology

Epidemiological studies show that the incidence of cardiovascular disease (CVD) is higher in men than in premenopausal women. The incidence of such events rises following menopause, suggesting that, because of reduced estrogen levels after menopause, women lose an important cardiovascular protective mechanism and are at greater risk of developing CVD.[1-3] It is well known that estrogens have multiple protective effects on the cardiovascular system.[4,5] In animal models it has been demonstrated that estrogens reduce cardiac ischemia/reperfusion injury, improving the functional response after such a protocol,[6-8] and mitigate angiotensin II–induced cardiac hypertrophy in female rats.[9]

Cardiomyocytes possess 3 different and functional estrogen receptors (ERs): the 2 classic nuclear ERs (ERα and ERβ) and a G protein–coupled ER known as GPER. ERα and ERβ classically bind estrogen and translocate to the nucleus where they modulate transcription,[10] whereas GPER, which is localized at the plasma and intracellular membranes, activates several rapid and nongenomic intracellular signaling cascades.[11-14]

The sodium/proton exchanger (NHE) and the sodium/bicarbonate cotransporter (NBC) are the most important alkalinizing mechanisms in cardiac myocytes.[15,16] They not only regulate the intracellular pH (pH_i) but also modulate the intracellular sodium concentration ($[Na^+]_i$) and indirectly, through sodium/calcium antiporter activity, regulate the intracellular calcium concentration ($[Ca^{2+}]_i$).[17-20] It has been fully demonstrated that the overactivation of NHE and NBC is involved in Na^+ and Ca^{2+} overload,[17,20] leading to the development and progression of several CVD types.[21-29]

At present, it is known that at least 2 functional isoforms of NBC exist in ventricular myocytes: the electroneutral (stoichiometry $1Na^+/1HCO_3^-$), called NBCn1, and the electrogenic (stoichiometry $1Na^+/2HCO_3^-$), called NBCe1.[30] In our laboratory we demonstrated that NBCe1 generates an anionic

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Clinical Perspective

What Is New?

- The protein expression and the activity of the sodium/proton exchanger are increased in ovariectomized rats.
- The activity of the electrogenic sodium/bicarbonate cotransporter is decreased, whereas the activity of the electroneutral isoform is increased in ovariectomized rats.

What Are the Clinical Implications?

- These results represent a first step in the investigation of the association of the functional changes of these transporters to the cardiovascular risk during menopause and open the possibility of implementing new strategies to prevent or revert the development of cardiovascular pathologies during this period.

current that shortens the action potential duration in both rat and cat isolated ventricular myocytes.[31,32] In addition, NBCe1, because of its stoichiometry, promotes the influx of less Na^+ for each HCO_3^-. This allowed us and other groups to refer to this NBC isoform as a "Na^+-sparing" mechanism.[19,21] Interestingly, it was proposed that NBCe1 could be beneficial for heart function, and NBCn1 and NHE be harmful for it, especially when they are overactivated.[33] In this regard it was demonstrated that in spontaneously hypertensive rats (SHR) the NBCe1 activity is impaired, and this is compensated with an overexpression of NBCn1. This remodeling of NBC isoforms could explain, at least in part, the development of cardiac hypertrophy and the prolongation of the action potential duration of these rats.[21]

One of the most important pathways that mediate the cardiac protective effects of estrogens is the pathway involving PI3 kinase/Akt signaling.[34] However, the specific intracellular mechanism that generates the protection is not well known. In this regard, in 2008, Snabaitis et al demonstrated that Akt-mediated phosphorylation at Ser-648 inhibits NHE activity during intracellular acidosis.[35] Moreover, this inhibitory phosphorylation is involved in the physiological cardiac hypertrophy induced by aerobic exercise,[36] preventing the sodium overload observed in the pathologic cardiac hypertrophy. In addition, we have demonstrated that the activation of the Akt pathway stimulates NBCe1 activity.[37,38]

It has been demonstrated that ovariectomized (OVX) rats, as an animal model of menopause, may exhibit increased ischemia/reperfusion injury,[39,40] cardiac hypertrophy,[41] diastolic dysfunction, increase in systolic blood pressure,[42-44] and calcium abnormalities.[45,46] However, the mechanisms underlying these detrimental phenomena remain to be fully elucidated. Herein, we describe for the first time that OVX rats exhibit modifications of the NHE and NBC isoform activity, which can explain, at least in part, the increased CVD observed during menopause.

Material and Methods

The authors declare that all supporting data within this article are available.

Animals

All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of La Plata University. Three-month-old female Wistar rats were divided based on computer-generated numbers to achieve randomization and assigned to undergo either bilateral OVX (20 rats) or a sham operation (16 rats). The animals were anesthetized with isoflurane 3% for induction and 1.5% during the surgical procedure, which consisted of a bilateral removal of the ovaries. Briefly, a small incision was performed in the middle part of the abdomen, and the abdominal cavity was accessed through the *linea alba*. Adipose tissue was pulled away until the uterine horn and the ovaries were identified. The ovaric arteries were ligated, and both ovaries were extracted. The uterine horns were returned to the peritoneal cavity after the removal of the ovaries, and the wound was closed using sterile 4-0 Nylon sutures. (Surgikal Cardiopack Argentina SA, Buenos Aires, Argentina). The sham operation was performed by exposing the ovaries without isolation. Successful ovariectomy was confirmed by uterine atrophy. Thirty days after the surgery the animals were euthanized and their hearts and blood were used for different experiments. The physiological parameters (body weight and systolic blood pressure) were measured before the surgery and 30 days after it, before euthanasia of the animals. The systolic blood pressure was indirectly measured with the tail-cuff method as has been previously described.[47]

