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Cardiac hypertrophy reduction in SHR by specific silencing of myocardial Na^+/H^+ exchanger

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AQ:1 **Nolly MB, Pinilla AO, Ennis IL, Cingolani HE, Morgan PE.** Cardiac hypertrophy reduction in SHR by specific silencing of myocardial Na^+/H^+ exchanger. *J Appl Physiol* 118: 000–000, 2015. First published March 6, 2015; doi:10.1152/japplphysiol.00996.2014.—We examined the effect of specific and local silencing of sodium/hydrogen exchanger isoform 1 (NHE1) with a small hairpin RNA delivered by lentivirus (L-shNHE1) in the cardiac left ventricle (LV) wall of AQ: 2 spontaneously hypertensive rats, to reduce cardiac hypertrophy. Thirty days after the lentivirus was injected, NHE1 protein expression was reduced 53.3 ± 3% in the LV of the L-shNHE1 compared with the control group injected with L-shSCR (NHE1 scrambled sequence), without affecting its expression in other organs, such as liver and lung. Hypertrophic parameters as LV weight-to-body weight and LV weight-to-tibia length ratio were significantly reduced in animals injected with L-shNHE1 (2.32 ± 0.5 and 19.30 ± 0.42 mg/mm, respectively) compared with L-shSCR-injected rats (2.68 ± 0.06 and 21.53 ± 0.64 mg/mm, respectively). Histochemical analysis demonstrated a reduction of cardiomyocytes cross-sectional area in animals treated with L-shNHE1 compared with L-shSCR (309,81 ± 20,86 vs. 424,52 ± 21 μm^2, $P < 0.05$). Echocardiography at the beginning and at the end of the treatment showed that shNHE1 expression for 30 days induced 9% reduction of LV mass. Also, animals treated with L-shNHE1 exhibited a reduced LV wall thickness without changing LV diastolic dimension and arterial pressure, indicating an increased parietal stress. In addition, midwall shortening was not modified, despite the increased wall tension, suggesting an improvement of cardiac function. Chronic shNHE1 expression in the heart emerges as a possible methodology to reduce pathological cardiac hypertrophy, avoiding potentially undesired effects caused from a body-wide inhibition of NHE1.

hypertrophy; heart; NHE1; siRNA

SPONTANEOUSLY HYPERTENSIVE rats (SHR) are a model of genetic hypertension with an early development of high blood pressure and cardiac hypertrophy (14, 18, 26, 40). Na^+/H^+ exchanger isoform 1 (NHE1) is an integral membrane protein present in almost every tissue, including the heart, that, electroneutrally exchanging H^+ for Na^+, contributes to several processes, such as cellular growth and proliferation. The hypertrophic myocardium of SHR presents a hyperactive NHE1 without significant changes in myocardial intracellular pH (36). Compensatory activity of bicarbonate-dependent mechanisms, like the anion exchangers, prevents intracellular alkalinization as a triggering signal for protein synthesis (36). However, NHE1 hyperactivity increases intracellular Na^+ concentration, the signal responsible for intracellular Ca^{2+} rise through the Na^+/Ca^{2+} exchanger and calcineurin activation, leading to pathological cardiac hypertrophy and failure (3, 8, 12, 17, 33). AQ: 3

Sustained hypertensive cardiac hypertrophy involves a pathological remodeling with an altered molecular and structural phenotype (19, 20), becoming an independent risk factor for heart failure (10). Extensive amounts of data demonstrate that pharmacological inhibition of NHE1 was beneficial in preventing cardiac remodeling in different models of pathological cardiac hypertrophy and myocardial infarction (2, 6, 13, 15, 16, 24). Particularly, cariporide administration to SHR for 1 mo reduced cardiac hypertrophy with a slight reduction of arterial pressure (6, 9). Different clinical studies using NHE1 inhibitors obtained divergent results. Only the EXPEDITION and a subgroup of the GUARDIAN clinical trial reported positive results in analyzing myocardial ischemia (42). Unfortunately, the EXPEDITION study was prematurely ended because of an increased mortality in the cariporide-receiving group due to cerebrovascular events (30). It is possible that global inhibition of NHE1 at those doses could affect the exchanger in tissues other than myocardium, such as brain, or even inhibit unrelated proteins (4, 7, 23, 30, 45). Since the NHE family is composed of nine members with varying tissue and cellular distribution, with NHE1 being the main isoform in the heart, pharmacological inhibition could also extend to these isoforms, increasing the possibilities of secondary nondesired effects.

