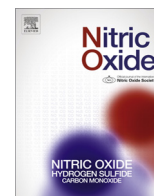


Contents lists available at [ScienceDirect](http://ScienceDirect)

## Nitric Oxide

journal homepage: [www.elsevier.com/locate/yniox](http://www.elsevier.com/locate/yniox)

## Cardioprotective mechanism of S-nitroso-N-acetylcysteine via S-nitrosated betadrenoceptor-2 in the LDLr<sup>-/-</sup> mice



Amarylis Claudine Bonito Azeredo Wanschel<sup>a,b,\*</sup>, Viviane Menezes Caceres<sup>c,1</sup>, Ana Iochabel Soares Moretti<sup>d</sup>, Alexandre Bruni-Cardoso<sup>b</sup>, Hernandes Faustino de Carvalho<sup>b</sup>, Heraldo Possolo de Souza<sup>d</sup>, Francisco Rafael Martins Laurindo<sup>e</sup>, Regina Célia Spadari<sup>c,1</sup>, Marta Helena Krieger<sup>b,1</sup>

<sup>a</sup> Marc and Ruti Bell Vascular Biology and Disease Program, Leon H. Charney Division of Cardiology, Department of Medicine, New York University School of Medicine, New York, NY, USA

<sup>b</sup> Department of Anatomy, Cellular Biology and Physiology, State University of Campinas (UNICAMP), Biology Institute, São Paulo, Brazil

<sup>c</sup> Department of Biosciences, Federal University of São Paulo (UNIFESP), São Paulo, Brazil

<sup>d</sup> Department of Emergency Medicine, University of São Paulo (USP), São Paulo, Brazil

<sup>e</sup> Heart Institute (InCor), University of São Paulo School of Medicine, São Paulo, Brazil

## ARTICLE INFO

## Article history:

Received 12 July 2013

Revised 16 October 2013

Available online 12 December 2013

## Keywords:

Hydrogen peroxide

Superoxide

S-nitroso-N-acetylcysteine

Ventricular hypertrophy

Betadrenoceptor-2

S-nitrosated

## ABSTRACT

Previous studies from our group have demonstrated the protective effect of S-nitroso-N-acetylcysteine (SNAC) on the cardiovascular system in dyslipidemic LDLr<sup>-/-</sup> mice that develop atheroma and left ventricular hypertrophy after 15 days on a high fat diet. We have shown that SNAC treatment attenuates plaque development via the suppression of vascular oxidative stress and protects the heart from structural and functional myocardial alterations, such as heart arrhythmia, by reducing cardiomyocyte sensitivity to catecholamines. Here we investigate the ability of SNAC to modulate oxidative stress and cell survival in cardiomyocytes during remodeling and correlation with  $\beta_2$ -AR signaling in mediating this protection. Ventricular superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) generation was measured by HPLC methods to allow quantification of dihydroethidium (DHE) products. Ventricular histological sections were stained using terminal dUTP nick-end labeling (TUNEL) to identify nuclei with DNA degradation (apoptosis) and this was confirmed by Western blot for cleaved caspase-3 and caspase-7 protein expression. The findings show that  $O_2^-$  and  $H_2O_2$  production and also cell apoptosis were increased during left ventricular hypertrophy (LVH). SNAC treatment reduced oxidative stress during on cardiac remodeling, measured by decreased  $H_2O_2$  and  $O_2^-$  production (65% and 52%, respectively), and a decrease in the ratio of p-Ser1177 eNOS/total eNOS. Left ventricle (LV) from SNAC-treated mice revealed a 4-fold increase in  $\beta_2$ -AR expression associated with coupling change to Gi;  $\beta_2$ -ARs-S-nitrosation ( $\beta_2$ -AR-SNO) increased 61%, while apoptosis decreased by 70%. These results suggest that the cardio-protective effect of SNAC treatment is primarily through its anti-oxidant role and is associated with  $\beta_2$ -ARs overexpression and  $\beta_2$ -AR-SNO via an anti-apoptotic pathway.

© 2013 Elsevier Inc. All rights reserved.

## Introduction

Left ventricular (LV) remodeling, often associated with heart failure (HF), can be triggered as a secondary response to abnormal cardiac pressure volume loading or neurohumoral stimuli. One common finding in HF is increased activation of the sympatho-adrenoceptor system [1] which mediates functional compensation

\* Corresponding author. Address: New York University School of Medicine, 522, First Avenue – Smilow 703, New York, NY 10016, USA. Fax: +1 212 263 9115.

E-mail address: [amarylisclaudine.wanschel@nyumc.org](mailto:amarylisclaudine.wanschel@nyumc.org) (Amarylis Claudine Bonito Azeredo Wanschel).

<sup>1</sup> Equal contribution.

and cardiac remodeling through the  $\beta$ -adrenoceptors [2]. Like  $\beta_1$ -,  $\beta_2$ -ARs are G protein coupled receptors that activate the adenylate cyclase/protein kinase A (AC-PKA) pathway. Interestingly,  $\beta_2$ -ARs also couples to the pertussis toxin-sensitive protein G inhibitor (Gi) pathway which eventually inhibits G stimulatory (Gs). Gi promotes cardiomyocyte survival through a protective PI3K-PKB pathway [3], that is involved in the  $\beta_2$ -AR-mediated cardiomyocyte protection from hypoxia and reactive oxygen species (ROS).

The role of oxidative stress as a pathophysiological mechanism in left ventricular remodeling and its participation in the progression of heart failure is well known [4]. Oxidative stress can induce many of the changes that contribute to myocardial remodeling. For example, the production of ROS [5] results in a phenotype

characterized by hypertrophy and apoptosis of isolated cardiac myocytes [6]. Increased apoptosis, or programmed cell death, plays a major role in cell survival signaling pathways involved in cardiac hypertrophy [7,8] and is one of the mechanisms that contributes to the transition from left ventricular hypertrophy (LVH) to heart failure [7,9,10].

Nitric oxide (NO<sup>•</sup>) has surfaced as an endogenous inhibitor of pathological hypertrophy [11] and treatments with NO<sup>•</sup> donors and S-nitrosothiols (RSNOs) have been shown to exhibit a diverse range of cardioprotective functions. We have previously shown that in low-density lipoprotein-receptor-knockout (LDLr<sup>-/-</sup>) mice that develop atheroma and left ventricular hypertrophy after 15 days on a high fat diet, treatment with an NO<sup>•</sup> donor, S-nitroso-N-acetylcysteine (SNAC), attenuates plaque development via the suppression of vascular oxidative stress [12]. We have shown that SNAC protects the heart from structural and functional myocardial alterations, such as heart arrhythmia, by reducing myocyte sensitivity to catecholamines [13].

NO<sup>•</sup> has been linked to G protein-coupled receptors (GPCRs) via S-nitrosation, which is a process of signal transduction resulting from the covalent modification of protein cysteine residue sites, [14–16] and can stimulate adrenoceptor-beta overexpression [17]. In this study we used LDLr<sup>-/-</sup> mice to assess the ability of SNAC to modulate oxidative stress and cell survival in cardiac remodeling and its correlation with  $\beta_2$ -AR signaling in mediating protection through an anti-apoptotic pathway.

