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Silencing of the Na^+/H^+ exchanger 1(NHE-1) prevents cardiac structural and functional remodeling induced by Angiotensin II

Andrés J. Medina[a], Oscar A. Pinilla[a], Enrique L. Portiansky[b], Claudia I. Caldiz[a], Irene L. Ennis[a,*]
iennis@med.unlp.edu.ar

[a]Centro de investigaciones Cardiovasculares "Dr. Horacio E, Cingolani" Facultad de Ciencias Médicas UNLP-CONICET, Argentina
[b]Laboratorio de análisis de imágenes, Facultad de Ciencias Veterinarias UNLP-CONICET, Argentina

[*]Corresponding author at: Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, UNLP.Calle 60 y 120, 1900, La Plata. Argentina.

ABSTRACT

Chronic activation of the renin angiotensin system (RAS) favors several cardiac diseases, among which myocardial hypertrophy occupies an outstanding place. In this context, the hyperactivity of the cardiac Na^+/H^+ (NHE-1) exchanger plays a key role. The pathologic remodeling of the myocardium constitutes an independent risk factor for morbidity and mortality with continuously increasing healthcare cost. Therefore, the development of better therapeutic strategies emerges as highly mandatory.

Our goal was to prevent angiotensin II (ANGII)-induced cardiac hypertrophy by NHE-1 gene silencing in Wistar rats. The intramyocardial injection of a lentivirus coding a specific small interference RNA (l-shNHE1) significantly reduced NHE-1 expression exclusively in the heart (~ 50 %) and prevented cardiac remodeling in rats exposed to chronic infusion of ANG II (heart weigh/tibia length: 24,03 ± 0,7915 mg/mm vs 28,45 ± 0,9779 mg/mm and collagen volume fraction 2,526 ± 0,5003 vs 5,982 ± 1,043 in l-shNHE1 + ANGII and ANGII, respectively). Interestingly, this was accompanied by an improvement in cardiac function determined by echocardiography even though blood pressure remained elevated (Fractional shortening 0,5960 ± 0,4228 vs -0,9567± 0,06888 and blood pressure at the end of ANGII treatment 141,2 ± 6,117 mm Hg vs 134,1 ± 6,723 mm Hg ; in l-shNHE1 + ANGII and ANGII, respectively). ANGII infusion increased myocardial NADPH oxidase activity but the l-shNHE1 injection prevented oxidative stress as revealed by the normalization of lipid peroxidation (T-BARS 12,40 ± 2,887.vs 23,05 ± 1,537 in l-shNHE1 + ANGII and ANGII, respectively).

These results allow as to propose the partial silencing of the cardiac NHE-1 through lentiviral injection as a promising tool in the prevention of ANGII-induced cardiac hypertrophy.

KEYWORDS: NHE-1; angiotensin II; cardiac hypertrophy, lentivirus, reactive oxygen species, small interference RNA.

1. INTRODUCTION

In the context of cardiovascular disease, when the heart is subjected to sustained hemodynamic overload, myocardial mass increases generating pathological cardiac hypertrophy (CH). This remodeling of the heart is characterized by an altered phenotype and constitutes an independent risk factor for morbidity and mortality that usually leads to heart failure (Levy, Garrison et al. 1990, Katholi and Couri 2011). In this sense, chronic activation of the renin angiotensin system (RAS) is responsible for several cardiac diseases, among which myocardial hypertrophy occupies an outstanding place (Lijnen and Petrov 1999). It is well documented that angiotensin II (ANGII) activates NADPH-oxidase increasing reactive oxygen species (ROS) production that play a crucial role in the development of CH(Garrido and Griendling 2009, Zhang, Perino et al. 2013).

The Na^+/H^+ exchanger (NHE) is a key transmembrane protein that catalyzes the extrusion of intracellular protons collaborating in the maintenance of intracellular pH (pH_i) in the physiological range. It is ubiquitously expressed in mammalian cells, including cardiomyocytes. Although nine NHE isoforms (termed NHE-1 through NHE-9) have been identified at present, cardiac cells primarily express NHE-1 (Fliegel and Karmazyn 2004). Each isoform represents distinct gene products and exhibit differences in their primary structure, membrane localization, functional properties, physiological roles, and sensitivity to pharmacologic inhibition.

It has been firmly proposed an important link between myocardial hypertrophy and NHE-1 activity by others and our own group (Cingolani and Ennis 2007, Karmazyn, Kilic et al. 2008). Not only an enhanced activity of the exchanger is detected in several models of CH but also its pharmacological inhibition probed to be effective to prevent or even induce the regression (Ennis, Escudero et al. 2003, Javadov, Choi et al. 2008, Wakabayashi, Hisamitsu et al. 2013). A number of factors that initiate the hypertrophic response has been involved in NHE stimulation. Activation of G protein coupled receptors by ANGII, endothelin (ET), and α1-adrenergic agonists, are among the best known (Dulce, Hurtado et al. 2006, Javadov, Baetz et al. 2006, Costa-Pessoa, Figueiredo et al. 2013). In this context, the increase in mitochondrial ROS induced by NADPH oxidase derived ROS (ROS induced-ROS release mechanism) may lead to NHE-1 activation through redox sensitive kinases (Caldiz, Garciarena et al. 2007, Garciarena, Caldiz et al. 2008). NHE-1 hyperactivity increases intracellular sodium ($[Na^+]_i$) and subsequently intracellular calcium ($[Ca^{2+}]_i$) through the reverse mode of the Na^+/Ca^{2+} exchanger (NCX). The latter would be the responsible for the activation of prohypertrophic phosphatases and kinases leading to CH (Perez, de Hurtado et al. 2001, Caldiz, Garciarena et al. 2007, Cingolani, Perez et al. 2008).

Based on the unequivocal success of NHE-1 pharmacologic inhibitors to prevent or treat cardiovascular diseases in experimental models, clinical trials were designed. However, although some positive outcomes were observed in certain subgroups of patients, overall results were not encouraging (Avkiran and Marber 2002, Boyce, Bartels et al. 2003, Mentzer, Bartels et al. 2008). Moreover, one of these trials was prematurely stopped because of an increased mortality observed in the treated group due to cerebrovascular events (Mentzer, Bartels et al. 2008). In this context and in order to avoid unwanted and non-specific effects of systemic pharmacologic inhibition of the exchanger, intramyocardial small interference RNA administration emerges as a

putative alternative. This strategy would allow specific silencing of myocardial NHE-1 without affecting other proteins, even with a high homology degree.

The aim of the present study was to evaluate the effectiveness of the specific silencing of cardiac NHE-1 to prevent the development of CH in rats subjected to chronic subcutaneous administration of ANGII.

2. MATERIALS AND METHODS

The study was performed according 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). All procedures followed during this investigation are 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.

2.1. Animals. Male Wistar rats, 5 month old, were randomly assigned to three experimental groups: 1- l-shNHE1 + ANGII (NHE1 with a small hairpin RNA delivered by lentivirus; (n=8); 2- ANGII (n=6); 3-sham operated (n=7)). Animals were anesthetized by intraperitoneal injection of ketamine (50 mg.kg^{-1} of body weight) diazepam (2.5 mg kg^{-1} of body weight) and hearts rapidly excised when plane 3 of stage III of anesthesia was reached. The rats were infused subcutaneously by mini-osmotic pumps (Alzet model 2004 with ANGII (200 µg /kg/day) (Baltatu, Silva et al. 2000)or vehicle (sterile physiologic solution) for 28 days.