RNA interference technology allows specific silencing of protein expression without affecting other proteins, even with a high homology degree. Working with normotensive nonhypertrophic animals, we have proved that short hairpin RNA (shRNA) could locally and specifically silence the cardiac NHE1 expression (37). The objective of the present study is to evaluate, in the SHR model, the cardiac structure and mechanics of the heart injected with a lentivirus carrying a shRNA capable of chronic silencing of NHE1. Specific and local NHE1 inhibition in the heart would provide the beneficial effects obtained with pharmacological inhibitors, such as prevention of cardiac hypertrophy development, avoiding nondesired effects caused by unlimited access of the inhibitor to every organ.

MATERIALS AND METHODS

Animals. 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 Argentine Republic Law no. 14346 concerning animal protection. The experimental protocol was approved by the Animal Welfare Committee of La Plata School of Medicine. Male SHR, 4 mo of age, were randomly divided in three groups corresponding to L-shNHE1 (NHE1 with a small hairpin RNA delivered by lentivirus; $n = 10$), L-shSCR (NHE1 scrambled sequence; $n = 6$), and sham operated ($n = 4$). Before death, animals were anesthetized by

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intraperitoneal injection of pentobarbital sodium (35 mg/kg body wt), and hearts rapidly excised.

Construction and production of lentiviral vector. A third generation lentiviral vector capable of expressing a reporter gene under the cytomegalovirus promoter and shRNA under the RNA polymerase III H1 promoter was used, as previously described (5). The shRNA sequence against rat NHE1 described earlier (37) was 5′-GATAGGTTTCCATGTGATC-3′. For production of lentivirus that carries shNHE1 (L-shNHE1), human embryonic kidney-293T cells were plated in T75 flasks to obtain 80-90% confluence on the transfection day, and cotransfected with a four plasmid vector system by the calcium phosphate method. The crude viral suspension was harvested from 293T cell cultures 48 and 72 h after transfection, filtered (0.45 μm), and concentrated using Centricon Plus-70 filter columns (100,000 molecular weight cutoff; Millipore) at 2,000 *g* at 4°C for 2 h (37). Lentivirus aliquots were subsequently stored at −80°C until use. Lentivirus titers were determined measuring fluorescence of positive human embryonic kidney-293 cells transduced with serial viral dilutions in 293T cells, in the presence of 10 μg/ml of polybrene (Sigma). The same lentiviral vector coding for a NHE1 disorganized nonsilencing nucleotide sequence, 5′-GGCATGTCGTCTAGTATTA-3′ (scramble), was used as control (L-shSCR).

Intramyocardial injection of the lentivirus vector. SHR were anesthetized with sevofluorane (~4% for induction and 2–3% for maintenance) used in a gas mixture with oxygen and delivered through ventilation by using a positive-pressure respirator (model 680, Harvard, South Natick, MA). After deep anesthesia was reached, a left thoracotomy was performed via the fourth intercostal space, and the lungs retracted to expose the heart. Following this, the L-shNHE1 or L-shSCR (~2×10^7 transducing units in 200 μl volume) were intramyocardially injected at one site in the free wall of the left ventricle (LV) using a 30-G needle (37). After injection, the lungs were inflated by increasing positive end-expiratory pressure, and the thoracotomy site was closed in layers. Immediately after surgery, rats were returned to their cages and carried to a recovery room and subsequently returned to the animal facility until death (1 mo later). Rats had ad libitum access to food and water.

Determination of NHE1 protein expression. One month after lentiviral injection, freshly isolated LV were homogenized (Brinkmann Homogenizer, Brinkmann Instruments, Westbury, NY) in four volumes of ice-cold IPB buffer (1% Igepal, 5 mM EDTA, 0.15 M NaCl, 0.15% deoxycholate, 10 mM Tris, pH 7.5), supplemented with protease inhibitors (Mini Complete, Roche Molecular Biochemical). The homogenates were centrifuged at 13,000 rpm for 10 min, and the supernatant set aside for Bradford protein analysis before being suspended in an equal volume of SDS-PAGE sample buffer and used for immunoblots. Sample lysates (100 μg of protein) were resolved by SDS-PAGE on 7.5% acrylamide gels, transferred to polyvinylidene fluoride membranes, and then incubated with monoclonal mouse anti-NHE1 (MAB3140, Chemicon) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-47724, 1:2,000, Santa Cruz). Immunoblots were then incubated with horseradish peroxidase-linked anti-mouse IgG (sc-2314, Santa Cruz). Blots were visualized with enhanced chemiluminescence reagent (Millipore) using a Chemidoc Station (Bio-Rad) and quantified by Image-J analysis software.