We demonstrated that SNAC treatment attenuated oxidative stress and apoptosis responses induced by the hypertrophic phenotype and these protective mechanisms appear to be associated with increased  $\beta_2$ -AR expression/nitrosation mediated by Gi-coupling. This study demonstrates cross-talk between hypertrophic signaling and  $\beta_2$ -AR signaling, which may represent an important mechanism in the transition from compensatory myocardial hypertrophy to ventricular dysfunction and heart failure.

## Methods

### Left ventricular hypertrophy model

Low-density lipoprotein-receptor-knockout (LDLr<sup>-/-</sup>) mice on an atherogenic diet for 15 days have increased left ventricular mass that was characterized by increased LV weight (mg) per body weight (g) ratio, increased cardiomyocyte diameter and interstitial/perivascular collagen deposition which was prevented by SNAC treatment [13].

### Animals

Three-month-old male C57BL6 and low-density lipoprotein-receptor-deficient (LDLr<sup>-/-</sup>) mice (24 ± 3 g, n = 60) from Jackson Laboratory (Bar Harbor, ME) were used in the experiments. The Institutional Committee for Ethics in Animal Experimentation (CEEA/IB 2044-1-UNICAMP) approved the experimental protocols in agreement with the guidelines of the Brazilian College for Animal Experimentation (COBEA). The 3 months old male mice were randomly allocated to one of 3 groups and received food and water ad libitum for 15 days: (i) control LDLr<sup>-/-</sup> mice fed a standard diet (Nuvital CR1) and injected i.p. with a daily dose of 0.1 ml of PBS (C; n = 20); (ii) hypercholesterolemic LDLr<sup>-/-</sup> mice fed a high fat diet (containing: 20% fat, 1.25% cholesterol and 0.5% cholic acid) and injected i.p. with a daily dose of 0.1 ml PBS (H; n = 20) and (iii) hypercholesterolemic LDLr<sup>-/-</sup> mice fed a high fat diet (containing: 20% fat, 1.25% cholesterol and 0.5% cholic acid) but injected i.p. with a daily dose of 0.51 mmol/kg of SNAC (H+S; n = 20) (Fig. 1). The mice were anesthetized with xylazine (Coopers, São Paulo, Brazil) and

ketamine (Parke–Davis, Argentina), 6 and 40 mg/kg, respectively, IP. The heart was gently perfused with PBS/DTPA buffer composed of (in mM) 7.78 Na<sub>2</sub>HPO<sub>4</sub>, 2.20 KH<sub>2</sub>PO<sub>4</sub>, 140 NaCl, and 2.73 KCl, pH 7.4, to remove the blood. The left ventricles were removed and cut into segments which were used immediately for HPLC analysis and DHE-derived fluorescence. Other left ventricle segments were used for Western blotting analysis and TUNEL assays.

### Synthesis of SNAC and in vitro stability of SNAC solution

The SNAC synthesis process, stability solution, and calculation of the concentration and dose adopted were performed as described [18].

### In vitro analysis of inotropic responses to isoprenaline

After being anesthetized, each mouse was euthanized by cervical dislocation, the heart was removed, and the left atria were isolated. The atria were suspended in 20 ml organ baths containing Krebs–Henseleit solution with the following composition: 115 mM NaCl, 4.6 mM KCl, 2.5 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 12.4 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 25.0 mM NaHCO<sub>3</sub>, 11 mM glucose and 0.11 mM ascorbic acid. This solution was warmed (36.5 ± 0.1 °C) and continuously gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The atria were attached to isometric force transducers (Narco F-60, Narco Biosystem, Houston, TX, USA) under a resting tension of 4.9 mN and contractile responses were recorded on a Narco Biosystem polygraph. The left atria were electrically paced at 1 Hz and 5 ms using a voltage stimulus 20% above the threshold [19]. The length of the left atrium was set to obtain 80% of the resting tension associated with the maximum developed force. The tissues were allowed to stabilize for 60 min. Following stabilization, the atria were incubated with phenoxybenzamine (10 μM) for 15 min to block  $\alpha$ -adrenoceptors, extraneuronal uptake, and muscarinic receptors [20]. This period was followed by 45 min of thorough washing [19]. After recovery of the basal frequency and tension, corticosterone (30 μM) and desipramine (0.1 μM) were added to the bath and maintained throughout the experiment to inhibit extraneuronal uptake and neuronal reuptake, respectively. After this treatment, cumulative concentration–response curves for isoprenaline (ISO) were obtained. A selective  $\beta_2$ -AR antagonist, 50 nM ICI118,551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) [21], was then added and left in contact with the tissue for 2 h before another concentration–response curve was obtained using the same agonist (ISO) in the presence of antagonist (ICI118,551). A maximum response was reported when a 0.5 log unit increase in the agonist concentration produced no additional increase in the atrial tension.

Sensitivity to isoprenaline was evaluated by determining the concentration that produced 50% of the maximum response (EC<sub>50</sub>), and it was expressed as the negative logarithm of the EC<sub>50</sub> (pD<sub>2</sub>).

### Treatment with pertussis toxin

In order to examine the involvement of Gi proteins in the response to isoprenaline, C, H and H+S mice were treated with pertussis toxin (PTX; 30 μg/kg, i.p. 3 days before sacrifice) [22]. The right and left atria were isolated and prepared for analysis of the chronotropic and inotropic responses to isoprenaline, respectively, as described below. To assess the effectiveness of the treatment with PTX, the atria were incubated with 20 μM carbachol for 5 min, followed by washing and equilibration for 90 min [19,23,24].

### Tissue extracts analysis by HPLC (separation of DHE, 2-hydroxyethidium (EOH), and ethidium)

Left ventricle segments (~3 mm in length) were incubated in 0.5 ml of PBS/DTPA buffer containing 100  $\mu$ M diethylenetriamine pentaacetic acid (Sigma, St. Louis, MO, USA) (PBS/DTPA) for 15 min in a 1.5-ml Eppendorf vial. A volume of 2.5  $\mu$ l of DHE 10 mM stock solution was added to the buffer to achieve a final concentration of 50  $\mu$ M and a final DMSO concentration of 0.5% vol/vol and further incubation in the dark was carried out for 30 min at 37 °C. The segments were washed in PBS, transferred to liquid nitrogen, and homogenized with mortar and pestle. The homogenate was resuspended in acetonitrile (0.5 ml), sonicated (3 cycles at 8 W for 10 s), and centrifuged (12,000g for 10 min at 4 °C). The supernatant was dried under vacuum (Speed Vac Plus model SC-110A, Thermo Savant) and the resulting pellets were maintained at –20 °C in the dark until analysis when the samples were resuspended in 120  $\mu$ l PBS/DTPA and injected (100  $\mu$ l) into the HPLC system. Positive controls, elaboration of this method and HPLC conditions of analysis, was performed as described previously [15]. Simultaneous detection of DHE and its derived oxidation products (EOH and ethidium) using, respectively, ultraviolet and fluorescence detection, allowed the use of DHE as an internal control during organic extraction. Thus, DHE-derived products were expressed as a ratio of EOH and ethidium generated per DHE consumed (initial DHE concentration minus remaining DHE; EOH/DHE and ethidium/DHE, respectively). The data were also normalized for tissue weight.