Ventricular Myocyte Isolation

Rat ventricular myocytes were isolated according to the technique previously described with some modifications.[48] Briefly, the hearts were attached via the aorta to a cannula, excised, and mounted in a Langendorff apparatus. They were then retrograde perfused at 37°C with Krebs-Henseleit solution (K-H) of the following composition (in mmol/L): 146.2 NaCl, 4.7 KCl, 1 $CaCl_2$, 10 HEPES, 0.35 NaH_2PO_4, 1 $MgSO_4$, and 10 glucose (pH adjusted to 7.4 with NaOH). The solution was continuously bubbled with 100% O_2. After a stabilization period of 4 minutes, the perfusion was switched to a nominally Ca^{2+}-free K-H for 5 minutes. The hearts were

then recirculated with collagenase (138 units/mL) in K-H containing 60 μmol/L $CaCl_2$. Perfusion continued until the hearts became flaccid (15 minutes). The hearts were then removed from the perfusion apparatus by cutting at the atrioventricular junction. The desegregated myocytes were separated from the undigested tissue and rinsed several times with a K-H solution containing 1% BSA. The $CaCl_2$ concentration of K-H solution was increased in 4 steps to 1 mmol/L. Myocytes were kept in 1 mmol/L $CaCl_2$ K-H solution at room temperature (20°C to 22°C) until use.

Platelet Isolation and Platelet pH_i Measurements

Blood (7 mL) was extracted directly from the rat's heart, mixed with 2 mL of anticoagulant (2.5% sodium citrate, 1.5% citric acid, and 2.0% glucose) and centrifuged at 300*g* for 15 minutes. The supernatant (platelet-rich plasma) was collected and centrifuged (400*g*, 15 minutes). The pellet obtained was washed twice with a calcium-free buffer (mmol/L: NaCl 140, KCl 5, EGTA 0.5, acetylsalicylic acid 1, glucose 10, and HEPES 10 as well as 1% BSA; pH 7.4). After resuspension in the same solution but without EGTA, the platelets were incubated with the pH-sensitive fluorescent dye BCECF-AM (20 mmol/L) for 20 minutes at 37°C. The dye-loaded platelets were subjected to intracellular acidification by preincubation with NH_4Cl (20 mmol/L, 20 minutes) and then transferred to a sodium-free solution (mmol/L: N-methyl-D-glucamine 140, KCl 5, EGTA 0.1, $MgSO_4$ 1, glucose 10, HEPES 10, pH 7.4) until use. The experiments were performed in the nominal absence of bicarbonate (HEPES buffer) to ensure that the pH_i recovery after the acidic load was entirely due to NHE-1 activation. NHE-1 was activated by reintroduction of external Na^+. Aliquots of 50 μL of acidified platelet suspension were diluted in 2 mL of HEPES buffer (mmol/L: NaCl 140, KCl 5, $CaCl_2$ 1, $MgSO_4$ 1, glucose 10, HEPES 10, pH 7.4) in the stirred and thermally stabilized cuvette of a spectrofluorometer (Aminco Bowman series 2, Thermo Fisher, Waltham, MA) and excited at 503 and 440 nm; the emitted fluorescence was collected at 535 nm. The signal at 440 nm (pH_i insensitive) was used as an internal control of platelet aggregation, and it did not show significant changes during the experiments. The pH_i recovery rate was calculated to express NHE activity.

Calibration of the fluorescence signals was carried out in a high-potassium nigericin solution (135 mmol/L KCl replaced the same concentration of NaCl in the HEPES buffer) with 10 μmol/L nigericin, which balances intracellular and extracellular pH.[49]

pH_i Measurements in Isolated Myocytes

pH_i was measured in single myocytes with an epifluorescence system (Ion Optix, Milton, MA), using the previously described BCECF technique.[50] Briefly, myocytes were incubated at room temperature for 10 minutes with 10 μmol/L BCECF-AM followed by a 30-minute washout. Dye-loaded cells were placed in a chamber on the stage of an inverted microscope (Nikon TE 2000-U, Nikon, Tokyo, Japan) and continuously superfused with 2 different solutions, 1 to measure NBC activity, containing (mmol/L) 5 KCl, 118 NaCl, 1.2 $MgSO_4$, 0.8 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 20 $NaHCO_3$, pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2 and in the continuous presence of cariporide (HOE 642) 10 μmol/L (NHE blocker) and the other to measure NHE activity, containing (mmol/L) 146.2 NaCl, 4.69 KCl, 11 glucose, 10 HEPES, 0.35 $NaH_2PO_4.H_2O$, 1.05 $MgSO_4.7H_2O$, 1 $CaCl_2$, pH 7.4. The myocytes were stimulated via 2 platinum electrodes on either side of the bath at 0.5 Hz. Dual excitation (440 and 495 nm) was provided by a 75-W xenon arc lamp and transmitted to the myocytes. Emitted fluorescence was collected with a photomultiplier tube equipped with a band-pass filter centered at 535 nm. The 495- to 440-nm fluorescence ratio was digitized at 10 kHz (ION WIZARD fluorescence analysis software, IonOptix, Westwood, MA). At the end of each experiment the fluorescence ratio was converted to pH by calibrations using the high-K^+–nigericin method.[49]

Ammonium Pulse

The alkalinizing transporters' activity was assessed by evaluating the pH_i recovery from an ammonium prepulse-induced acute acid load. The NHE activity was evaluated using an external solution free of bicarbonate (HEPES), whereas NBC activity was evaluated in the presence of bicarbonate bubbled with 95% O_2/5% CO_2 and HOE 642 10 μmol/L (NHE blocker). Transient (3-minute) exposure of myocytes to 20 mmol/L NH_4Cl was used for this purpose. The dpH_i/dt at each pH_i, obtained from an exponential fit of the recovery phase, was analyzed to calculate the net H^+ efflux (J_H) using the following equation: $J_H=\beta_{tot}\ dpH_i/dt$, where β_{tot} is total intracellular buffering capacity. For HCO_3^- solution, β_{tot} was calculated by the sum of the intracellular buffering due to CO_2 (β_{CO2}) plus the intrinsic buffering capacity (β_i). β_{CO2} was calculated as $\beta_{CO2}=2.3\ [HCO_3^-]_i$, where $[HCO_3^-]_i=[HCO_3^-]_o\ 10^{pHi-pHo}$.[51,52] β_i of the myocytes was measured by exposing cells to varying concentrations of NH_4Cl in Na^+-free HEPES bathing solution. pH_i was allowed to stabilize in Na^+-free solution before application of NH_4Cl. β_i was calculated from the equation $\beta_i=\Delta[NH_4^+]_i/\Delta pH_i$ and referred to the midpoint values of the measured changes in pH_i. β_i at different levels of pH_i were estimated from the least-squares regression lines of β_i-versus-pH_i plots. In order to distinguish the NBC isoforms, we used a noncommercial selective functional antibody against the extracellular loop 3 of the NBCe1 (a-L3) generated in our laboratory.[30] In these experiments nonimmune serum was

used as control, and serum containing the a-L3 was used to discriminate 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.[30]