Echocardiography. Rats were monitored echocardiographically under light anesthesia with sevoflurane ~4% by two-dimensional M-mode echocardiography with a 7-MHz transducer at the beginning and at the end of protocol. Measurements were performed according to the American Society of Echocardiography leading-edge method (28).

Morphological studies. Ventricular tissue was fixed in buffered 10% formaldehyde and paraffin embedded. LV coronal sections (4 μm thick) at the equator were stained with hematoxylin-eosin for determining cardiomyocyte cross-sectional area (CSA) or with Gomori trichrome technique for quantifying LV collagen volume fraction. All of the stained sections were observed under the microscope (Olympus BX-50, Tokyo, Japan). The images were captured using an analog video camera, digitized and processed by a computer morphometry program (Image-Pro Plus, Media Cybernetics, Silver Spring, MA). CSA was determined in no less than 100 randomly selected cells with rod-shape, clear cross-striations and visible round nucleus per rat. Each cell was individually traced, and its CSA directly determined. Collagen volume fraction was calculated as the sum of all connective tissue areas of the coronal sections, divided by the total surface of the section. Perivascular collagen was excluded from this measurement. The investigator responsible for the morphological analysis was blinded as to each experimental group.

Statistics. Data are expressed as means ± SE. Student *t*-test or one-way ANOVA test followed by Student-Newman-Keuls were used when appropriate. P value < 0.05 was considered of statistical significance.

RESULTS

The LV wall of 4-mo-old SHR hearts was injected with lentiviral particles carrying either a NHE1-silencing shRNA (L-shNHE1) or the shNHE1 scrambled sequence (L-shSCR) (Fig. 1*A*). After 30 days, LV samples were homogenized, electrophoresed, and immunoblotted for NHE1 protein amount (Fig. 1*B*). NHE1 protein expressed as NHE1-to-GAPDH ratio, was 53 ± 3% in L-shNHE1- compared with L-shSCR-injected rats (Fig. 1*C*). Although lentivirus suspension was injected inside the myocardial wall, the possibility of NHE1 silencing outside the heart was assessed. NHE1 protein expression was studied in liver and lung, as previously done with the heart LV. Figure 1, *D–G*, shows that there was no significant change in the amount of NHE1 expression in these tissues, suggesting that the lentivirus was mainly retained in the heart. F1

Cardiac NHE1 silencing effect on the hypertrophic heart was analyzed by morphometric parameters like heart weight, LV weight (LVW), body weight (BW), and tibia length. L-shNHE1 injection induced a reduction of LVW-to-BW ratio and LVW-to-tibia length ratio indexes compared with the aged-matched SHR injected with L-shSCR or sham operated (Fig. 2, *A* and *B*). Also, heart weight-to-BW ratio in L-shNHE1- vs. L-shSCR-injected SHR was 2.98 ± 0.2 mg/g ($n = 10$) vs. 3.34 ± 0.19 mg/g ($n = 6$, *t*-test, $P < 0.05$), indicating cardiac hypertrophy reduction. Cardiac hypertrophy and high blood pressure are two linked phenomena that could be affected by NHE1 inhibition (6). Thirty days after L-shNHE1 injection in the heart LV, average arterial blood pressure values were not statistically different between the three groups (Fig. 2*C*). Also, during this period, there was no variation among each group (data not shown), supporting that local silencing of cardiac NHE1 does not affect blood pressure. F2

Pathological cardiac hypertrophy is characterized by an increased cardiomyocyte size and LV fibrosis. Myocyte size was estimated analyzing the CSA of myocytes visualized in the photomicrograph with circular shape and a visible nucleus (Fig. 3*A*). CSA of SHR injected with L-shNHE1 was reduced 27% compared with L-shSCR (Fig. 3*B*), as it can be observed in a representative photomicrograph. The increased amount of interstitial fibrosis of hypertrophic heart affects cardiac performance (9, 13). Figure 3, *C* and *D*, shows the characteristically high percentages of interstitial collagen in heart LV slices of SHR (9, 13). Hearts with a reduced hypertrophy and silenced NHE1 have the same high extracellular collagen deposits than control hypertrophic hearts, as expected for a 30-day treatment (9). F3