### Western blotting

The frozen left ventricular tissue of the mice was pulverised in liquid nitrogen with a mortar and pestle, it was then resuspended in homogenization buffer, 1% Triton X-100 (Amresco, Solon, Ohio), 10 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10  $\mu$ g/ml Aprotinin (Amresco, Solon, Ohio), 1 mmol/l PMSF, 0.25 mmol/l sodium orthovanadate and 0.1% cocktail inhibitors protease. The samples were centrifuged for 20 min at 11,000g and the supernatant was collected and assayed for total protein concentration using the Bradford method (Bio Rad, Hercules, CA, USA). Samples were stored at –80 °C until assay. Protein expression was determined via SDS–polyacrylamide gel electrophoresis under reducing conditions. Left ventricular tissue extracts (30  $\mu$ g/ml) from at least four animals of each group were boiled in equal volumes of loading buffer (150 mM Tris–HCl, pH 6.8; 4% SDS; 20% glycerol; 15%  $\beta$ -mercaptoethanol; and 0.01% bromophenol blue) and subjected to electrophoresis on 10% polyacrylamide gels. Following electrophoretic separation, proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Buckinghamshire, England). Membranes were blocked with 5% non-fat dry milk or bovine serum albumin (Sigma, St. Louis, MO, USA) in buffer containing 10 mM Tris–HCl (pH 7.6), 10 mM NaCl, and 0.1% Tween 20 (Calbiochem, Darmstadt, Germany) (TBST) for 1 h. Primary antibodies against the following were employed: eNOS (rabbit polyclonal, 1:1000, 610299; BD Transduction Laboratories); eNOSpS1177 (mouse monoclonal, 1:500, 612392; BD Transduction Laboratories); betadrenoceptor-1 (rabbit polyclonal, 1:100, sc-568, Santa Cruz Biotechnology, Santa Cruz, CA, USA), betadrenoceptor-2 (rabbit polyclonal, 1:100, sc570, Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-3 (rabbit polyclonal, 1:1000, sc7148, Santa Cruz Biotechnology, Santa Cruz, CA, USA), nitrotyrosine (mouse monoclonal, 1:1000, clone 1A6), and GAPDH (rabbit polyclonal, 1:2000, sc25778, Santa Cruz Biotechnology, Santa Cruz, CA, USA). All antibodies were incubated at 4 °C overnight. After the blots had been washed twice with TBST, secondary antibody horseradish peroxidase conjugate (goat anti-rabbit polyclonal, 1:10,000,

G21234 or goat anti-mouse 81–6520, Invitrogen, Molecular Probes, Oregon, USA) was applied at 1:10,000 for 1 h. Blots were washed in TBST twice over 30 min, incubated using an enhanced Super Signal chemiluminescent reagent detection kit (Pierce, Rockford, IL, USA), and exposed to Kodak O-OMAT-AR photographic film (Kodak, Rochester, NY, USA). Band intensity of original blots was quantified using Image J software.

### Assessment of S-nitrosation using chemical derivatization biotin-switch (BST) coupled to immunoprecipitation and Western blotting

S-nitrosated proteins were labeled with biotin in the lysates, as previously described [25,26]. Left ventricles were rinsed with PBS containing 0.1 mM EDTA and 0.01 mM neocuproine (Sigma, St. Louis, MO, USA), cut into segments, which were pulverised in liquid nitrogen with a mortar and pestle, immediately resuspended in HEN lyses buffer containing 0.1% SDS, 0.5% CHAPS, and 20 mM NEM (N-ethylmaleimide) (Sigma, St. Louis, MO, USA), and lysed by rocking for 30 min, at 4 °C. The lysates were centrifuged for 10 min at 14,000g and 4 °C and the excess NEM used to block sulfhydryl groups [4] was removed by protein precipitation with acetone. The resulting pellets were resuspended in HEN buffer containing 1% SDS (HENS) and the S-nitrosothiols were reduced and biotinylated by the simultaneous addition of 10 mM sodium ascorbate and 0.05 mM of the sulfhydryl-specific biotinylating agent, MPB [N-(3-maleimidylpropionyl) biocytin, Molecular Probes], for 1 h at room temperature (RT). The extra label was removed by a second acetone precipitation, and the proteins resuspended in HENS buffer and assayed for total protein concentration then a small amount was saved to perform GAPDH expression by Western blot and 100  $\mu$ g were used to immunoprecipitate the biotinylated proteins. Left ventricular tissue extracts (100  $\mu$ g/ml) were incubated overnight in 50  $\mu$ l streptavidin–agarose. Immunoprecipitates were washed three times with 800  $\mu$ l of HEN buffer and resuspended in 25  $\mu$ l of HEN, followed by the addition of 20  $\mu$ l of 2 $\times$  Laemmli sample buffer (150 mM Tris–HCl, pH 6.8; 4% SDS; 20% glycerol and 0.01% bromophenol blue); Western blotting was then performed as described above and revealed with antibodies against betadrenoceptor-2 (rabbit polyclonal, 1:100, sc570, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or caspase-3 (rabbit polyclonal, 1:1000, sc7148, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Control experiments were also performed in which the sodium ascorbate was omitted, thus preventing the reduction of S-nitrosothiols. All samples were protected from light during all procedures prior to electrophoresis; densities were analyzed by Image J software.

### DNA fragmentation detection by terminal deoxynucleotidyl transferase mediated-dUTP nick end labeling (TUNEL)

Each left ventricle was weighed, and the ratio of LV weight (mg) per body weight (g) was calculated. Briefly the mice were anesthetized, and their hearts were perfused *in situ* with PBS followed by 10% PBS buffered formaldehyde. The LVs were fixed in 10% formaldehyde for at least 2 days and then washed in 70% alcohol, they were then processed for paraffin inclusion using standard methods: cross-sections of 3  $\mu$ m were cut and mounted on silanized glass slides, dewaxed with xylene, and rehydrated in a decreasing ethanol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water for 30 min and assayed for DNA fragmentation using a TUNEL assay (*In Situ* Cell Death Detection Kit, POD-Roche Mannheim, Germany) according to the manufacturer's instructions. After extension of the fluorescein-labeled deoxy-UTP tail with the TdT enzyme, a peroxidase-labeled anti-fluorescein antibody was used and peroxidase activity was revealed with 3,3'-diaminobenzidine. Twenty microscopic fields

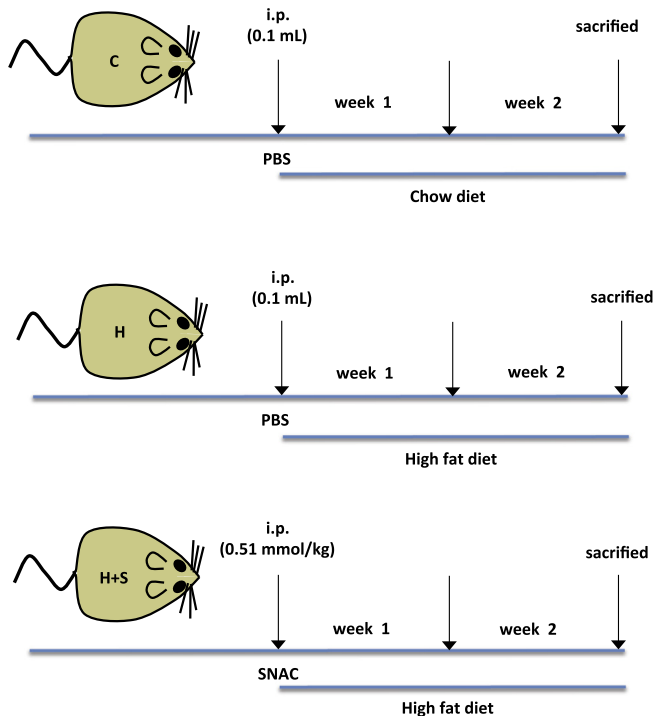


Fig. 1. Experimental outline of SNAC treatment in LDLr<sup>-/-</sup> mice ( $n = 12$ ).

from the LV of three animals per group were selected randomly at  $100\times$  objective. The frequency of apoptotic cells was counted and expressed as a percentage of the total. Observations and photomicrographs were made with an Olympus microscope equipped for fluorescence microscopy (Fig. 1).