Potassium Pulse

To investigate the NBCe1 activity in isolation we performed a potassium pulse. Increasing extracellular K^+ ($[K^+]_o$) isotonically from 5 to 45 mmol/L produced a depolarization of ≈60 mV that enhanced the NBCe1 activity and in turn elevated pH_i.[30,38] The high K^+ was applied for 15 minutes, and during this period the pH_i was recorded. The HCO_3^--buffered solution used in the K^+-induced depolarization experiments contained (mmol/L) 118 NaCl, 5 KCl, 1 $MgSO_4$, 0.35 NaH_2PO_4, 10 glucose, 40 choline chloride, 20 $NaHCO_3$, pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2. K^+-induced depolarization was assessed by replacing 40 mmol/L choline chloride with 40 mmol/L KCl, maintaining ionic strength. The alkalization induced by hyperkalemic solution only occurred in the presence of extracellular sodium and bicarbonate.[30] Moreover, the NHE blocker HOE 642 could not prevent the increase in the intracellular pH_i. Only the preincubation of the myocytes with S0859 (an NBC blocker) or with the selective NBCe1 inhibitory antibody a-L3 abolished the hyperkalemia-induced alkalization.[30]

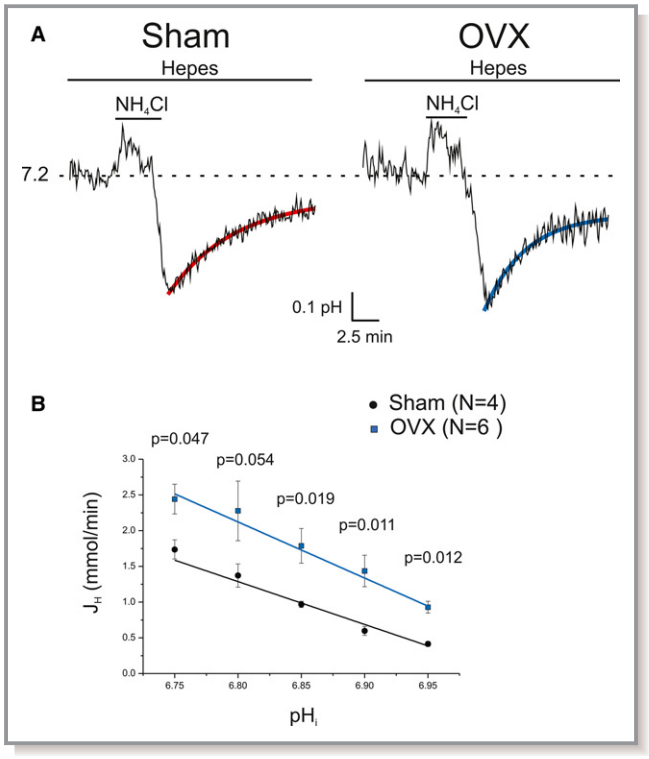

Figure 1. NHE function in OVX myocytes is increased in comparison to sham myocytes. **A**, Representative traces of intracellular pH (pH_i) during the application of an ammonium pulse (20 mmol/L NH_4Cl) in sham and OVX ventricular myocytes perfused with HEPES buffer. **B**, Average proton efflux (J_H) carried by NHE calculated at different pH_i values during the recovery from acidosis in sham and OVX myocytes. The values were fitted with the following linear regression (sham: $JH = -6.00pH_i + 42.07$; R^2, 0.97; and OVX: $J_H = -7.85pH_i + 55.53$; R^2, 0.99). *P*-value vs sham is depicted above each average point. N indicates number or rats for each group; NHE, sodium-proton exchanger; R^2, R-Squared.

Table. Model Characterization

	Sham	OVX
ΔBW, g	29.2±3.02 (n=16)	57.1±4.2 (n=14)*
ΔSBP, mm Hg	−2.7±2.9 (n=14)	10.23±3.9 (n=12)†
UW/TL, mg/cm	0.15±0.01 (n=13)	0.08±0.01 (n=14)*
HW, mg	0.88±0.05 (n=6)	0.89±0.09 (n=6)
HW/BW, mg/g	0.0043±0.0002 (n=6)	0.0040±0.0002 (n=6)

ΔBW indicates change in body weight between presurgical measurement and after 30 days (before use); ΔSBP, change in systolic blood pressure between presurgical measurement and after 30 days (before use); HW, heart weight after euthanasia; HW/BW, heart weight/body weight in sham and OVX rats; UW/TL, uterine weight/tibia length.
*P<0.0001 vs sham.
†P<0.05 vs sham.