2.2. Systolic Blood Pressure measurement and ANGII infusion. Systolic blood pressure (SBP) was measured before and weekly after the implant of mini-osmotic pumps by tail-cuff plethysmography according to the procedure described (Bunag 1973).

2.3. Cell Culture and transfections. HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 g/ml streptomycin in 5% CO_2 air-equilibrated (control) 37°C incubator. Plasmids used were expressed by transient transfection of HEK293T cells using the calcium phosphate method (Jordan and Wurm 2004).

2.4. Construction and Production of Lentiviral Vectors. A third generation lentiviral vector capable of expressing a reporter gene under the CMV promoter and shRNA under the RNA polymerase III H1 promoter was produced as previously described (Barth, Kizana et al. 2008). Briefly, the shRNA against the NHE-1 was subcloned into a third generation lentiviral vector plasmid that expresses DsRed under the CMV promoter and shRNA under the RNA polymerase III H1 promoter (PPT.C.DsRed.H1). The shRNA sequence against NHE-1 was constructed inserting 5`-GATAGGTTTCCATGTGATC-3` (positive strand), followed by 5`-GTTCTTCAAGAGAGAAC-3` (loop), 5`-ATCACATGGAAACCTATC-3= (negative strand), and 5`-ATCACATGGAAACCTATC-3` (terminator), at the BamHI cloning site (5´) and PacI cloning site (3`), following the H1 RNA polymerase promoter, to generate PPT.C.DsRed.H1.shNHE1 (l-shNHE1). This shRNA against NHE-1 was previously proven effective in reducing whole NHE-1 protein expression in cultured fibroblasts (Akram, Teong et al. 2006). Lentiviral particles were obtained by co-transfecting HEK293T cells with the four different

plasmids. Three of these plasmids encode the gene sequence of lentivirus structural proteins and the other have the sequence of the shNHE1 or shSCR. HEK293T cell media culture was collected at 24, 48, and 72 hours after transfection and immediately centrifuged for 10 minutes at 1000 g, virus containing supernatant was centrifuged for 1.5 hours at 30 000 g. Viral pellets were resuspended in sterile PBS overnight at 4°C, aliquoted, and stored at -80°C. Lentivirus titer was determined measuring fluorescence of positive HEK293T cells transduced with serial dilutions of the viral stock in the presence of 10 ug/mL of polybrene (Sigma). Lentivirus encoding the nonsilencing sequence was used as control in HEK293T.

2.5. Intramyocardial injection of the lentivirus vector. Wistar rats 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 ($2x10^7$ transducing units in 200 µl volume) or sterile PBS (200 µl) were intramyocardially injected at two sites in the free wall of the left ventricle (LV) using a 30-G needle (Perez, Nolly et al. 2011). Immediately after surgery, rats were returned to their cages and carried to a recovery room and subsequently returned to the animal facility until death. Rats had ad libitum access to food and water.

2.6. Western blotting. Left ventricles and HEK293T cells overexpressing NHE-1 and transduced with l-shNHE1 or l-shSCR were homogenized in RIPA buffer (Santa Cruz Biotechnology, sc-24948) with protease inhibitors cocktail (Roche), PMSF and sodium orthovanadate. After a brief centrifugation the supernatant was kept and protein concentration determined by the Bradford method (Bio-Rad dye reagent) as described by the manufacturer with BSA as a standard. Samples were denatured and equal amounts of protein were subjected to PAGE and electrotransferred to PVDF membranes. Membranes were then blocked with non-fat-dry milk and incubated overnight with antibody against NHE-1 (Santa Cruz Biotechnology sc-28758). The detection of GAPDH (Millipore MAB374) was used as loading control. Peroxidase-conjugated anti rabbit (NA934, GE Healthcare Life Sciences), anti-mouse (NA931, GE Healthcare Life Sciences) were used as secondary antibodies. Bands were visualized with enhanced chemiluminescence reagent (ECL, Millipore) and a Chemidoc Station (Bio-Rad) and quantified using Image J analysis software.

2.7. 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 (Lang, Bierig et al. 2005).

2.8. Cross Sectional Area (CSA) analysis. Coronal sections of obtained from the equator of the left ventricle were prepared for conventional histological techniques. Thus, tissue was fixed in buffered formalin for 24 h. For CSA determinations, five µm thick paraffin-embedded sections were stained with Hematoxylin and eosin. Histological (40x magnification) images were digitized using a digital video camera (Olympus DP73, Japan) mounted on a widefield microscope (Olympus BX53, Japan). Captured images were saved in TIF format using an image analysis software

(Olympus cellSens Dimension V1.7, Japan) automatically coordinated with the camera for later analysis. Only round cells with visible round nucleus were considered for CSA measurements. Each cell was individually traced, and its cross-sectional area was directly determined.

CSA determinations were done using another image analysis software (Image-Pro Plus v6.3 - Media Cybernetics, USA

2.9. Collagen Volume Fraction (CVF) determination. Five 5 µm thick coronal sections of the left ventricle were stained using the Picrosirius red technique (Direct Red 80, Aldrich, Milwaukee, WI 53233, USA) for collagen evaluation as described elsewhere (Yeves, Garciarena et al. 2010). Samples were observed under polarized light, using an analyzer (U-ANT, Olympus) and a polarizer (U-POT, Olympus) was used to study the birefringence of the stained collagen.

Histological (20x magnification) images were digitized using a digital video camera (Olympus DP73, Japan) mounted on a widefield microscope (Olympus BX53, Japan). Captured images were saved in TIF format using an image analysis software (Olympus cellSens Dimension V1.7, Japan) automatically coordinated with the camera for later analysis.

CVF was calculated as the sum of all connective tissue areas of the coronal sections (type I and type III collagen), divided by the total surface of the section. Perivascular collagen was excluded from this measurement.

2.10. NHE-1 activity. Intracellular pH (pH_i) was measured in three groups of HEK293T cells, two of these were infected with l-shNHE1 or l-shSCR for 72 h and 48 h before the measurement were transfected with a plasmid that overexpress rat NHE-1 (pNHE1). The other group was only transfected with pNHE1 (control group). Cells were grown in coverslips and superfused with the K-H solution (bicarbonate free) with an epi-fluorescence system (Ion Optix, Milton, MA) following the previously described BCECF epifluorescence technique (Yeves, Garciarena et al. 2010). At the end of each experiment, the fluorescence ratio was converted to pH by in vivo calibrations using the high K^+-nigericin method (Perez, Alvarez et al. 1995).

NHE-1 activity was assessed by evaluating the rate of pH_i recovery from an ammonium pre-pulse induced acid load. The duration of intracellular acidosis was extended for 2 min by washout of NH_4Cl with Na^+-free solution and NHE-1 was reactivated by reintroduction of extracellular Na^+ (Yeves, Garciarena et al. 2010).