#### Statistical analysis

The results were expressed as mean  $\pm$  SEM and were compared by Analysis of Variance (ANOVA) followed by the Tukey test, with  $P < 0.05$  indicating a statistically significant difference.

## Results

#### ROS species reduction and eNOS activation

Because oxidative stress generates a wide array of deleterious processes that synergize to contribute to adverse cardiac remodeling, we investigated superoxide and hydrogen peroxide amounts in the LV by using HPLC analysis and quantification of the DHE-derived fluorescent products to increase our understanding of redox events during LV remodeling. HPLC analysis of the left ventricle revealed an increase in the EOH/DHE (Fig. 2a) and ethidium/DHE (Fig. 2b) ratio for the hypertrophied left ventricles from animals of the H group compared to those in the control group. SNAC treatment revealed decreased ratios of EOH/DHE and ethidium/DHE (52% and 65% respectively) in relation to those of the H group (Fig. 2a and b).

We also examined the expression of activated eNOS in this situation. The ratio of p-Ser1177 eNOS/total eNOS on the left ventricle from control and H mice was not changed. However, SNAC treatment revealed approximately a 50% reduction of this ratio on H+S mice, as seen in Fig. 2c.

To check for the possibility of toxicity caused by SNAC-donated nitric oxide reacting with superoxide resulting in peroxynitrite (ONOO<sup>-</sup>) formation, we analyzed the expression of nitrotyrosine (NT) which is used as a biomarker of reactive nitrogen species

formation [27]. No changes in NT expression were observed in the left ventricle from animals treated with SNAC compared to those in the H group (Fig. 2d).

#### $\beta$ -Adrenoceptors involvement

To explore the pathway mediating the pro-apoptotic effect in the cardiomyocytes of the left ventricle, we first analyzed initial  $\beta_1$ -AR protein expression by Western blot, and found no significant differences (Fig. 3a). We then evaluated  $\beta_2$ -AR protein expression to verify the effects of SNAC treatment in the mediation of the anti-apoptotic effect. We found that 15 days on a high fat diet (LVH) plus treatment with SNAC lead to a 4-fold increase in  $\beta_2$ -AR expression ( $P < 0.001$ ;  $n = 7$ ; Fig. 3b).

#### $\beta_2$ -ARs coupled to Gi proteins are active by SNAC in the left atrium

Left atria of LDLr<sup>-/-</sup> mice (C group) exhibit lower basal tension and maximum response to isoprenaline than left atria of C57BL6 mice. Feeding them with high-fat diet with or without SNAC treatment (H+S and H groups, respectively) did not modify the atrial contractile performance.

In left atria of LDLr<sup>-/-</sup> mice, the sensitivity to isoprenaline was not different from that of the C57BL6 mice. However, when LDLr<sup>-/-</sup> mice were fed with cholesterol-enriched diet (H), the concentration–response curves to isoprenaline were shifted to the left revealing that the atria were supersensitive to isoprenaline when compared with C57BL6. The treatment with SNAC of LDLr<sup>-/-</sup> mice fed with cholesterol-enriched diet cancelled the left atria supersensitivity to isoprenaline (Table 1).

In vitro incubation of left atria with 50 nM ICI118,551, a selective  $\beta_2$ -adrenoceptor antagonist, had no effect on the basal tension or the response to isoprenaline in left atria isolated of C57BL6 mice. Nevertheless, the  $\beta_2$ -adrenoceptor antagonist reversed the contractile deficit in left atria of LDLr<sup>-/-</sup> mice fed or not with cholesterol-enriched diet as well as the supersensitivity to isoprenaline in left atria of mice of the H group. In left atria of LDLr<sup>-/-</sup> mice fed with cholesterol enriched diet and treated with SNAC, the presence of ICI118,551 shifted to the left the concentration response curve to isoprenaline (Table 1).

These data suggest that in left atria of LDLr<sup>-/-</sup> mice fed with cholesterol-enriched diet, the response to isoprenaline is mediated by a mixed population of  $\beta_1$ -ARs coupled to Gs protein and  $\beta_2$ -ARs coupled to Gi protein.

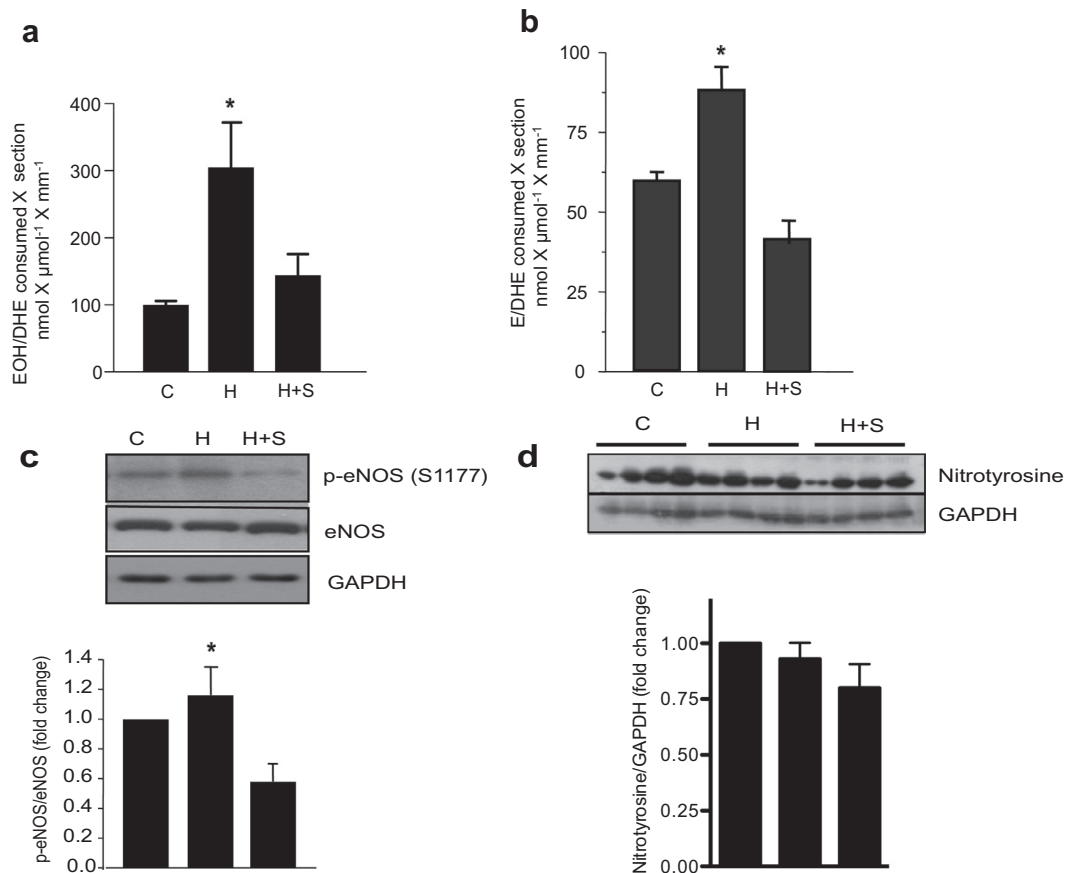
In order to test the hypothesis that LDLr<sup>-/-</sup> mice fed with commercial diet or cholesterol enriched diet and treated or not with SNAC were also treated with PTX. In left atria of C, H and H+S mice treated with PTX there was an increase in the basal tension and to isoprenaline (Fig. 3c) so that the contractile deficit previously detected was eliminated. The sensitivity to isoprenaline was not different between C ( $pD_2$  value  $8.44 \pm 0.10$ ) and H mice ( $pD_2$  value  $8.74 \pm 0.11$ ). On the other hand, in the H+S group, treatment with PTX revealed supersensitivity to the inotropic effect of isoprenaline in the left atria ( $pD_2$  value  $9.11 \pm 0.11$ ).