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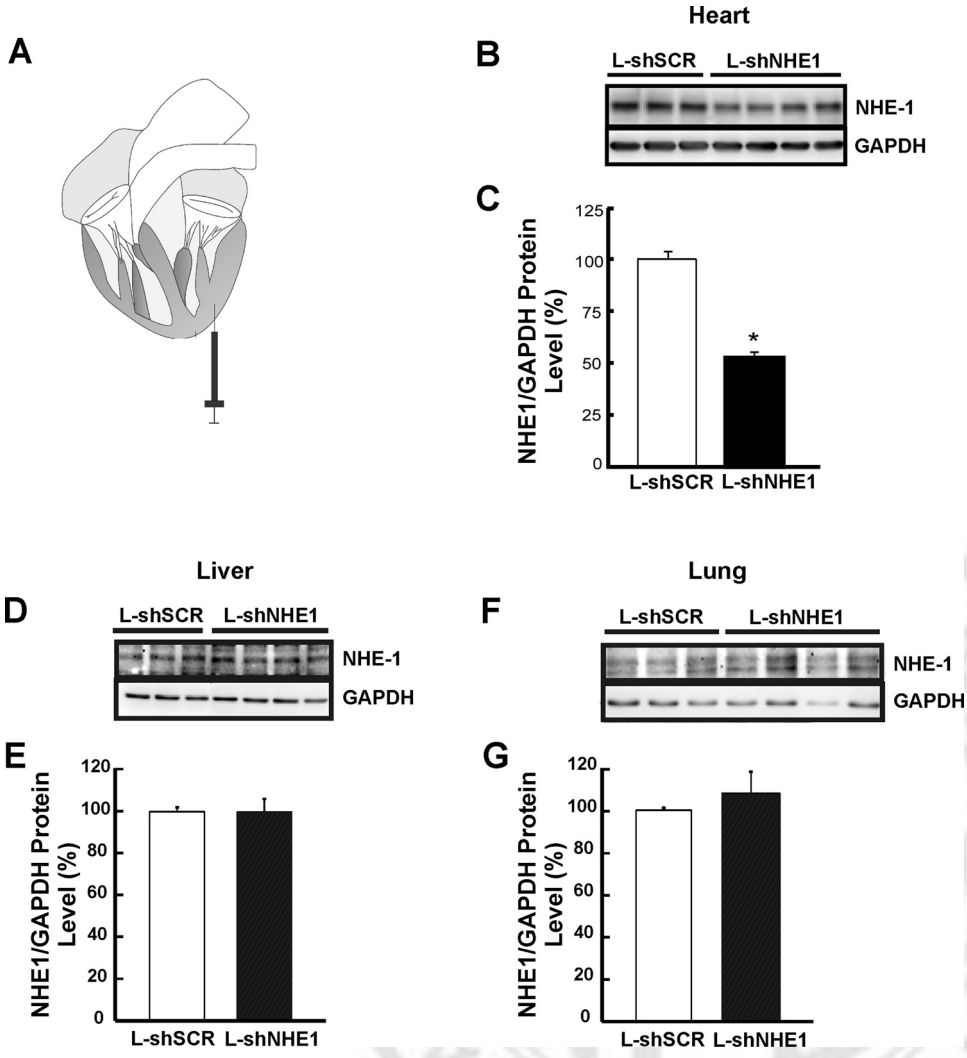

Fig. 1. Na^+/H^+ exchanger isoform 1 (NHE1) protein and mRNA expression in heart, liver, and lung. Left ventricular (LV) myocardium, liver, and lung lysates were resolved by SDS-PAGE, blotted, and probed with an anti-NHE1 antibody and with an anti-GAPDH to indicate amount of material loaded. *A*: scheme of lentivirus injection into the heart. *B*, *D*, and *F*: representative immunoblots (heart, liver, and lung, respectively) corresponding to rats injected with L-shNHE1 (NHE1 with a small hairpin RNA delivered by lentivirus; $n = 10$) or L-shSCR (NHE1 scrambled sequence; $n = 6$). *C*, *E*, and *G*: bar graph shows average expression of NHE1 normalized to GAPDH expression, both quantified by densitometry of the specific immunoblots (heart, liver, and lung, respectively) (*t*-test). Values are means ± SE. $*P < 0.05$.