2.11. Quantitative real-time PCR. NHE-1 and brain natriuretic peptide (BNP) mRNA expression was assessed by real-time RT-PCR and normalized to GAPDH following the procedure described previously (Ennis, Garciarena et al. 2005). Primers used for amplification are listed in **Table 1**

Table 1

Gene	Forward	Reverse
NHE-1	5`-TCACCACAGCTCCATCAGAG-3`	5`-GATCTCGAAGGGTGTCCGTA-3`
BNP	5`-CCCAGATGATTCTGCTCCTG-3`	5`-TTCTGCATCGTGGATTGTTC-3`
GAPDH	5`-GGGTGTGAACCACGAGAAAT-3`	5`-CCACAGTCTTCTGAGTGGCA-3`

2.12. Assessment of lipid peroxidation. We used the thiobarbituric acid reactive substances (T-BARS) spectroscopic technique(Buege and Aust 1978) to evaluate lipid peroxidation as index of ROS formation. Briefly, cardiac tissue from different experimental groups was homogenized in physiological saline solution and centrifuged at 2500 rpm to allow measuring T-BARS in the supernatant. Absorbance at 535 nm was measured and T-BARS expressed in nmol/g of tissue using an extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$.

2.13. NOX Activity. NADPH oxidase (NOX)–dependent superoxide production was measured in left ventricle homogenates from rats of all experimental groups, using a previously described lucigenin-enhanced chemiluminescence method(Bendall, Cave et al. 2002). Proteins were diluted in modified HEPES buffer was added to wells just before reading. NOX activity measured by $O_2^{\cdot}$ production was expressed in cpm/mg of protein over 15 minutes.

2.14. Epifluorescence microscopy. DsRed fluorescence images from HEK293T cells were obtained with an inverted Olympus 1X71 microscope equipped with a 100 W halogen lamp. Fluorescence emission was detected using 575 nm filter. The magnification of the images was 100X.

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

3. RESULTS

The silencing efficiency of the l-shNHE1 coding for the siRNA against NHE-1 was evaluated in HEK293T cells. Because the basal expression of the exchanger is low in this cell line (Villa-Abrille, Cingolani et al. 2011, Odunewu and Fliegel 2013), we induced its overexpression by transfecting the cells with a plasmid carrying the sequence of rat NHE-1 (**Fig 1A**)**.** As shown in **Fig 1B,** the l-shNHE1 significantly decreased the NHE-1 protein expression (~ 35%) compared to control. In order to corroborate these results, the activity of NHE-1 was measured in the same experimental groups. The rate of intracellular pH_i recovery from acidosis induced by the NH_4^+ prepulse technique was significantly diminished in l-shNHE1-infected cells (**Fig 1C**). The experiments were performed in HCO_3^- free solution since under this condition the NHE-1 is the only alkalinizing mechanism active. These results confirmed the l-shNHE1 as an adequate tool to experimentally reduce NHE-1 expression and activity.

Because we were essentially interested in evaluating the capability of myocardial NHE-1 specific silencing to prevent CH development, Wistar rats were injected with the l-shNHE1 and after seven days of the injection the animals were subjected to continuous long-term delivery of ANGII by subcutaneous osmotic minipumps. First, the *in vivo* silencing efficacy of the l-shNHE1 (200 µl) injected into the left ventricle (LV) wall was explored. After 35 days of injection, the l-shNHE1 succeeded to persistently decrease myocardial NHE-1 expression both at the mRNA and protein levels (~ 50%) (**Fig 2A-B**). Importantly, this effect was specific for the myocardium, since no differences in the expression of the NHE-1 were observed in other tissues such as lung and liver among groups (**Fig 2C-D**).

Since ANGII is a pressor peptide, its effect upon SBP was determined. Rats chronically exposed to ANGII increased their SBP compared to control. The injection of the l-shNHE1 did not significantly modify this effect (**Fig 3A**). As expected, ANGII administration induced CH, as revealed by the HW/TL ratio, the echocardiographic determination of the interventricular septum (IVSd) and the posterior wall thickness (PWd) in diastole as well as the up-regulation of myocardial BNP expression. However, this was not the case for the rats exposed to the l-shNHE1, supporting a key role of the NHE-1 in CH development (**Fig 3B-E**). This effect was confirmed at the histological level by determining the cross sectional area of cardiomyocytes and collagen abundance. Rats that were intramyocardially injected with the l-shNHE1 showed a smaller area of cardiomyocytes as well as a reduced collagen fraction (**Fig 3F-G**). Even more interesting was the finding that the l-shNHE1 significantly improved cardiac contractile function measured by echocardiography (**Fig 3H**), even though blood pressure continued elevated and cardiac mass was decreased. This result is in agreement with a previous report of our group (Ennis, Garciarena et al. 2007).

As we have already mentioned, ANGII increases the production of ROS, and these molecules are key players in CH development. Therefore, we tested the effect of the l-shNHE1 intramyocardial injection on ROS production. As it can be appreciated in **Fig 4A** the activity of NADPH oxidase was significantly increased in the hearts exposed to ANGII compared to control, even in the hearts injected with the l-shNHE1. Subsequently, we measured the consequences of the increase in ROS production by determining lipid peroxidation (T-BARS). Interestingly, only in the rats not exposed to the l-shNHE1 a significant increase in lipid peroxidation was evident (**Fig 4B**).

4. DISCUSSION

The main finding of the present study is the demonstration that the partial silencing of the myocardial NHE-1 is a suitable strategy to prevent ANGII-induced CH. It is worth to highlight that this antihypertrophic effect, as well as the improvement in cardiac function, were obtained without normalization of SBP. **Figure 5** summarizes the sequence of events proposed.

As mentioned in the Introduction, NHE-1 pharmacologic inhibitors have been clinically tested in order to reproduce the benefits observed in the experimental models (Theroux, Chaitman et al. 2000, Zeymer, Suryapranata et al. 2001, Mentzer, Bartels et al. 2008). Unfortunately, the outcomes of these clinical studies were not as good as expected. Only the EXPEDITION and a subgroup of the GUARDIAN clinical trial reported benefits with the NHE-1 inhibitor cariporide when dealing with patients with myocardial ischemia (Theroux, Chaitman et al. 2000, Mentzer, Bartels et al. 2008). Awkwardly, the EXPEDITION study was ended up prematurely due to an increased incidence of cerebrovascular events and mortality in the treated group (Mentzer, Bartels et al. 2008). A possible explanation for these findings is that systemic pharmacologic inhibition usually affects not only the intended target but also some related or unrelated proteins in many tissues. At present, nine members of the NHE family have been described with varying tissue and cellular distribution. Current pharmacological inhibitors are not highly selective for isoforms, therefore the inhibitors evaluated could not only affect NHE-1 activity but also extend to other isoforms increasing the possibilities of non-desire effects. On the other hand, small interference RNA technique permits the silencing of a specific protein expression without interfering with the