The effect of C, H mice treatment with PTX in the tension developed by left atria before the agonist has been added to the organ bath (basal tension) suggested that Gi-coupled- $\beta_2$ -ARs might exhibit constitutive activity. However the effect of treatment with PTX in the sensitivity to isoprenaline developed by left atria was only observed in the H + S mice, what suggest that in this group SNAC might be induced Gi-coupled- $\beta_2$ -ARs additional activity.

#### $\beta$ -Adrenoceptor S-nitrosation

Since S-nitrosothiols (RSNOs) are reported to modulate G-protein coupled receptor signaling via reversible, thiol-sensitive





**Fig. 2.** Production of reactive oxygen species is downregulated during left ventricle hypertrophy in the presence of SNAC. (a) Ratio of 2-hydroxyethidium/dihydroethidium (EOH/DHE) and (b) ethidium/dihydroethidium (E/DHE). (c) Western blot of phosphorylated and unphosphorylated endothelial nitric oxide synthase (eNOSpSer1177 and eNOS) in tissue lysates of left ventricle. (d) Western blot of Nitrotyrosine from tissue lysates from left ventricle. Data are the mean of quadruplicate samples  $\pm$  S.E.M. and are representative of 3 independent experiments. \* $P < 0.05$ .

mechanisms, we assessed the S-nitrosation status of  $\beta_2$ -AR at cysteine residues in heart tissue by a biotin-switch followed by immunoprecipitation and western blotting.  $\beta_2$ -AR nitrosation in the H+S group exceeded that of the H group by 61% (Fig. 3d).

Left ventricles were subject to the biotin-switch and probed for S-nitrosated GAPDH (GAPDH-SNO), along with an ascorbate control. Notably, omission of ascorbate lead to a nearly complete loss of the biotinylation signal. (Supplemental Fig. 1).

#### Inhibition of apoptosis

To investigate the viability of myocardial cells in the left ventricular regions, and the *in vivo* anti-apoptotic effect of SNAC, we performed TUNEL staining on the different experimental groups. Representative photographs of TUNEL-positive nuclei in the heart are shown in Fig. 4a. Quantitative analysis showed a significantly higher proportion of TUNEL-positive cells in the myocardia of H mice those in that of control mice whereas very few or no TUNEL-positive cells could be detected in the hearts the H mice after SNAC treatment (Fig. 4b). These cell apoptosis results were confirmed by western blotting with a specific antibody to cleaved caspase-3 (Fig. 4c) and caspase-7 (Fig. 4d). Cell apoptosis was confirmed by cleaved caspase-3 protein expression since it increases in response to diverse intrinsic and extrinsic death stimuli. As expected, LVH increased levels of cleaved caspase-3 and caspase-7 protein in the mouse hearts (Fig. 4c and d). However, this increase was significantly blocked by treatment with SNAC.

As the Bax translocation to the mitochondria is a key event, which leads to downstream apoptotic events, we next investigated

whether SNAC altered Bax protein in the LVH. SNAC treatment inhibited expression of Bax (Fig. 4e).

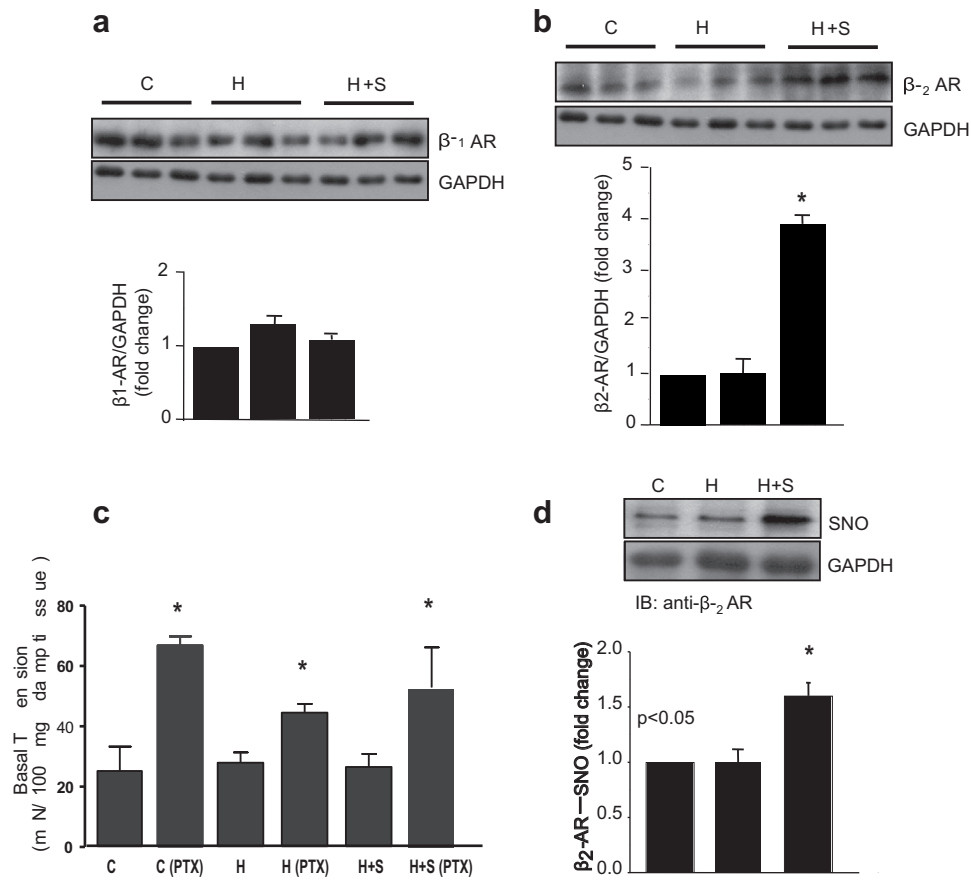
Because ER stress induced apoptosis is partly mediated by the transcription factor CHOP, we evaluate the effects of SNAC treatment on ER stress-induced apoptosis. We found that 15 days on a high fat diet (LVH) plus treatment with SNAC lead to a decreased CHOP expression (Fig. 4f). Collectively, these data indicated that SNAC played an antiapoptotic role at the mitochondrial and the ER level by reducing Bax and CHOP expression (see Fig. 6).