The effect of silencing cardiac NHE1 on the heart LV was also assessed by echocardiography to estimate LV mass at the beginning and at the end of a 30-day treatment period. Figure 4, *A–C*, represents individual data of animals injected with L-shSCR, L-shNHE1, and sham operated, respectively. Local silencing of NHE1 for 30 days induced a 9% reduction in the original mass, in contrast to L-shSCR-injected rats ($P < 0.05$) (Fig. 4, *B* and *D*). At this time point, the LV mass of SHR injected with L-shNHE1 compared with L-shSCR-injected heart was reduced ~11% ($P < 0.05$). On the other hand, LV mass of sham-treated SHR showed

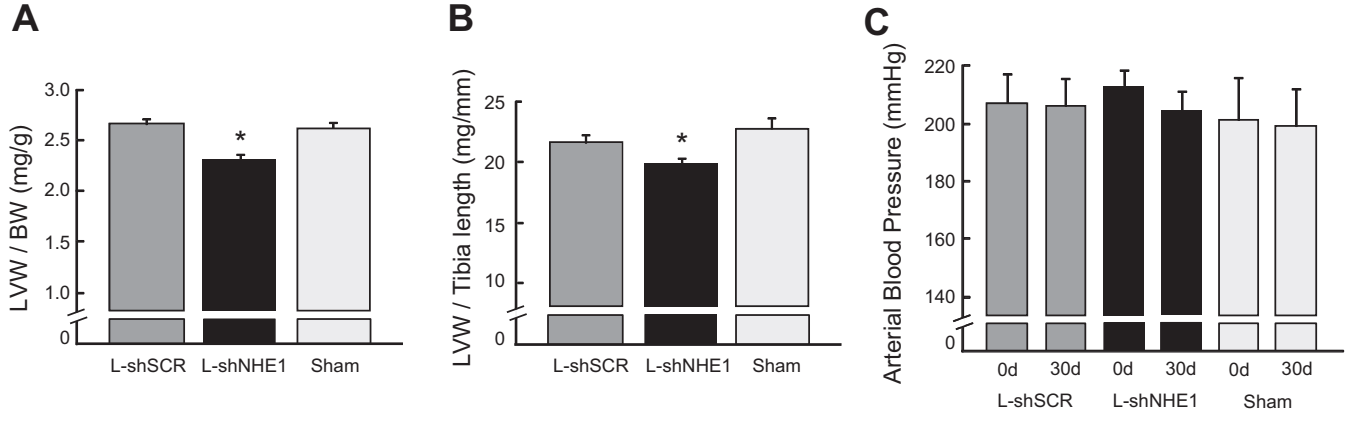

Fig. 2. Cardiac hypertrophy indexes of spontaneously hypertensive rats (SHR). After 30 days of treatment, animals were killed, and the heart LV were isolated and weighed. Cardiac hypertrophy was expressed as ratio of LV weight (LVW) to body weight (BW) (*A*) or as ratio of LVW to tibia length (*B*) (ANOVA). $*P < 0.05$. *C*: average arterial blood pressure values measured at the beginning [0 days (0d)] and at the end of the treatment [30 days (30d)].

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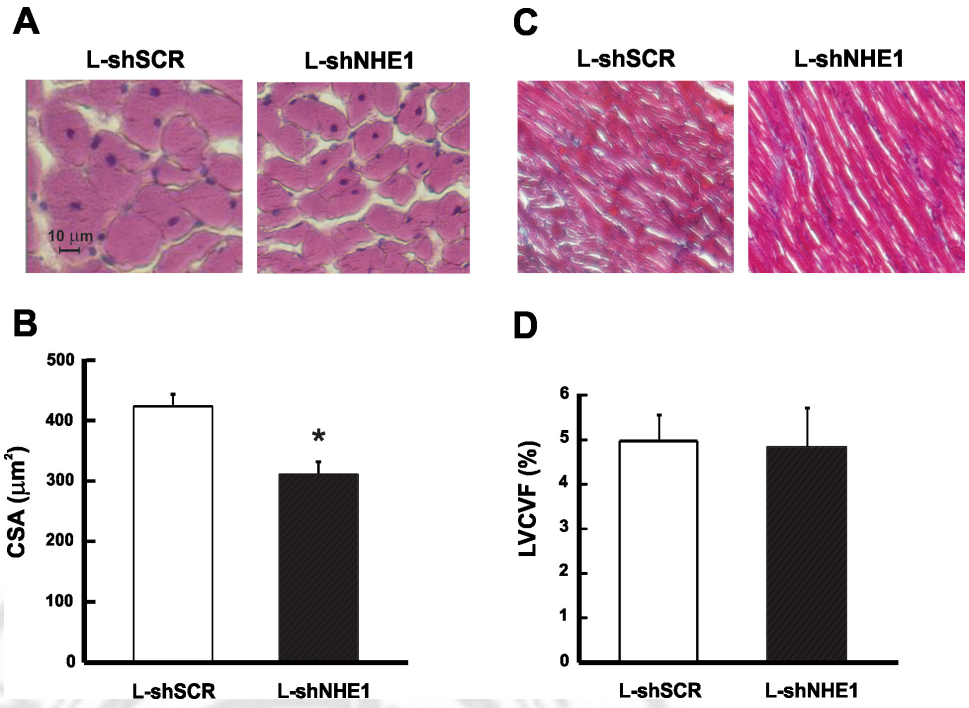