expression of other proteins even with a high homology degree. Therefore, we consider that intramyocardial injection of the l-shNHE1 represents a good option to obtain protein specific and organ restricted NHE-1 inhibition **(Figure 2)**. Not only this approach allows stable gene silencing at least for one month (Diaz, Perez et al. 2014, Nolly, Pinilla et al. 2015) but also it should provide the benefits of pharmacological inhibitors avoiding non-desire effects due to wide access of the inhibitor to every isoform and organ. In connection with this, in previous works of our group the shNHE1 naked sequence (Morgan, Correa et al. 2011) or contained into a lentivirus (Perez, Nolly et al. 2011, Nolly, Pinilla et al. 2015) was administered to silence NHE-1 expression and function in normal and hypertrophied rat hearts. Remarkably, the silencing effect obtained was restricted to the heart but widely extended through the LV wall, farther to the injection site, as other authors have also prove (Kizana, Cingolani et al. 2009, Schuman, Landa et al. 2011, Diaz, Perez et al. 2014). Interestingly, by this intervention we were able to reduce the left ventricular mass in adult spontaneously hypertensive rats in a previous work (Nolly, Pinilla et al. 2015). However, we should keep in mind that the clinical use of viral vectors is not deprived of certain safety and ethical issues yet unresolved. In this regard, it is necessary to consider the possibility that replication-competent viruses can be generated during vector production, and insertional mutagenesis could occur leading to cancer, among the most relevant (Thomas, Ehrhardt et al. 2003)

ANGII is a well-known octapeptide capable of increasing blood pressure and inducing CH development (Mehta and Griendling 2007). Moreover, it has been demonstrated that this hormone stimulates NHE-1 activity through activation of the AT1 receptor(Matsui, Barry et al. 1995). Among the intracellular pathways triggered by ANGII, the stimulation of the NADPH oxidase has been propose to be critical for CH development (Garrido and Griendling 2009) . We have previously demonstrated that NADPH oxidase-derived ROS prompt the release of greater amounts of ROS from the mitochondria by a process named "ROS induced- ROS release" (Garciarena, Caldiz et al. 2008). The last ones activate a redox-sensitive kinase pathway that ends up with the phosphorylation of the cytosolic regulatory tail of the NHE-1 triggering its hyperactivity. The consequence of this mechanism is the augmentation of intracellular Na^+ leading to a secondary increase in intracellular Ca^{2+} through the reverse mode of the NCX. The rise in intracellular Ca^{2+} finally favors calcineurin activation and the transcription of hypertrophic genes (Ennis, Garciarena et al. 2007, Hisamitsu, Nakamura et al. 2012). Importantly, a negative inotropic effect of calcineurin through different mechanisms has been described (Sah, Oudit et al. 2002, Li, Yatani et al. 2003). Therefore, the decrease in cardiac NHE-1 protein expression by l-shNHE1 intramyocardial administration emerges as a good strategy to prevent intracellular Na^+ accumulation, calcineurin activation, hypertrophy development and its negative impact on cardiac contractility. The preservation of cardiac function after regression of CH seems not to be unique to the regression of CH by NHE-1 inhibition (Esposito, Rapacciuolo et al. 2002). It seems that in the myocardium, intracellular Ca^{2+} is compartmentalized in such way that the contractile pool is different from the pool that regulates reactive signaling. In agreement with this, it has been suggested that calcineurin is activated preferentially by specific sub-cellular Ca^{2+} pools (Frey, Barrientos et al. 2004, Wu, Zhang et al. 2006). Therefore we can speculate that the decrease in diastolic [Ca^{2+}] might be sensed by the Ca^{2+} calmodulin-calcineurin pathway, but not by the

contractile machinery. It deserves to be noted that the prevention of CH development by l-shNHE-1 administration occurs without normalizing high blood pressure and NADPH oxidase activity but with normalization of lipid peroxidation The reason for this would be that mitochondria is the main source of intracellular ROS favored by NHE-1 hyperactivity. Therefore, a diminish activity of the exchanger will result in a decrease production of ROS. These results are in accordance with previous reports of our laboratory indicating that pharmacological specific inhibition of NHE-1 prevents oxidative stress (Garciarena, Caldiz et al. 2008).

5 CONCLUSIONS

In the present work, we succeeded to prevent pathological CH induced in rats by chronic administration of ANGII, even though the persistence of increased afterload (high SBP). This result was obtained by cardiac restricted in vivo partial silencing of the NHE-1. We used a lentiviral vector coding for a specific shRNA against the exchanger directly injected into the left ventricular wall. This maneuver has the potential advantage of not interfering with NHE-1 expression in other organs and therefore limiting the possibilities of non-desired effects.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Figure 1. Lentiviral silencing of the Na^+/H^+ exchanger 1 in HEK293T cells transiently transfected with pNHE1 plasmid. ***A***. Bright-field and fluorescent images of cell cultures; I and II non-infected cells; III and IV cells infected with l-shNHE1; V and VI, cells infected with l-shSCR. (magnification: 100X). ***B***. NHE-1 protein expression. Top: representative Western blot bands. Bottom: average results from each experimental group. ***C.*** Na^+/H^+ exchanger 1 activity measured during intracellular acidosis recovery by BCECF epifluorescence. Left typical registers during pHi recovery. Right: average values of dpHi/dt at a common pH_i 6.8. Bars represent mean ± SEM, the number of individual experiments for each group is depicted inside bars. * correspond to p<0.05 One-way *ANOVA* analysis, l-shNHE1+pNHE1 vs pNHE1 and l-SCR+pNHE1 groups.

Figure 2. Intramyocardial injection of the l-shNHE1 in Wistar rats. ***A.*** NHE-1 protein expression in left ventricles of Wistar rats. Top: representative Western blot bands. Bottom: average results from each experimental group. ***B.*** Myocardial NHE-1 mRNA abundance quantificated by RT-qPCR. ***C and D.*** Lung and liver protein expression of NHE-1. Bars represent mean ± SEM, the number of individual experiments for each group is depicted inside bars. * correspond to p<0.05 One-way *ANOVA* analysis, l-shNHE1+ANGII vs Ctrl and ANGII groups.

Figure 3. ANGII chronic infusion by osmotic mini pumps. ***A***. After 28 days of ANGII administration a significant increase in SBP was observed. * correspond to p<0.05, t-test, before (b) vs. after (a) in each group. ***B-D.*** Cardiac hypertrophy estimated by heart weight/tibia length ratio (HW/TL), the interventricular septum (IVSd) and posterior wall thickness (PWd) in diastole determined by echocardiography ***E***. BNP mRNA myocardial expression. ***F-G.*** Chronic administration of ANGII significantly increased CSA and interstitial collagen, effects prevented by the l-shNHE1 (magnification: 40X and 20X, respectively). ***H.*** Cardiac function assessed by fractional shortening was improved by the l-shNHE1 injection. Bars represent mean ± SEM, the number of individual experiments for each group is depicted inside bars. # indicate p<0,05, ANGII vs Ctrl and l-shNHE1+ANGII groups; * indicate p<0.05; l-shNHE1+ANGII vs Ctrl and ANGII groups. † indicate p<0,05, l-shNHE1+ANGII vs ANGII; & indicate p<0,05, Ctrl vs l-shNHE1+ANGII; ††† indicate p<0.001, Ctrl vs ANGII, One-way *ANOVA* analysis.