To confirm whether the protective effect of SNAC may be partially mediated through the inhibition of caspase-3 activity by NO-mediated S-nitrosation, we assessed S-nitrosation of procaspase-3 and cleaved caspase-3. The results indicated no changes in caspase-3 S-nitrosation after treatment with SNAC (Fig. 5).

#### Discussion

In the present study, we set out to elucidate the cardio protective role of SNAC and its role in cell survival in the hypertrophied heart. Our results provide evidence that SNAC protects the heart by reducing sensitivity to catecholamines thereby preventing excessive adrenergic stimulation due to an increase in  $\beta_2$ -AR coupling to Gi. It also increases S-nitrosation of the  $\beta_2$ -AR, which may explain the anti-apoptotic effect of SNAC treatment.

The development of oxidative stress in LVH is a multifactorial process caused by variety of mechanisms. Superoxide anions  $\text{O}_2^-$  are probably the most important free oxygen radicals generated *in vivo*, and it is highly likely that they are derived from more than one source. One major source is NADPH oxidase, but ROS can also



**Fig. 3.** SNAC decreases ISO sensitivity by promoting  $\beta$ 2AR-Gs uncoupling and  $\beta$ 2AR-Gi coupling. (a) Western blot of betadrenoceptor-1 from tissue lysates from left ventricle. (b) Western blot of betadrenoceptor-2 from tissue lysates from left ventricle. (c) Baseline tension to isoprenaline (ISO) in the left atria of LDLr<sup>-/-</sup> mice fed with commercial diet C, or cholesterol enriched diet H. Where indicated, mice were treated with Pertussis toxin (PTX) 30  $\mu$ g/kg, i.p., 3 days before sacrifice. (d) Left ventricular tissue was subjected to the Biotin Switch and western blotting was then performed against betadrenoceptor-2. Data are the mean of triplicate samples  $\pm$  S.E.M. and are representative of 3 independent experiments. \* $P$  < 0.05.

**Table 1**

Baseline tension (mN/mg wet tissue, BT) and  $pD_2$  values of isoprenaline (ISO) in the left atrium of C57BL6 and LDLr<sup>-/-</sup> (C) mice fed with cholesterol enriched diet (H) and treated with SNAC (H+S).

	Without ICI118,551		With ICI118,551		With PTX	
	BT	$pD_2$	BT	$pD_2$	BT	$pD_2$
C57BL6	51.93 $\pm$ 3.79	8.66 $\pm$ 0.10	58.63 $\pm$ 5.54	8.87 $\pm$ 0.17	–	–
C	25.21 $\pm$ 8.06 <sup>†</sup>	9.05 $\pm$ 0.17	71.56 $\pm$ 9.45 <sup>#</sup>	9.05 $\pm$ 0.23	–	–
H	27.93 $\pm$ 3.44 <sup>†</sup>	9.56 $\pm$ 0.22 <sup>†</sup>	62.25 $\pm$ 5.46 <sup>#</sup>	9.07 $\pm$ 0.17	54.90 <sup>#</sup>	9.45
H+S	26.55 $\pm$ 4.31 <sup>†</sup>	8.79 $\pm$ 0.10 <sup>°</sup>	72.09 $\pm$ 14.56 <sup>#</sup>	9.48 $\pm$ 0.26 <sup>#</sup>	35.26 $\pm$ 2.51 <sup>#</sup>	9.11 $\pm$ 0.11 <sup>#</sup>

$pD_2$  = ( $-\log M$  of the isoprenaline concentration that gives a response equal to 50% of the maximum response).

<sup>†</sup>  $P$  < 0.05 in compared to C57BL6 mice.

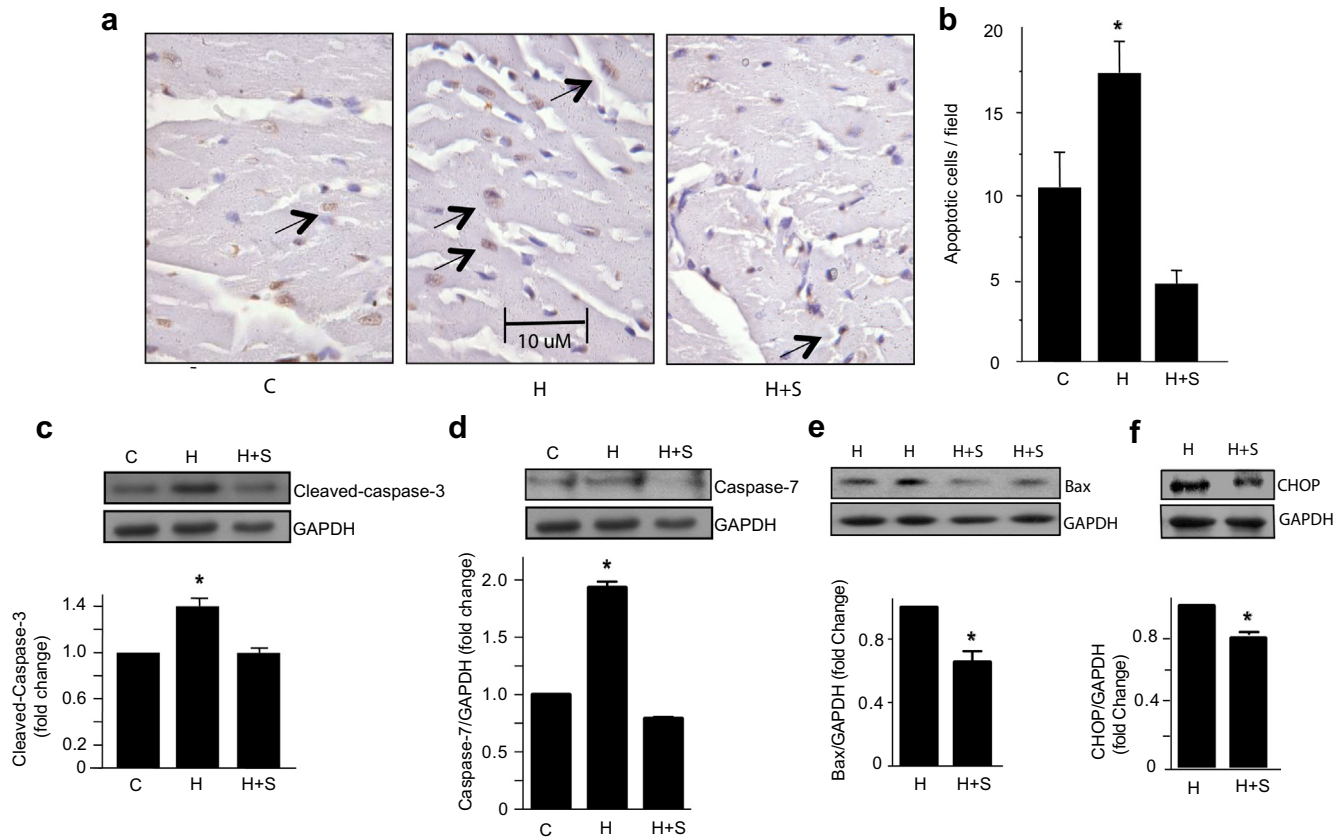
<sup>°</sup>  $P$  < 0.05 vs H mice.