Fig. 3. Myocyte cross-sectional area (CSA) and collagen volume fraction of cardiac LV (LVCVF). *A*: representative microphotographs of hematoxylin- and eosin-stained LV sections of age-matched SHR injected with L-shSCR or L-shNHE1. Magnification, ×20. Bar indicates 10 μm. *B*: mean values of CSA determined in L-shSCR (open bar, $n = 6$) and L-shNHE1 (solid bar, $n = 10$) injected rats (*t*-test). $^*P < 0.05$. *C*: representative microphotographs of the LV of age-matched SHR injected with L-shSCR or L-shNHE1 and stained with Gomori trichrome technique. *D*: mean values of LVCVF determined in L-shSCR (open bar, $n = 6$) and L-shNHE1 (solid bar, $n = 10$) injected rats. Magnification, ×4. Values are means ± SE.

a significant increase, which did not differ from that of L-shSCR injected (Fig. 4, *C* and *D*).

SHR cardiac performance was estimated by the analysis of echocardiographic images. Accordingly, with the reduction of LV mass and myocyte size, LV wall thickness was reduced in L-shNHE1- vs. L-shSCR-injected SHR (Fig. 5*A*). These same animals do not show any significant change in the LV diastolic diameter at the end of the 30-day period (Fig. 5*B*). LV systolic diameter revealed no significant changes over this period for L-shNHE1 [2.32 ± 0.08 mm (0 days) vs. 2.53 ± 0.10 mm (30 days)], or L-shSCR [2.20 ± 0.07 mm (0 days) vs. 2.29 ± 0.05 mm (30 days)] injected SHR (*t*-test $P < 0.05$). Parietal wall stress is a parameter that opposes ventricular contraction. The reduction of LV wall thickness in the absence of changes in pressure and diameter determined an increase in LV parietal stress (Fig. 5*C*). This increment was not accompanied by a reduction of midwall shortening, as could be expected; on the contrary, L-shNHE1- vs. L-shSCR-injected SHR at the 30-day period did not show any significant change (Fig. 5*D*). The fact that midwall shortening remains preserved, despite an increased parietal stress, suggests an improvement in cardiac contractility.

DISCUSSION

Hypertrophic myocardium of SHR and other pathological cardiac hypertrophy models are characterized by a hyperactive NHE1 (8, 24, 36). Also, transgenic mice with the sole transformation of NHE1 into a hyperactive exchanger was enough to generate cardiac hypertrophy (32, 33). In the present study, specific and local silencing of the NHE1 in the heart LV of SHR with a lentivirus-borne shNHE1 produced a reduction of both the NHE1 protein expression and LV hypertrophy.

Our laboratory has previously used the shNHE1 sequence as an oligonucleotide duplex (31) or incorporated into a lentivirus (37) to silence NHE1 expression and function in the heart of normotensive animals. Herein, after 1 mo of expressing shNHE1 in the hypertrophic myocardium of SHR, the reduction of NHE1 protein expression was 50% of the control group. This reduction suggests that the silencing effect was not restricted to the single injection area, but extended into the LV mass. It was demonstrated that lentivirus transduction takes place with a nonuniform pattern, and that shRNA molecules can have a limited spread throughout the LV (11, 25, 31). In consequence, NHE1 protein determination in the whole LV homogenate included regions that could have not received the shNHE1. Also, other possible explanations for the partial reduction in NHE1 expression could be an insufficient amount of lentiviral particles injected or a limited silencing effect of shNHE1 within the cell. Nevertheless, this reduction of NHE1 expression was enough to induce a significant decrease in LV hypertrophy without altering blood pressure values. Furthermore, echocardiography estimations showed that silencing NHE1 over 30 days limited the expected normal LV mass increment of 5-mo-old SHR, and even induced a small reduction. In consequence, cardiac shNHE1 expression alters the normal course of the LV growth in the SHR, preventing an excessive mass development. Similar results were obtained specifically silencing in vivo other genes by chronic and local expression of shRNA in the heart (11, 22, 35, 37, 39). Expression of shRNA-NF-κB/p65 (22) or shRNA-thyrotropin releasing hormone (39), delivered with a lentivirus into the LV wall of a hypertrophic heart, rendered a 49.6 and 53% reduction of mRNA expression, respectively. This gene silencing was associated with a significant reduction of hypertrophic parameters. Previously, in our laboratory, cardiac injection of lentivirus-borne shRNA directed to NHE1 or mineralocorticoid receptor in normotensive rats also reduced, by approximately one-half, the protein expression and prevented the NHE1/mineralocorticoid receptor-dependent contractile slow force response to myocardial stretch (11, 37).