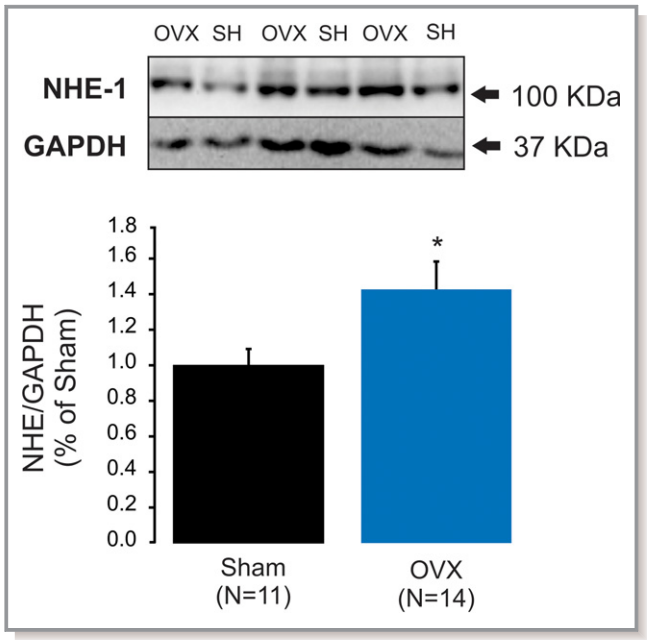

Figure 2. NHE protein expression in OVX hearts is greater than in sham hearts. Representative immunoblots showing the increased expression of NHE in OVX heart homogenates in comparison to sham (SH). Average data are depicted in the bar graphs as percentage of NHE/GAPDH respect to Sham. *P<0.05 vs Sham. N indicates number of hearts for each group; NHE, sodium/proton exchanger; OVX, ovariectomized rats.

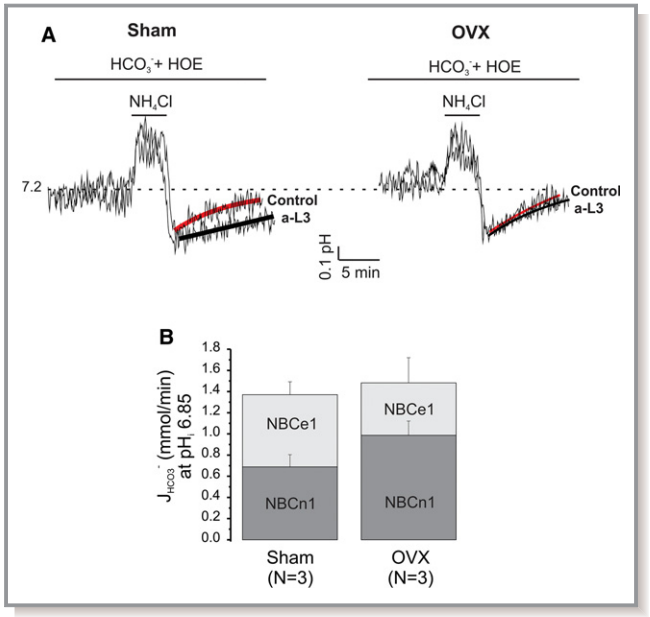

Figure 3. NBC isoform activity. **A**, Superimposed representative traces of intracellular pH (pH_i) during the application of an ammonium pulse in the absence (nonimmune serum as control) or presence of the serum containing the functional inhibitory antibody against NBCe1 (a-L3; 1:500). The experiments were performed in the continuous presence of the NHE inhibitor HOE 642 (10 μmol/L). **B**, Average bicarbonate influx (J_{HCO3}) calculated at pH_i 6.85 in the absence (control) or presence of a-L3. 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. N indicates number of rats for each group; NBCe1, electrogenic sodium-bicarbonate cotransporter; NBCn1, neutral sodium-bicarbonate cotransporter; OVX, ovariectomized rats.

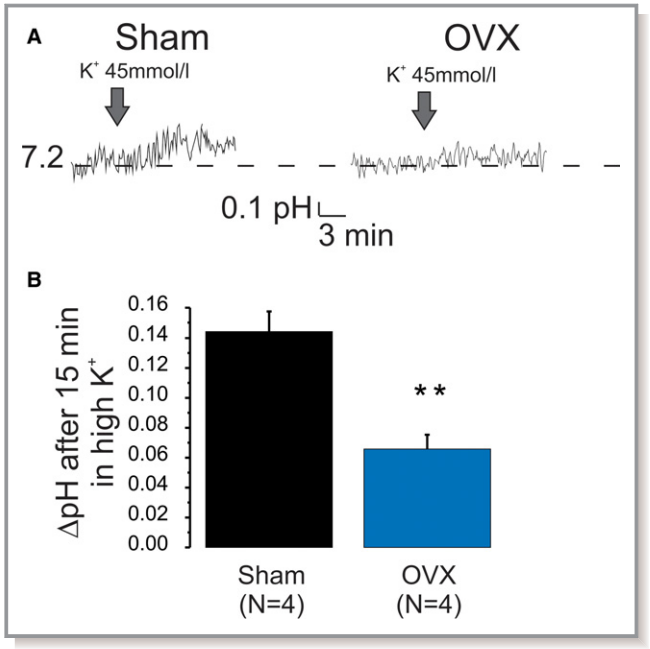

Figure 4. NBCe1 activity is impaired in OVX myocytes. **A**, Representative traces of intracellular pH (pH_i) recorded in sham and OVX ventricular myocytes during the application of a potassium pulse (45 mmol/L K^+). **B**, Average data of pH_i alkalinization induced by the hyperkalemic solution in sham and in OVX myocytes. Data are expressed as increase of pH_i units after 15 minutes in comparison to the 0 time point in high-K^+ solution. $^{**}P<0.01$ vs sham. N indicates number of rats for each group; NBCe1, electrogenic sodium-bicarbonate cotransporter; OVX, ovariectomized rats.

Immunodetection

Rat hearts were homogenized, and protein was measured by the Bradford method using BSA as standard. Samples (equal amounts of proteins) were seeded in 8% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Blots were probed overnight with antibodies raised against Akt (1:1000; Cell Signaling, Danvers, MA); phospho-Ser473 Akt (P-Akt, 1:1000; Cell Signaling), NHE-1 (1:1000; Santa Cruz Biotechnology, Dallas, TX); NBCe1 (1:1000; Millipore, Burlington, MA), and NBCn1 (1:1000; Santa Cruz Biotechnology). GAPDH detection (1:1000; Santa Cruz Biotechnology) was used as a loading control. Immunoreactivity was visualized by a peroxidase-based chemiluminescence detection kit (Immobilon Western, Millipore) using a Chemidoc Imaging System (Bio-Rad, Hercules, CA). The signal intensity of the bands in the immunoblots was quantified by densitometry using Image J software (National Institutes of Health, Bethesda, MD).