Figure 4. ANGII induced myocardial production of reactive oxygen species (ROS). ***A.*** ANGII infusion increased myocardial NADH oxidase (NOX) activity measured by the lucigenin method. ***B.*** Lipid peroxidation in left ventricular samples was measured by TBARS technique. ANGII increased TBARS, effect prevented by the intramyocardial injection of l-shNHE1. Bars represent mean ± SEM, the number of individual experiments for each group is depicted inside bars. * indicate p<0.05, Ctrl vs l-shNHE1+ANGII and ANGII groups; # indicate p<0,05, ANGII vs Ctrl and l-shNHE1+ANGII groups; One-way *ANOVA* analysis.

Figure 5. Schematic summary of the sequence of events proposed to underline the prevention of CH development by the intramyocardial injection of the l-shNHE1 in Wistar rats subjected to sustained subcutaneous delivery of ANGII.

Suppl. Figure 1: Representative image of the intramyocardial injection of the l-shNHE1 in a Wistar rat.

Suppl.Figure 2: Representative Western Blot of NHE-1 expression in non-transfected and 48 hs. after transfection with pNHE1 HEK293T cell homogenates.

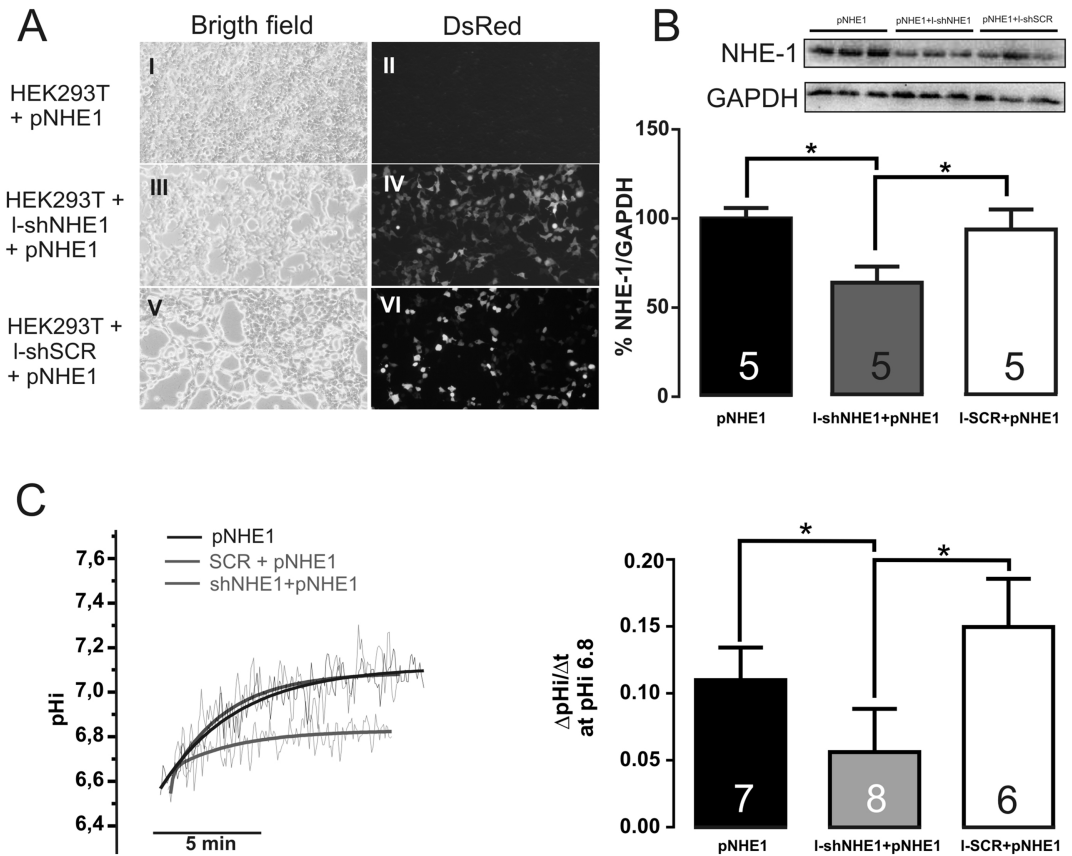
A
Brigth field
DsRed
HEK293T + pNHE1
HEK293T + l-shNHE1 + pNHE1
HEK293T + l-shSCR + pNHE1
I
II
III
IV
V
VI
B
pNHE1
pNHE1+l-shNHE1
pNHE1+l-shSCR
NHE-1
GAPDH
% NHE-1/GAPDH
150
100
50
0
*
*
5
5
5
pNHE1
l-shNHE1+pNHE1
l-SCR+pNHE1
C
pNHE1
SCR + pNHE1
shNHE1+pNHE1
pHi
7,6
7,4
7,2
7,0
6,8
6,6
6,4
5 min
ΔpHi/Δt at pHi 6.8
0.20
0.15
0.10
0.05
0.00
*
*
7
8
6
pNHE1
l-shNHE1+pNHE1
l-SCR+pNHE1