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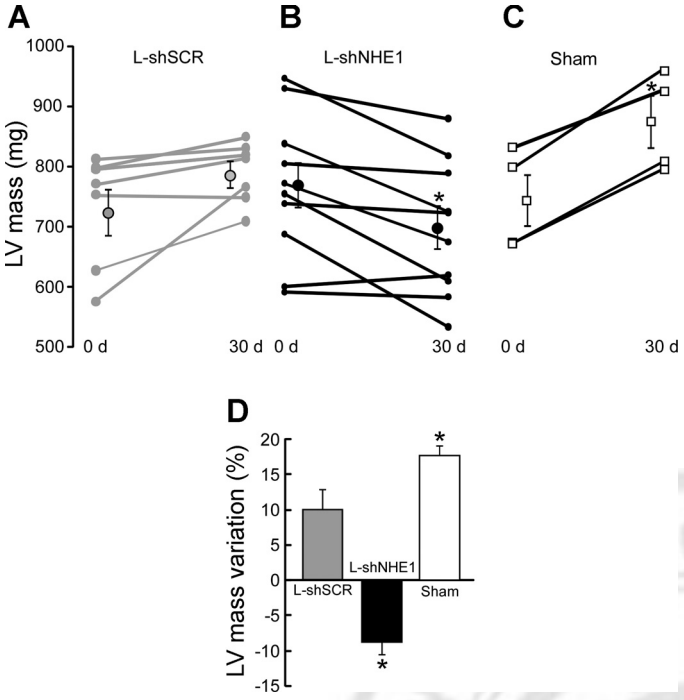

Fig. 4. Time-based variation of cardiac LV mass. LV mass was estimated by echocardiography at the beginning of the treatment (0d) and before death (30d), as described in MATERIALS AND METHODS. LV mass individual recordings and average values (±SE) of SHR injected with L-shSCR (shaded circle; *A*), L-shNHE1 (solid circle; *B*), and sham injected (open square; *C*) are shown. *D*: average values corresponding to LV mass difference between the injection day and the death day (0d vs. 30d, *t*-test). $*P < 0.05$. Values are means ± SE; L-shNHE1 ($n = 10$), L-shSCR ($n = 6$), and sham ($n = 4$).

It is possible that some amount of the lentivirus injected in the heart could have leaked into the bloodstream, reducing the NHE1 expression and activity in other organs. NHE1 protein expression in the lung or liver remains unaltered, confirming a local silencing effect restricted to the heart, similarly to a previous report using the same technique (11). The present study also shows that 1 mo of treatment with L-shNHE1 did not significantly affect the increased myocardial interstitial fibrosis of SHR. Since collagen turnover rate corresponds to ~80–120 days half-life, treatment periods longer than 30 days would be required to evidence a reduction of collagen deposits (6, 9, 41).

Cardiac hypertrophy could be interpreted as an initial adaptive response, allowing the heart to maintain cardiac output under conditions of hemodynamic overload. According to Laplace's law, LV wall thickness increases in hypertension as a compensatory mechanism to decrease wall stress, restoring muscle economy and preserving LV function (1). Several studies suggest that pathological cardiac hypertrophy exceeds this adaptation, becoming itself an independent risk factor for heart failure (10) and mortality (27, 29). Cardiac remodeling that takes place in response to pathological stimulus sustained over time (e.g., hypertension, neurohormonal activation such as angiotensin II/endothelin-1) leads to changes, including altered myocyte size and contractility, reexpression of fetal genes, loss of myocytes with fibrotic replacement, and changes in the microcirculation, which are detrimental for cardiac performance. Our results showed that, despite the increased pressure and parietal stress, reduction of cardiac hypertrophy in the SHR did not result in LV dysfunction, as midwall shortening remained invariable. These findings are comparable with previous studies in animal models of pressure overload, where inhibition of cardiac hypertrophy did not impair heart performance (19, 21, 38).