Statistics

GraphPad Prism 6 (GraphPad, San Diego, CA) was used for all the statistics analysis, and OriginPro 8 (OriginLab, Northampton, MA) was used for linear fitting in Figure 1B. Data were expressed as means±SEM. The Shapiro-Wilk test was used to test normality. Data were compared with Student t test or t-test with Welch's correction if variances could not be assumed to be equal. The number of animals (N) was used for the statistics analysis. For the functional experiments, each value represents an average of 1 to 5 individual measurements (myocytes or platelets). A value of $P<0.05$ was considered statistically significant (2-tailed test).

Results

Physiological Parameters

The differences in body weight and baseline systolic blood pressure between the presurgical values and the values 1 month after surgery were significantly higher in OVX than in sham animals (Table). As previously reported, no significant differences in heart weight or in heart weight/body weight

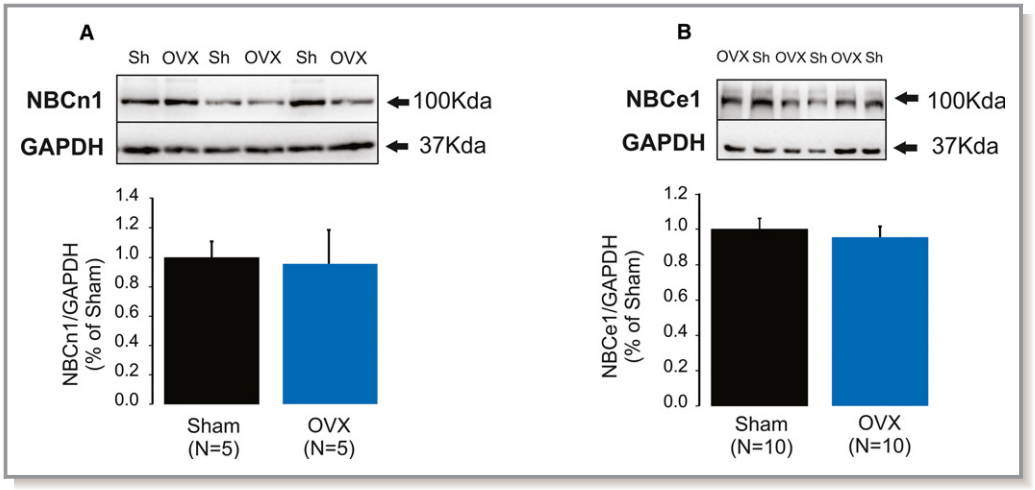

Figure 5. NBC protein expression did not change between sham and OVX hearts. **A**, Representative immunoblots showing the expression of NBCn1 and NBCe1 in sham and OVX heart homogenates. **B**, Average data are depicted in the bar graphs as percentage of NBCn1/GAPDH or NBCe1/GAPDH relative to sham. N indicates number of hearts for each group; NBCe1, electrogenic sodium-bicarbonate cotransporter; NBCn1, neutral sodium-bicarbonate cotransporter; OVX, ovariectomized rats; Sh, sham.

index were evident between groups.[43,46] Uterine weight was decreased in OVX, consistent with the lack of circulating estrogen (Table).

Activity and Expression of NHE Are Increased in OVX Rats

Because NHE hyperactivity or its overexpression[49,53-55] has been widely demonstrated to be involved in cardiac pathologies, we decided to investigate if the function and/or protein expression of NHE in the ventricular myocytes of the OVX rats were also altered. As shown in the representative traces of pH_i (Figure 1A) and in the proton efflux (J_H) during the recovery from acidosis (Figure 1B), the NHE activity was significantly increased in OVX myocytes in comparison to sham myocytes.

In agreement with these functional results, W-B (Western-Blot) analysis demonstrated that NHE protein expression in OVX was significantly higher than that in sham rats (Figure 2).

Total NBC Activity Is Similar Between Sham and OVX Myocytes, But There is a Remodeling of NBC Isoforms

Figure 3A illustrates representative superimposed traces of sham and OVX myocytes in the presence and absence of the inhibitory antibody against the extracellular loop 3 of the NBCe1 (a-L3, dilution 1/500). Figure 3B compares the J_{HCO3-} calculated at pH_i of 6.85 in both conditions. Interestingly, total NBC activity was similar in sham and OVX. However, whereas NBCn1 and NBCe1 showed equivalent activity in sham myocytes, the OVX myocytes exhibited a predominance of NBCn1 activity. These results are in concordance with our own data in SHR rats, which exhibit a remodeling in cardiac NBC isoforms, resulting in a decrease of NBCe1 expression in the cell surface and a compensatory upregulation of NBCn1.[21]

In order to confirm if NBCe1 activity is impaired in OVX myocytes, sham and OVX myocytes were exposed to a high extracellular K^+ solution. This hyperkalemic solution induced a depolarization of the membrane potential, which, as we have previously demonstrated, selectively stimulates NBCe1, leading to cellular alkalization.[30,38] Figure 4A shows representative traces of continuous pH_i recordings in sham and OVX myocytes. As observed in the average data, NBCe1 activity is significantly reduced in OVX myocytes (Figure 4B).

In contrast to what happened with NHE, the functional modification in NBC isoforms was not correlated with a variation in their total protein expression (Figure 5A and 5B).