Figure 1

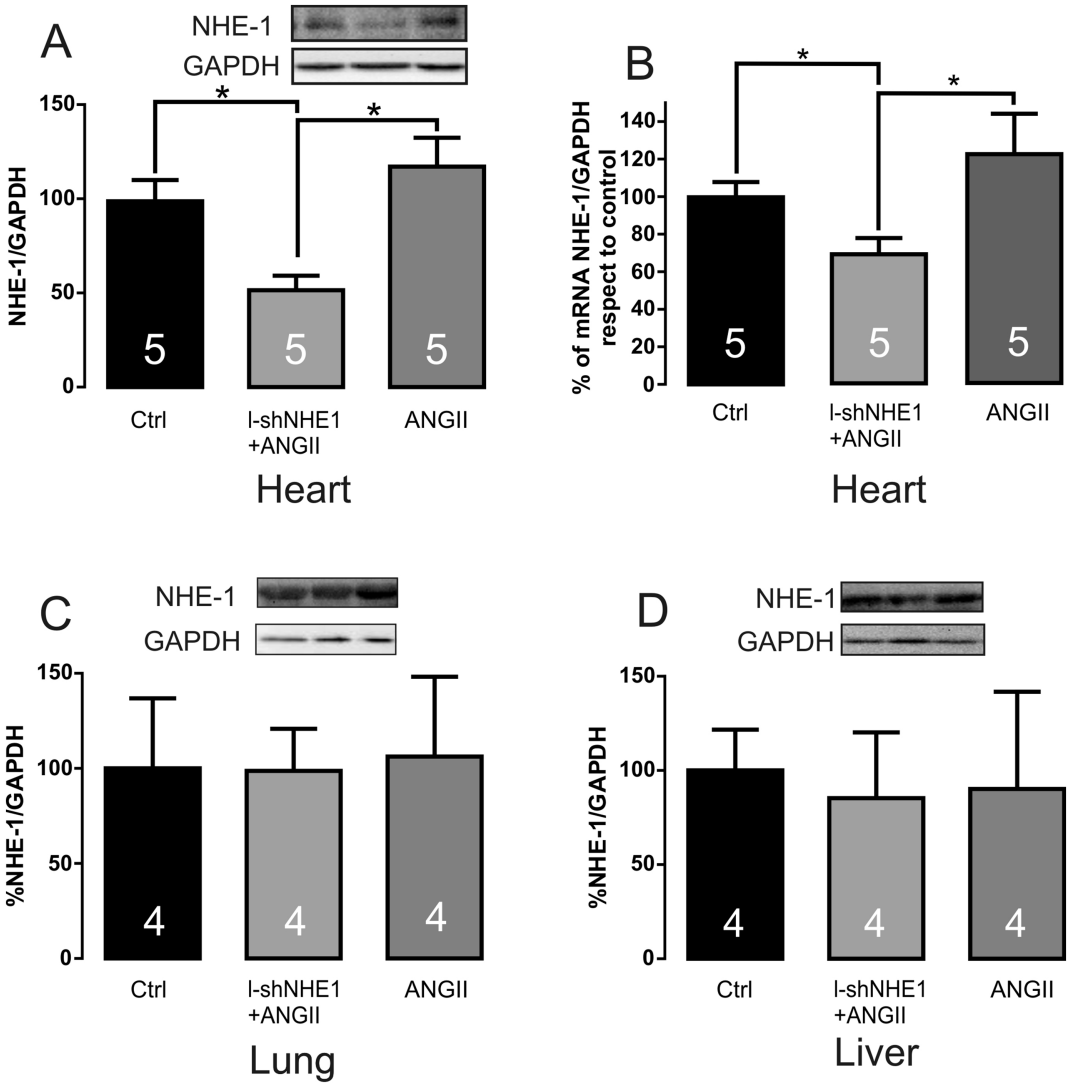
A
NHE-1
GAPDH
NHE-1/GAPDH
150
100
50
0
*
*
5
5
5
Ctrl
l-shNHE1 +ANGII
ANGII
Heart
B
% of mRNA NHE-1/GAPDH respect to control
140
120
100
80
60
40
20
0
*
*
5
5
5
Ctrl
l-shNHE1 +ANGII
ANGII
Heart
C
NHE-1
GAPDH
%NHE-1/GAPDH
150
100
50
0
4
4
4
Ctrl
l-shNHE1 +ANGII
ANGII
Lung
D
NHE-1
GAPDH
%NHE-1/GAPDH
150
100
50
0
4
4
4
Ctrl
l-shNHE1 +ANGII
ANGII
Liver

Figure 2

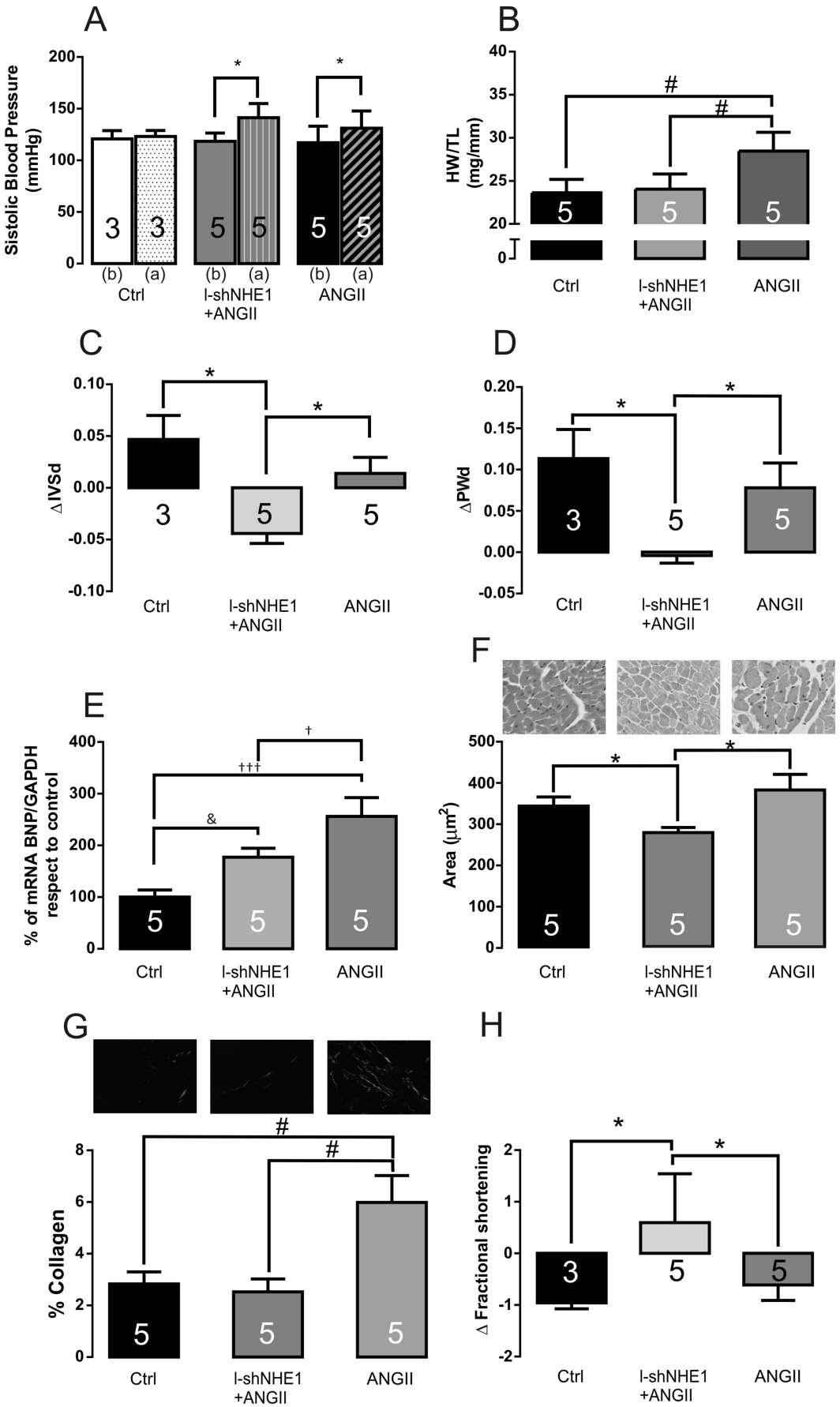
A
Sistolic Blood Pressure (mmHg)
200
150
100
50
0
*
*
3
3
5
5
5
5
(b)
(a)
(b)
(a)
(b)
(a)
Ctrl
l-shNHE1 +ANGII
ANGII
B
HW/TL (mg/mm)
40
35
30
25
20
0
#
#
5
5
5
Ctrl
l-shNHE1 +ANGII
ANGII
C
ΔIVSd
0.10
0.05
0.00
-0.05
-0.10
*
*
3
5
5
Ctrl
l-shNHE1 +ANGII
ANGII
D
ΔPWd
0.20
0.15
0.10
0.05
0.00
-0.05
*
*
3
5
5
Ctrl
l-shNHE1 +ANGII
ANGII
E
% of mRNA BNP/GAPDH respect to control
400
300
200
100
0
†
†††
&
5
5
5
Ctrl
l-shNHE1 +ANGII
ANGII
F
Area (μm²)
500
400
300
200
100
0
*
*
5
5
5
Ctrl
l-shNHE1 +ANGII
ANGII
G
% Collagen
8
6
4
2
0
#
#
5
5
5
Ctrl
l-shNHE1 +ANGII
ANGII
H
Δ Fractional shortening
2
1
0
-1
-2
*
*
3
5
5
Ctrl
l-shNHE1 +ANGII
ANGII