<sup>#</sup>  $P$  < 0.05 vs the same group without ICI 118,551 (ANOVA followed by Tukey test). Data are the mean  $\pm$  S.E.M. of 5–7 experiments done in the absence or the presence of 50 nM ICI118,551.

be produced intracellularly through electron leakage from the mitochondria during oxidative phosphorylation and through the activation of various cellular enzymes including xanthine oxidase, nitric oxide synthase uncoupling, and/or cyclooxygenase [28–32]. Superoxide is subject to dismutation by superoxide dismutase (SOD) into  $H_2O_2$ , a compound that may mediate the compensatory responses involved in cardiac remodeling. Our data showed increased levels of oxidative stress in left ventricular hypertrophy, demonstrated by the presence of  $O_2^-$  and  $H_2O_2$  in the LV of LDLr<sup>-/-</sup> mice fed a high fat diet. However, DHE oxidized to a compound characterized as ethidium represents the overlapping of oxidation products due to specific ( $H_2O_2$ ) and nonspecific sources

(heme proteins) [33,34]. SNAC treatment resulted in a decrease in oxidative stress levels by decreasing  $H_2O_2$  and  $O_2^-$  production by approximately 65% and 52%, respectively. Similar levels in the cardiac remodeling of the ROS reduction by antioxidants have been shown *in vivo* [35,36].

Our previous studies [13] in LDLr<sup>-/-</sup> mice have shown that SNAC can suppress cardiac remodeling; and in the present study we have demonstrated that these effects are due to suppression of ROS generation in the LV of this animal model fed a high fat diet. We suggest that this reduction in ROS may be due to the well-known scavenging action of RSNOs or the N-acetylcysteine per se activity as an antioxidant *in vivo* and *in vitro*. N-acetylcysteine also

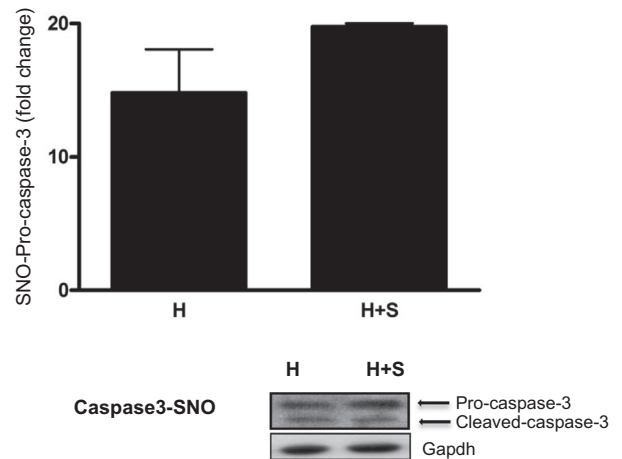


**Fig. 4.** SNAC inhibits apoptosis triggered by left ventricular remodeling. (a) Immunohistochemical staining for apoptosis by TUNEL (brown) in left ventricle of LDLR<sup>-/-</sup> mice. (Bar, 10  $\mu$ m). (b) Index of apoptotic cells per field expressed as percentage of apoptotic/total cells ratio. (c) Western blot of Cleaved-caspase-3, (d) caspase-7, (e) Bax or (f) CHOP in tissue lysate from left ventricle. Data are mean of triplicate samples  $\pm$  S.E.M. for at least 3 independent experiments.  $P < 0.05$ .

reacts with hydroxyl radicals at a rate constant of  $[1.36 \times 10(10) \text{M}^{-1} \text{s}^{-1}]$  [37]. But reacts slowly with  $\text{H}_2\text{O}_2$  and superoxide ( $\text{O}_2^-$ ).

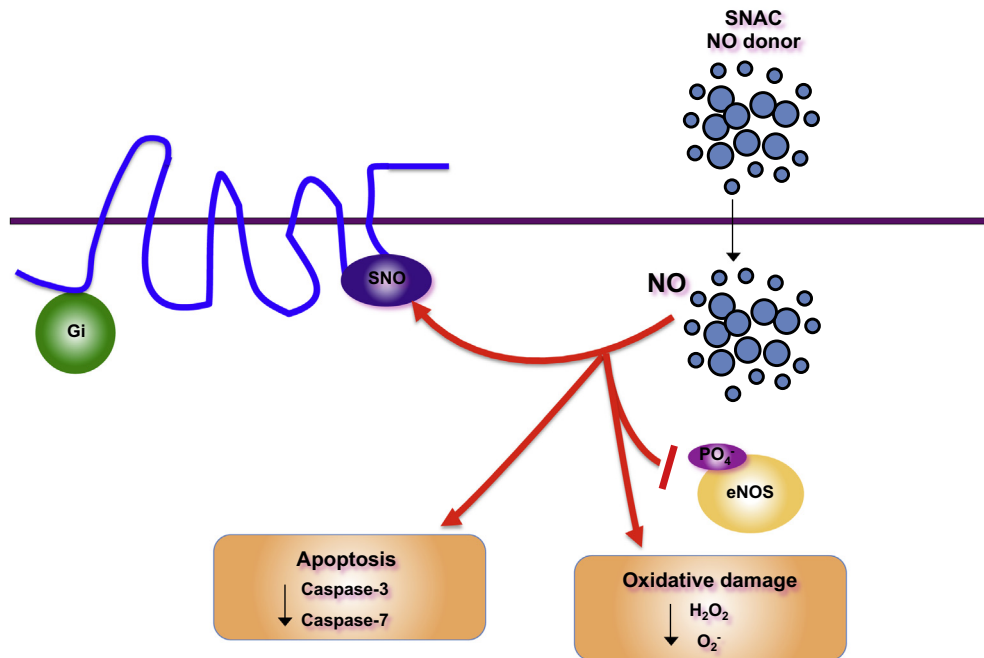
The induction of eNOS phosphorylation and its consequent activation through the Akt-phosphorylation pathway [38] can be induced by a range of factors, including  $\text{H}_2\text{O}_2$  [38–41]. Oxidative stress in the myocardium, represented by  $\text{H}_2\text{O}_2$  levels in the LV, were decreased due to SNAC treatment, concomitant with a decrease in the ratio of p-Ser1177 eNOS/total eNOS may be due a feedback mechanism after treatment with NO $\cdot$  donor (SNAC). Previous studies in the aortas of these animals showed that the location of elevated superoxide levels is associated with constitutive NOS overexpression [12].

S-nitrosothiols are known to induce betadrenoceptor overexpression, and these RSNOs are able to prevent agonist-stimulated receptor downregulation [42] as reported by Whalen [15] et al. in mice. The cardioprotective action of SNAC in the left ventricle (LV) can be attributed to the induction of the overexpression of  $\beta_2$ -AR. Moreover, in cardiomyocytes, S-nitrosothiols can also be responsible for changes in G-protein coupling. For example, Whalen et al. showed a decrease  $\beta$ -AR phosphorylation mediated by the G-protein coupled receptor kinase 2 (GRK2) and the subsequent recruitment of  $\beta$ -arrestin to the receptor, resulting in the attenuation of receptor desensitization and internalization. This kind of coupling change on G-proteins from a  $\beta_2$ -adrenergic receptor mechanism was also seems to be induced by 3-morpholinylsindonimine (SIN-1), which releases NO $\cdot$  and superoxide simultaneously [43]. A similar mechanism may be responsible for the S-nitrosation of  $\beta_2$ -ARs by SNAC, which would increase the coupling of these adrenoceptors to Gi.