Implications of NHE and NBC After Intracellular Acidosis in Bicarbonate Solution

On the basis of previous experiments, we decided to investigate if the total alkalizing transporter activity (in a medium with bicarbonate) is increased in OVX myocytes in comparison to the shams. Figure 6A shows representative traces during an ammonium pulse. Figure 6B shows that the total J_H at pH_i 6.85 in OVX myocytes is significantly higher in comparison to sham ones, and, more relevant, it shows the relative contribution of the alkalinizing transporters to the recovery from acidosis. The NHE activity is calculated as the

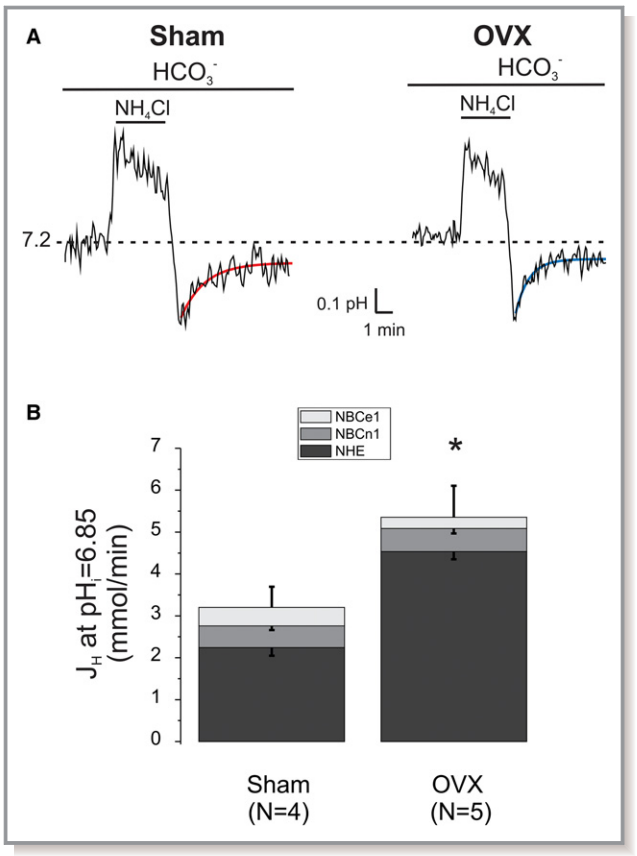

Figure 6. Relative contribution of the transporters to the pH_i recovery in bicarbonate solution. **A**, Representative traces of intracellular pH (pH_i) during the application of an ammonium pulse (20 mmol/L NH_4Cl) in sham and OVX ventricular myocytes perfused with bicarbonate buffer. **B**, Average bicarbonate influx (J_{HCO3-}) at pH_i 6.85 in sham and OVX myocytes, evidencing the relative contributions of NHE, NBCn1, and NBCe1 to the total recovery. **P*<0.05 vs sham. N indicates number of rats for each group; NHE, sodium/proton exchanger; NBCe1, electrogenic sodium-bicarbonate cotransporter; NBCn1, neutral sodium-bicarbonate cotransporter; OVX, ovariectomized rats.

J_H at pH_i 6.85 in HCO_3^- minus the J_H in HCO_3^- plus HOE 642. Then, the NBCn1 activity is calculated as the J_H at pH_i 6.85 in the presence of HCO_3^- plus HOE 642 and a-L3; and finally, the NBCe1 activity is calculated as the J_H at pH_i 6.85 in HCO_3^- plus HOE 642 minus that in HCO_3^- plus HOE 642 and a-L3.

Implications of NHE and NBC During Steady-State pH_i After Recovery From Intracellular Acidosis

We next wanted to investigate the consequence of the modification of the alkalinizing transporters in pH_i during the steady state. As shown in Figure 7A, the final pH_i reached after recovering from the intracellular acidosis (steady state) in extracellular HEPES solution (where the only active alkalinizing transporter is the NHE) is significantly higher in OVX myocytes in comparison to the shams. On the other hand, the steady-state pH_i in the presence of extracellular HCO_3^-, where both the NHE and the NBC isoforms are active, is similar between those 2 groups (Figure 7B). Note that the maximal acidification with or without bicarbonate was similar in OVX and sham rats (Figure 7C and 7D), ruling out that the activity of the alkalinizing transporters could be influenced by an initial different pH_i.

Role of Akt in NHE and NBC Isoform Remodeling

Because Akt is an important molecule that has been involved in several protective intracellular pathways of estrogens, we aimed to investigate whether the amount of p-Akt is impaired in OVX hearts and if this impairment is interfering in the physiologic Akt-induced NHE and NBCe1 regulation. We measured p-Akt in sham and OVX hearts. Figure 8 exhibits representative W-B and the average data, which clearly illustrate that p-Akt is decreased in OVX hearts.

NHE Activity in Platelets as a Potential Biomarker

We finally attempted to further evaluate if NHE activity in platelets might reflect the cardiac NHE performance. Figure 9A shows representative traces during the pH_i recovery. The average data demonstrated that, in concordance with the cardiac NHE, the NHE activity in OVX platelets is significantly higher than that observed in sham platelets (Figure 9B).

Discussion

The present work demonstrated for the first time that cardiac ventricular myocytes of OVX rats have several alterations in the major alkalinizing transporters (NHE and NBC) involving an increase in NHE activity and protein expression and a remodeling of NBC isoforms (decreased NBCe1 activity and higher NBCn1 activity).

It is well known that the NHE overexpression and/or its hyperactivity are associated with the development and progression of cardiac diseases. It has been demonstrated that the activation of NHE leads to an increase in intracellular sodium concentration, which activates the reverse mode of the Na^+/Ca^{2+} exchanger (rNCX), enhances the intracellular calcium concentration ($[Ca^{2+}]_i$), and finally leads to cell growth.[24,25,56] NBC has been associated with cardiac pathologies as a consequence of a similar pathway.[57-59] Interestingly, the existence of 2 functional isoforms (NBCn1 and NBCe1) makes it necessary to evaluate them as independent transporters. In this regard it has been proposed that NBCn1 hyperactivity, similarly to NHE hyperactivity, is harmful because it leads to sodium and calcium overload. In opposition, NBCe1 activity might be beneficial for myocyte