Figure 3

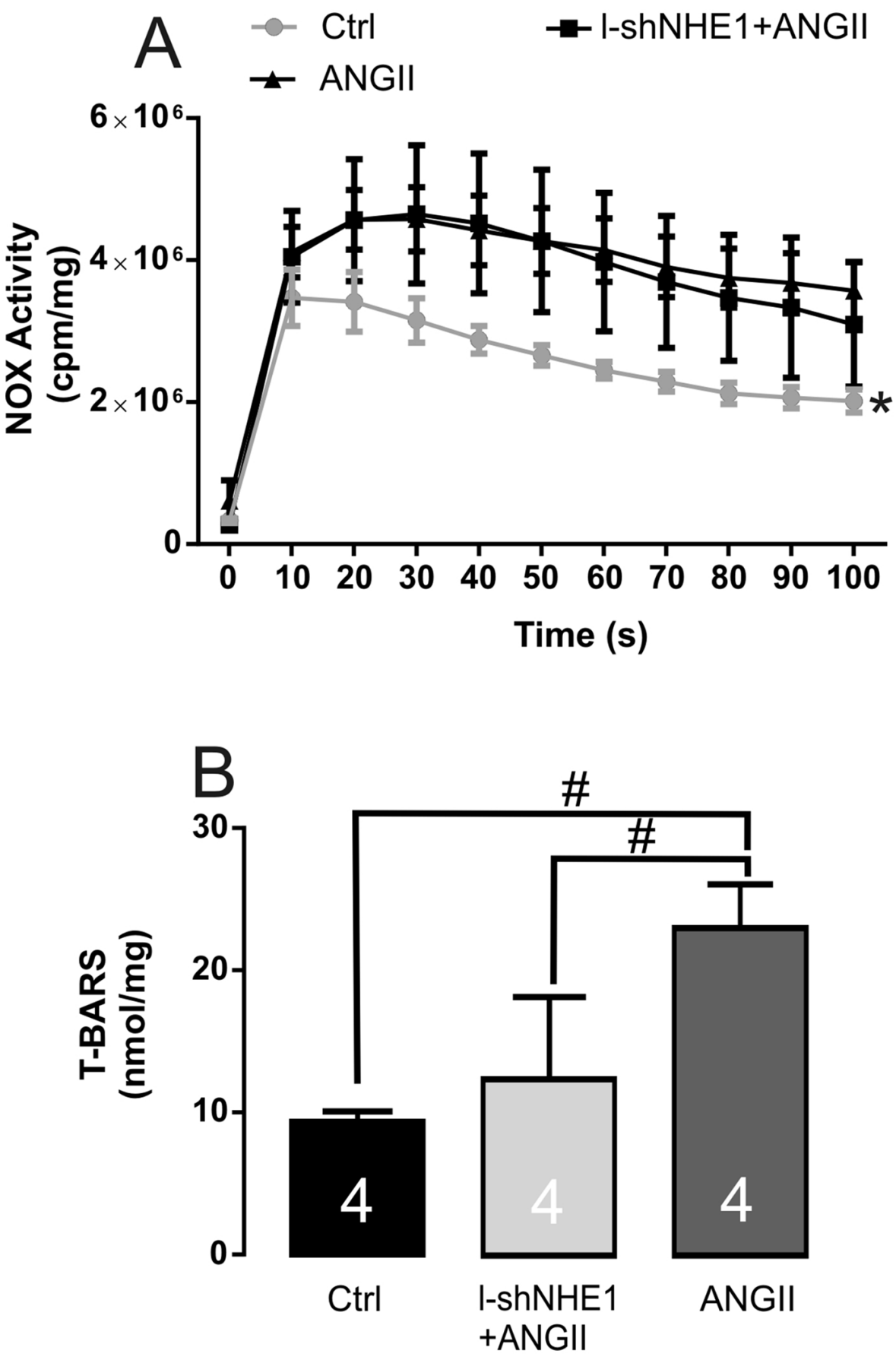
A
Ctrl
ANGII
l-shNHE1+ANGII
NOX Activity (cpm/mg)
6×10⁶
4×10⁶
2×10⁶
0
0
10
20
30
40
50
60
70
80
90
100
Time (s)
*
B
T-BARS (nmol/mg)
30
20
10
0
#
#
4
4
4
Ctrl
l-shNHE1 +ANGII
ANGII

Figure 4

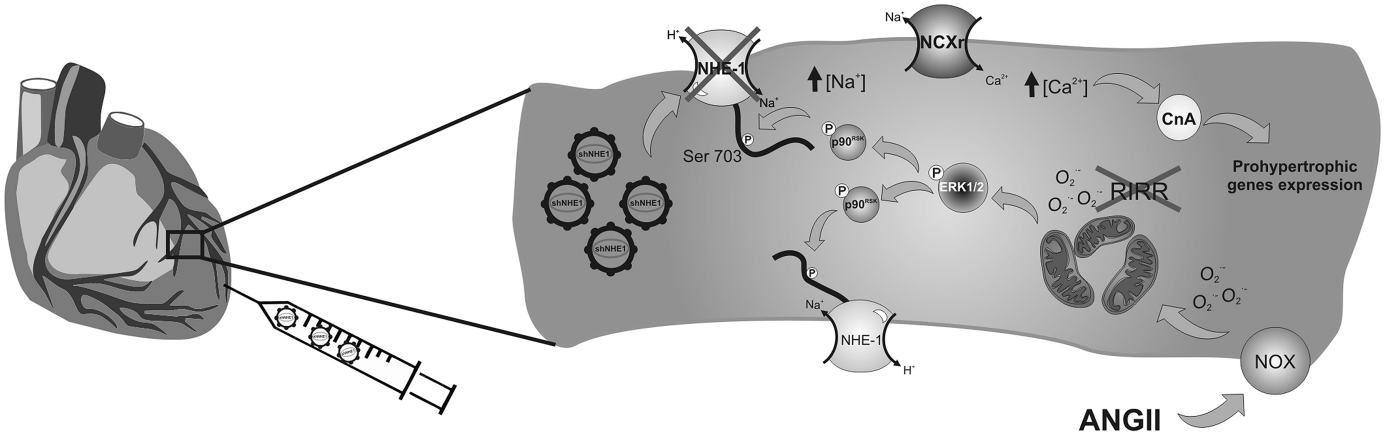
H+
NHE-1
Na+
[Na+]
NCXr
Ca2+
[Ca2+]
CnA
Prohypertrophic genes expression
shNHE1
shNHE1
shNHE1
shNHE1
Ser 703
P
p90rsk
ERK1/2
RIRR
O2-
Na+
NHE-1
H+
NOX
ANGII