Pharmacological inhibition of cardiac NHE1 has been demonstrated to be a very effective strategy to reduce cardiac hypertrophy and myocardial infarction in experimental animals (8, 24). The GUARDIAN clinical trial showed positive results injecting 120 mg of the specific NHE1 inhibitor cariporide [in vitro IC_{50} for NHE1 (0.02- 3.4 μM)], which is able to inhibit 85% levels of this protein (43). In the EXPEDITION clinical trial, the use of 180 mg of cariporide was associated with an increased death rate related to cerebrovascular events. Although the reason for the neurotoxicity of cariporide is not clear, it seems possible that high drug concentration or accumulation in specific tissues may affect the activity of other proteins. Cariporide can affect other proteins, such as carbonic anhydrase II (44), persistent sodium channels (7), and other members of the SLC9 family, such as as NHE2, NHE3, and NHE5, with an in vitro IC_{50} of 12–62 μM [PubChem, NCBI (34)]. These potential nondesired effects could be avoided by the use of a more precise method to inhibit the myocardial NHE1, such as the interference RNA.

In summary, the aim of this work was to analyze the in vivo effect of local and specific silencing of cardiac NHE1 for a long period of time, on pressure overload-induced cardiac hypertrophy. The use of a lentiviral vector to deliver the shNHE1 provides a potential long-term and stable expression. This methodology is free of the disadvantages associated with the use of pharmacological drugs. Our results suggest that the use of a shRNA could represent a therapeutic strategy to

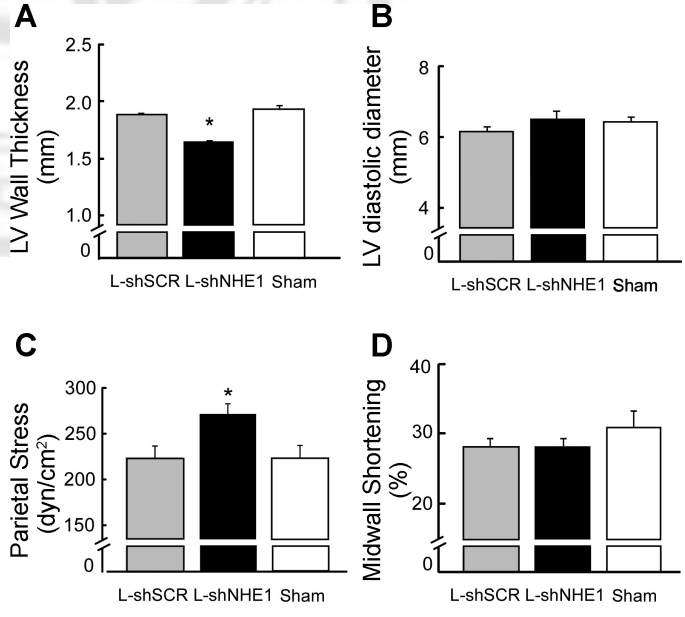

Fig. 5. Cardiac dimensions and function estimated by echocardiography. Average values of LV wall thickness (*h*; *A*) and LV diastolic diameter (LVDd; *B*) were determined after 30 days of injection of L-shNHE1 (solid bar, $n = 10$) and L-shSCR (shaded bar, $n = 6$) or sham operation (open bar, $n = 10$). *C*: cardiac stress (*T*) was calculated using Laplace formula: $T = [P \times (LVDd)/2]/2 \times h$. *D*: midwall shortening was calculated as shown elsewhere (18). h = (interventricular septum thickness + posterior wall thickness)/2. Values are means ± SE. $*P < 0.05$ for L-shNHE1 vs L-shSCR only (ANOVA). AQ: 5

silence the myocardial NHE1, a well-known participant of pathological cardiac hypertrophy and failure development.

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GRANTS

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DISCLOSURES

AQ: 4 No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.B.N., A.O.P., and P.E.M. performed experiments; M.B.N., A.O.P., and P.E.M. analyzed data; M.B.N., I.L.E., H.E.C., and P.E.M. interpreted results of experiments; M.B.N., H.E.C., and P.E.M. prepared figures; M.B.N., H.E.C., and P.E.M. drafted manuscript; M.B.N., I.L.E., H.E.C., and P.E.M. edited and revised manuscript; M.B.N., I.L.E., H.E.C., and P.E.M. approved final version of manuscript; I.L.E., H.E.C., and P.E.M. conception and design of research.

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