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

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Submitted 6 November 2014; accepted in final form 26 February 2015

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 alkalization as a triggering signal for protein synthesis (36). However, NHE1 hyperactivity increases intracellular Na⁺ concentration, the signal responsible for intracellular Ca²⁺ rise through the Na⁺/Ca²⁺ exchanger and calcineurin activation, leading to pathological cardiac hypertrophy and failure (3, 8, 12, 17, 33).

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 undesired 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
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 promoter was used, as described previously (5). The shRNA sequence against rat NHE1 described earlier (37) was 5’-GATAG-GTTTCCATGTGCTGATC-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 Centricron 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 a reporter gene in positive human embryonic kidney-293 cells transduced with serial 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’-GGCATGTGCTGATC-3’ (scramble), was used as control (L-shSCR).

Intramyocardial injection of the lentivirus vector. SHR were anesthetized with sevoflurane (~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 site was closed in layers. Immediately after surgery, rats were inflated by increasing positive end-expiratory pressure, and the thoracotomy site was closed 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).

RESULTS

The LV wall of 4-mo-old SHR hearts was injected with lentiviral particles containing either a NHE1-silencing shRNA (L-shNHE1) or the shNHE1 scrambled sequence (L-shSCR) (Fig. 1A). After 30 days, LV samples were homogenized, electrophoresed, and immunoblotted for NHE1 protein amount (Fig. 1B). NHE1 protein expressed as NHE1-to-GAPDH ratio, was 53 ± 3% in L-shNHE1- compared with L-shSCR-injected rats (Fig. 1C). 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.

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 ± 0.3 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. 2C). 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.

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 in a representative photomicrograph (Fig. 3A). CSA of SHR injected with L-shNHE1 was 27% compared with L-shSCR (Fig. 3B), 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).
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...
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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. 5A). These same animals do not show any significant change in the LV diastolic diameter at the end of the 30-day period (Fig. 5B). 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. 5C). 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. 5D). 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).
pressure and parietal stress, reduction of cardiac hypertrophy in performance. Our results showed that, despite the increased in the microcirculation, which are detrimental for cardiac altered myocyte size and contractility, reexpression of fetal as angiotensin II/endothelin-1) leads to changes, including over time (e.g., hypertension, neurohormonal activation such that takes place in response to pathological stimulus sustained heart failure (10) and mortality (27, 29). Cardiac remodeling this adaptation, becoming itself an independent risk factor for studies suggest that pathological cardiac hypertrophy exceeds muscle economy and preserving LV function (1). Several a compensatory mechanism to decrease wall stress, restoring Laplace’s law, LV wall thickness increases in hypertension as under conditions of hemodynamic overload. According to 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 IC50 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 NHE2, NHE3, and NHE5, with an in vitro IC50 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

![Figure 4](image1.png)  
**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).

![Figure 5](image2.png)  
**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 × (LVDd)/2h] × 8. 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).
use of a shRNA could represent a therapeutic strategy to silence the myocardial NHE1, a well-known participant of pathological cardiac hypertrophy and failure development.

ACKNOWLEDGMENTS

We thank Juan Manuel Lofeudo, head of the Animal Care Facility of the Faculty of Medical Sciences, for assistance handling animals before and after surgery, and Leandro G. Di Ciani for technical assistance registering rat blood pressure.

GRANTS

This work was supported in part by grants PICT 25475 from Agencia Nacional de Promoción Científica de Argentina to H. E. Cingolani, and PIP 0249 and PIP 0433 from Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina to P. E. Morgan and I. L. Ennis, respectively.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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