Figure 5

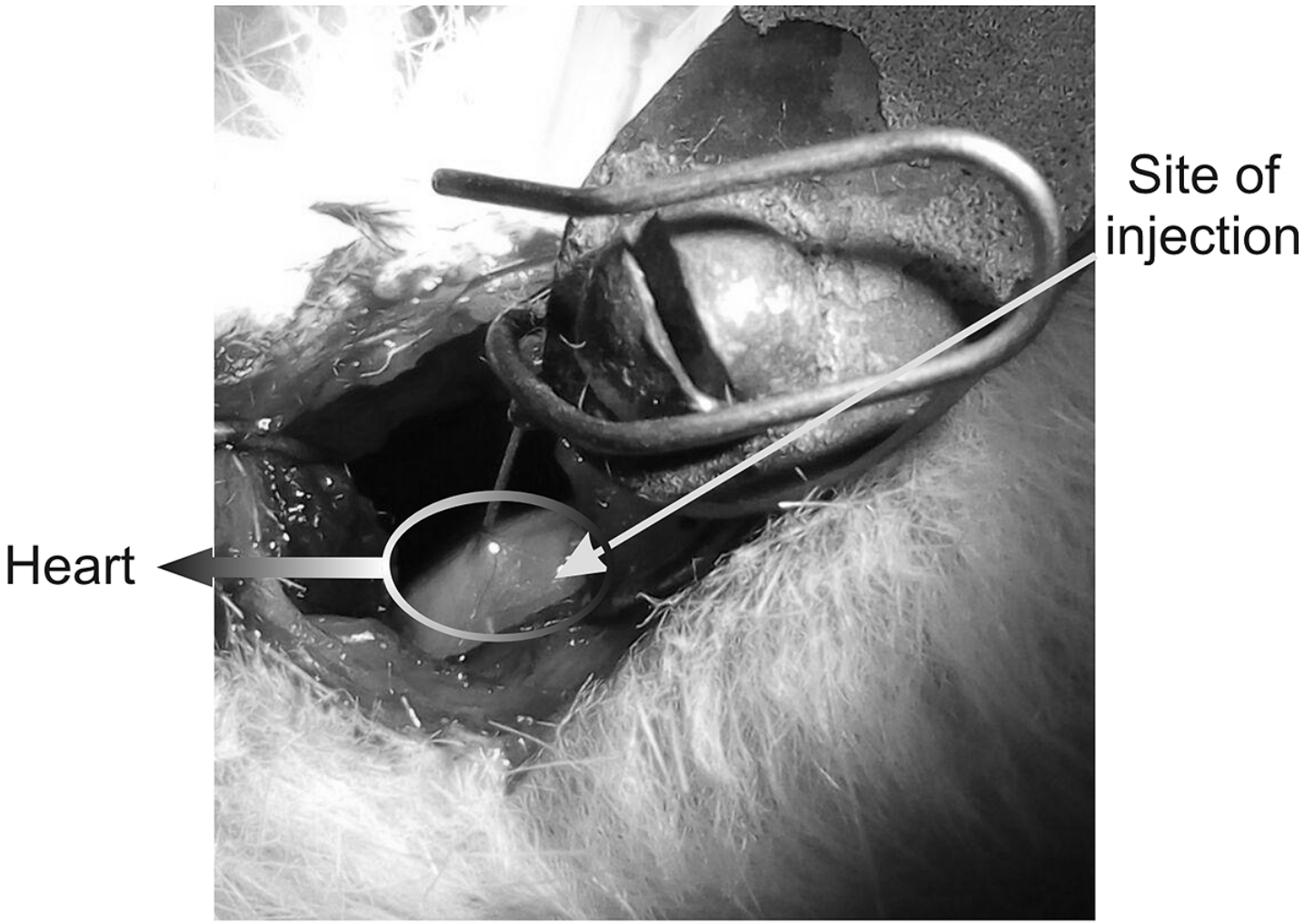

Figure 6

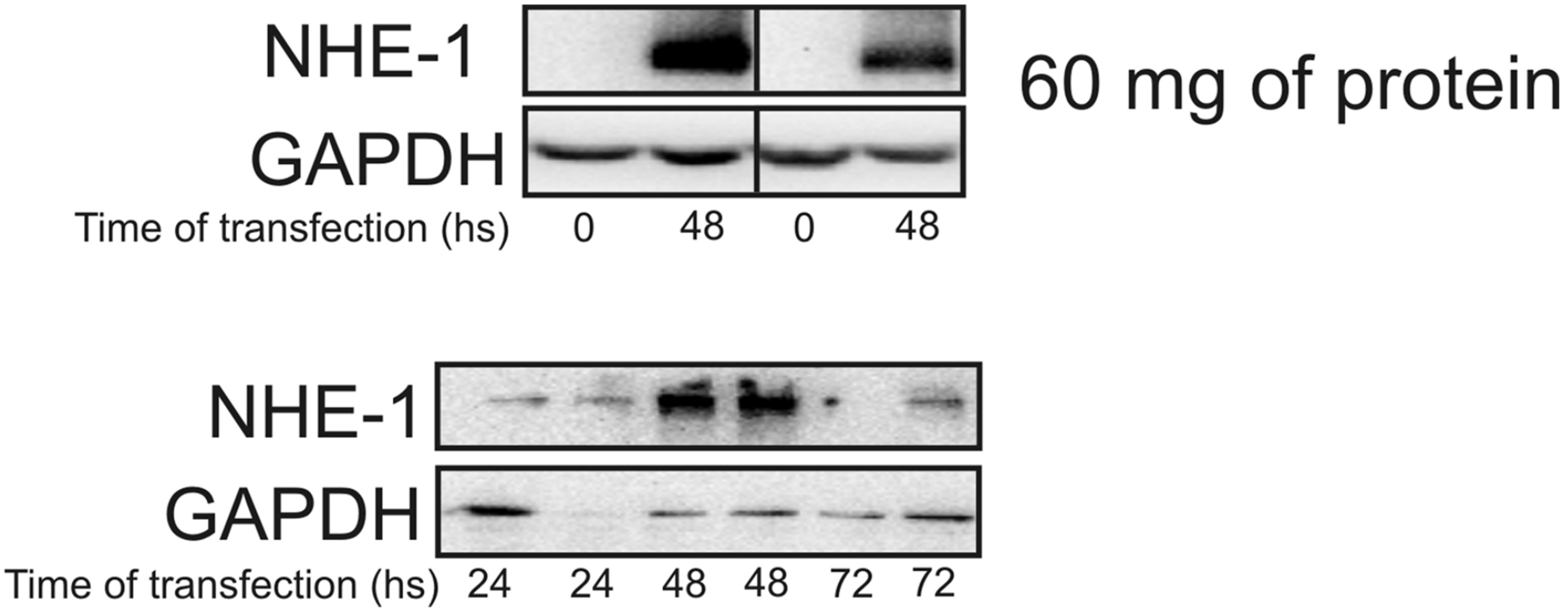
NHE-1
GAPDH
60 mg of protein
Time of transfection (hs) 0 48 0 48
NHE-1
GAPDH
Time of transfection (hs) 24 24 48 48 72 72

Figure 7