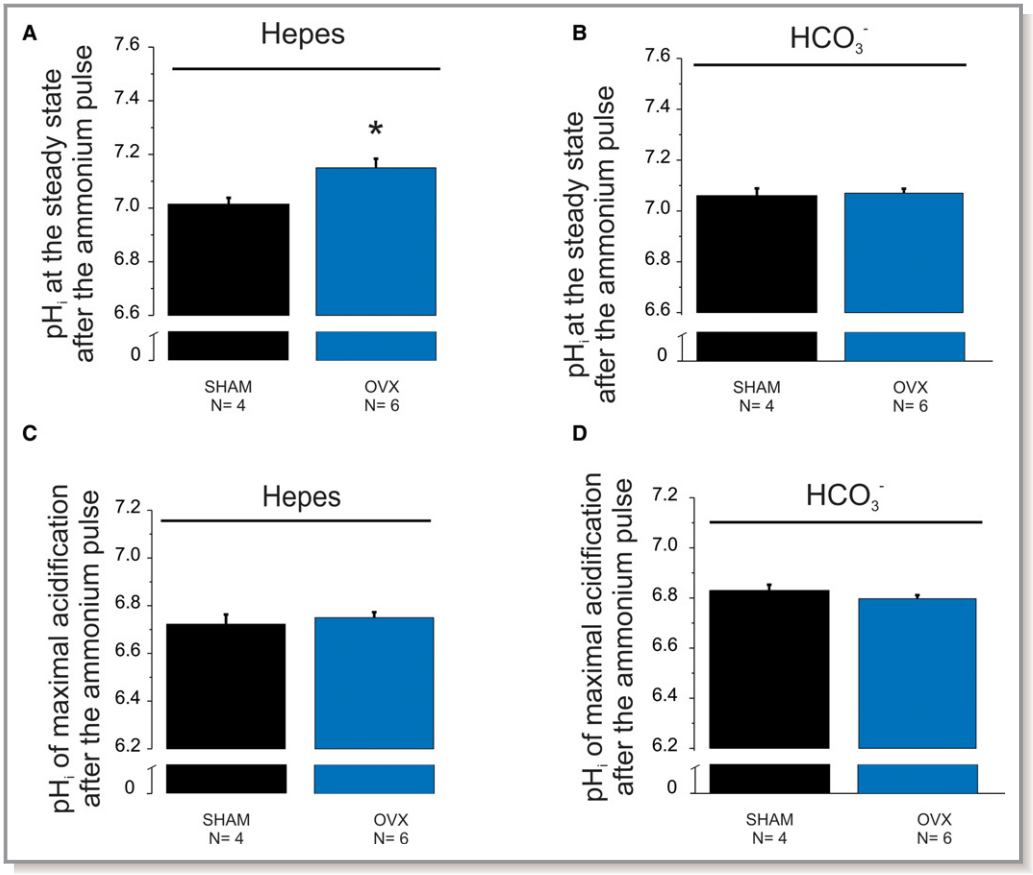

Figure 7. The modifications of the alkalinizing transporters lead to an increase in the pH_i during the steady-state only in HEPES buffer. **A**, Average final pH_i after the ammonium pulse in HEPES buffer in sham and OVX ventricular myocytes. **B**, Average final pH_i after the ammonium pulse in bicarbonate buffer in sham and OVX ventricular myocytes. **C**, Maximal acidification during the ammonium pulse in HEPES buffer in sham and OVX ventricular myocytes. **D**, Maximal acidification during the ammonium pulse in bicarbonate buffer in sham and OVX ventricular myocytes. *$P<0.05$ vs sham. N indicates number of rats for each group; OVX, ovariectomized rats.

physiology.[33] NBCe1 could be claimed as a "Na-sparing" mechanism because, based on its stoichiometry, it introduces less sodium into the myocyte per molecule of bicarbonate.[19,21] Moreover, because the NBCe1 generates an anionic current that shortens the action potential, the reduced duration of phase 2, the plateau, of the action potential would lead to less calcium entrance, preventing its overload and the development of cardiac hypertrophy.[60] In this regard we have provided evidence that in SHR rats the NBCe1 activity is decreased, and this is compensated for by an overexpression of NBCn1.[21] We suggested that this remodeling might explain the development and/or progression of the cardiac hypertrophy of SHR.[21] The results presented in this work allow us to speculate that the NHE overexpression, accompanied with the impairment of NBCe1 activity, might be deleterious for OVX myocytes and might explain, at least in part, several cardiovascular abnormalities reported in OVX rats.[39,40,45,46]

It has been previously demonstrated that NHE hyperactivity is associated with the development of cardiac hypertrophy.[23-25,54] In the present work we did not find a significant difference in heart weight between sham and OVX rats. One possibility of such a discrepancy is the difference in sex because the majority of research was done in male animals. Moreover, similar to our findings, it has been demonstrated that OVX rats did not develop cardiac hypertrophy.[43,46] In contrast, Wang et al, using oophorectomized mRen2.Lewis rats, demonstrated an increase in the ratio of heart weight/tibia length.[42] The explanation for this controversy is not apparent to us. It might be possible that the differential background of the rats affects the cardiac growth. Regarding this, the mRen2.Lewis strain has a genetic modification in the renin-angiotensin-aldosterone system that converts them in an estrogen-sensitive model. In our case we use young Wistar rats that do not have any pathology or alteration previous to the ovariectomy. Moreover, the time between the surgery and

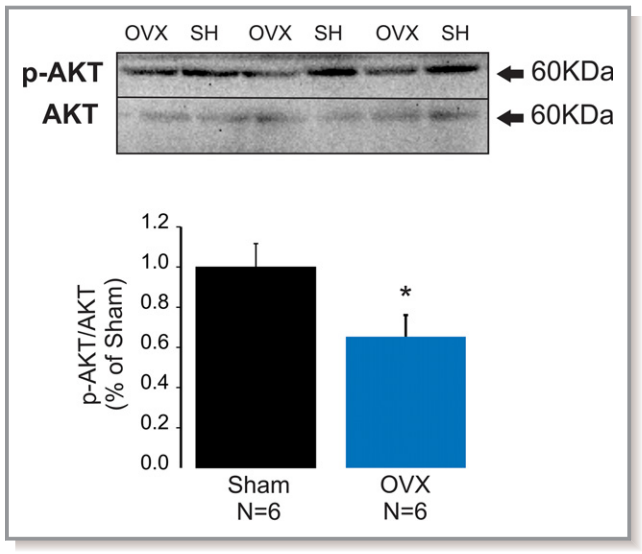

Figure 8. p-Akt expression: representative immunoblots showing the expression of p-Akt in sham and OVX heart homogenates. Average data are depicted in the bar graphs as percentage of p-Akt/Akt relative to sham. To normalize the amount of the phosphorylated proteins, the membranes were stripped and probed with an antibody that specifically detects total Akt. **P*<0.05 vs sham. N indicates number of hearts for each group; OVX, ovariectomized rats; SH, sham.

euthanasia might be important. Whereas Wang et al waited 3 months to use the rats after the ovariectomy, in the present work we used the rats only 1 month after the surgery. It might be possible that although NHE activity had already increased in our OVX rats, 1 month was not sufficient time for our OVX rats to develop cardiac hypertrophy. Last, although we found a significant increase in the systolic blood pressure in the OVX rats versus the shams, the increase did not reach hypertensive values.

The overexpression of the NHE accompanied with its upregulation in different models of CVD has been previously reported.[49,53-55] Interestingly, our results obtained in OVX rats showed the same behavior. On the other hand, in the present work, we observed a decreased activity of the NBCe1 that was not accompanied by a decrease in the protein expression. Similarly, in SHR rats the NBCe1 protein expression is higher in comparison to their control, whereas its activity is decreased, suggesting that there is a disturbance in NBCe1 protein trafficking.[21] More experiments are needed to clarify if this situation is taking place in the OVX rats.

Interesting as well, during the steady state reached after recovery from the intracellular acidosis, the pH_i is higher in the OVX rats only in the absence of extracellular bicarbonate, indicating that the NHE activity is increased. On the other hand, in the presence of bicarbonate the pH_i at the steady state is similar between OVX and sham rats, suggesting that the acidifying transporters (ie, the Cl^-/HCO_3^- exchanger or anion exchanger) might compensate for the increase in NHE activity. Although little is known about the regulation of the acidifying transporters during menopause, these results suggest that they might be upregulated, minimizing the NHE effect on pH_i. Similar results were demonstrated in SHR.[49] Nevertheless, the putative intracellular sodium overload due to the enhanced NHE activity cannot be compensated for by the activation of the anion exchanger, whose function is not coupled to sodium. Further experiments are needed in order to confirm this speculation.

It has been previously demonstrated that Akt is part of the intracellular signaling of estrogen-induced cardioprotection.[34] Moreover, it has been described that p-Akt inhibits NHE,[35,36] whereas it stimulates NBCe1.[37,38] Our results show that p-Akt is diminished in OVX hearts, perhaps due to the decrease in plasmatic estrogen levels after ovariectomy. Although the

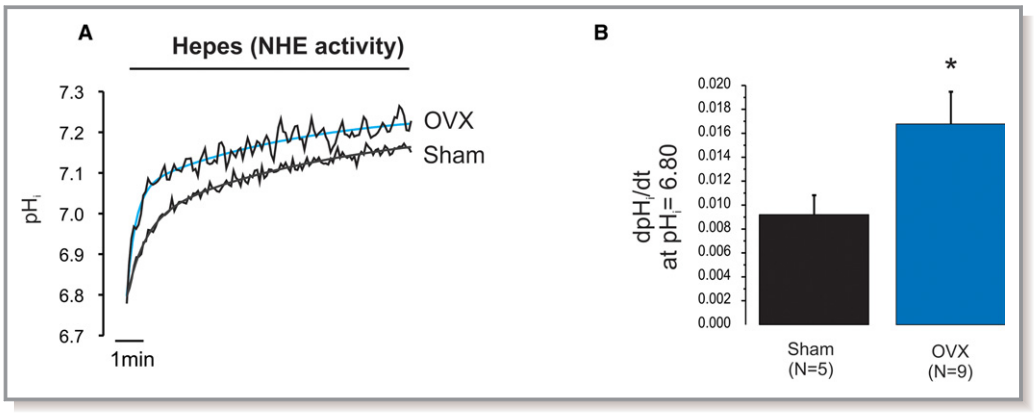

Figure 9. Platelets NHE activity. **A**, Representative traces of pH_i recorded in platelets during the recovery of an ammonium pulse in platelets perfused with HEPES buffer. **B**, Average rate of recovery from acidosis, depicted as change in pH units per minute at pH_i 6.8. **P*<0.05 vs sham. N indicates number of rats for each group; NHE, sodium/proton exchanger; OVX, ovariectomized rats.

results presented herein represent a potential chronic effect of the reduction of p-Akt levels, and the regulation of the alkalinizing transporters by this kinase was reported as an acute effect, we can speculate that the decrease in p-Akt levels in the OVX rats might explain, at least in part, the NHE upregulation and the impaired NBCe1 function.

Previous studies performed in hypertensive models reported enhanced NHE activity in different blood cell types, including platelets.[61,62] Furthermore, the enhanced activity of this transporter in these cells seems to be a reflection of the increased cardiac NHE activity that is associated with different cardiac pathologies, including the maladaptive hypertrophy induced by hypertension. Although the measurement of cardiac transporter activity in vivo is complicated, the possibility of having an indirect approximation that could be used as a potential biomarker of the progression of the cardiac alterations triggered by menopause deserves to be highlighted.

This is the first time that a cellular alteration in the cardiac alkalinizing transporters has been shown during menopause. The results presented herein might represent the first step in the investigation of the association of the functional changes of these transporters to cardiovascular risk during menopause. Moreover, we think that more studies evaluating the potential effect of chronic treatment with estrogens preventing these intracellular changes would be relevant to the knowledge about this physiological condition.

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Disclosures

None.

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