**Fig. 5.** SNO-Caspase-3 expression during left ventricle hypertrophy. Typical biotin switch detects both endogenous and exogenous S-nitrosation in left ventricle tissue. Left ventricular tissue was subjected to the Biotin Switch and Western blotting revealed with anti-caspase-3. Data are the means of triplicate samples and are representative of 2 independent experiments.

Betadrenoceptor overexpression can be associated with changes in coupling mechanisms. Studies have described that activation of the  $\beta_2$ AR protects myocytes against apoptosis induced by a wide array of assaulting factors such as enhanced  $\beta_1$ AR signaling, hypoxia, and ROS [44–46]. The activation of  $\beta_2$ AR-coupled Gi proteins has been described under various experimental conditions [47–50] and protects cardiac myocytes from apoptosis via the



**Fig. 6.** Potential role of SNAC preventing left ventricular hypertrophy. In cardiomyocytes, nitric oxide (NO) influx leading to betadrenoceptor-2 S-nitrosylation results in a Gi coupling. The activation of eNOS is negatively regulated. Therapeutic treatment with SNAC might result in conditions of low oxidative stress reducing levels of  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  and decreased cell death due to the reduced expression of caspase-3 and caspase-7.

downstream target (PI3K)-AKT (also known as protein kinase B) survivor pathway [44,45]. We have demonstrated that SNAC treatment promotes  $\beta_2\text{AR}$ -Gs coupling to Gi and also protects cardiomyocytes from apoptosis.

Studies have also shown exacerbated myocyte apoptosis during cardiac remodeling [51,52] and cardiomyocyte apoptosis increased 70% during LVH in our model. The prevention of cardiac remodeling by SNAC treatment led to a decrease in the number of apoptotic cardiomyocytes. We provided new information suggesting that SNAC regulates the mitochondrial apoptotic pathway likely also regulate apoptotic pathways at the ER.

It has been recognized that S-nitrosation (cGMP-independent) reactions can modulate a wide range of cell functions [53,54]. This is the result of the covalent modification of Cys thiols, which are important in cardio protection [5,55,56]. The present study has shown that SNAC treatment increases S-nitrosation in the  $\beta_2\text{AR}$ . To our knowledge, this is the first finding of  $\beta_2\text{AR}$  S-nitrosation. We understand the modification of this protein as evidence of coupling change. This is in agreement with the results of studies from Adam et al. [43] showing  $\text{NO}^\bullet$  effects on the depalmitoylation of  $\beta_2\text{AR}$  including reduction of the potency of a  $\beta$ -adrenergic agonist in the stimulation of adenylyl cyclase uncoupling  $\beta_2\text{AR}$  to the Gs pathway. Indeed, even  $\text{LDLr}^{-/-}$  mice show Gi-coupled- $\beta_2\text{AR}$ s constitutive activity, we have demonstrated that SNAC treatment decrease ISO sensitivity in left atria by promoting  $\beta_2\text{AR}$ -Gs uncoupling and  $\beta_2\text{AR}$ -Gi coupling. We suggest that this phenomenon may be mediated by  $\beta_2\text{AR}$ -SNO. Therefore, we investigated the mechanism of caspase inhibition because it is reported that caspases are also reversibly inhibited by  $\text{NO}^\bullet$  related S-nitrosation [57]. The caspases are a family of cysteine proteases and  $\text{NO}^\bullet$  can modify enzyme function by S-nitrosation of protein thiol groups [54,58]. However, our findings did not show differences in the S-nitrosated status of procaspase-3 under  $\text{NO}^\bullet$  donation from SNAC treatment.

In conclusion, the present study has, for the first time, demonstrated that administration of SNAC suppresses cardiac remodeling

in  $\text{LDLr}^{-/-}$  mice fed a high fat diet via the inhibition of oxidative stress and apoptosis, which is the result of  $\beta_2\text{AR}$  overexpression and coupling changes brought about by S-nitrosation. Moreover, we showed that treatment with SNAC leads to the coupling of  $\beta_2\text{AR}$  to Gi.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.niox.2013.12.003>.

#### References

- [1] J.N. Cohn, Abnormalities of peripheral sympathetic nervous system control in congestive heart failure, *Circulation* 82 (1990) 159–167.
- [2] M.R. Bristow, Beta-adrenergic receptor blockade in chronic heart failure, *Circulation* 101 (2000) 558–569.
- [3] I.N. Santos, R.C. Spadari-Bratfisch, Stress and cardiac beta adrenoceptors, *Stress* 9 (2006) 69–84.
- [4] H. Tsutsui, S. Kinugawa, S. Matsushima, Mitochondrial oxidative stress and dysfunction in myocardial remodeling, *Cardiovasc. Res.* 81 (2009) 449–456.
- [5] S. Atar, Y. Ye, Y. Lin, S.Y. Freeberg, S.P. Nishi, S. Rosanio, M.H. Huang, B.F. Uretsky, J.R. Perez-Polo, Y. Birnbaum, Atorvastatin-induced cardioprotection is mediated by increasing inducible nitric oxide synthase and consequent S-nitrosylation of cyclooxygenase-2, *Am. J. Physiol. Heart Circ. Physiol.* 290 (2006) H1960–H1968.
- [6] F.G. Spinale, M.L. Coker, C.V. Thomas, J.D. Walker, R. Mukherjee, L. Hebbard, Time-dependent changes in matrix metalloproteinase activity and expression during the progression of congestive heart failure: relation to ventricular and myocyte function, *Circ. Res.* 82 (1998) 482–495.
- [7] B. Ding, R.L. Price, E.C. Goldsmith, T.K. Borg, X. Yan, P.S. Douglas, E.O. Weinberg, J. Bartunek, T. Thielen, V.V. Didenko, B.H. Lorell, Left ventricular hypertrophy in ascending aortic stenosis mice: aortic and the progression to early failure, *Circulation* 101 (2000) 2854–2862.
- [8] E. Palojoki, A. Saraste, A. Eriksson, K. Pulkki, M. Kallajoki, L.M. Voipio-Pulkki, I. Tikkanen, Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats, *Am. J. Physiol. Heart Circ. Physiol.* 280 (2001) H2726–H2731.
- [9] E. Teiger, V.D. Than, L. Richard, C. Wisniewsky, B.S. Tea, L. Gaboury, J. Tremblay, K. Schwartz, P. Hamet, Apoptosis in pressure overload-induced heart hypertrophy in the rat, *J. Clin. Invest.* 97 (1996) 2891–2897.



- [10] P. Anversa, J. Kajstura, G. Olivetti, Myocyte death in heart failure, *Curr. Opin. Cardiol.* 11 (1996) 245–251.
- [11] L.A. Bouch, T.P. Cappola, R.W. Harrison, J.K. Crone, E.R. Rodriguez, A.L. Burnett, J.M. Hare, Combined loss of neuronal and endothelial nitric oxide synthase causes premature mortality and age-related hypertrophic cardiac remodeling in mice, *J. Mol. Cell. Cardiol.* 35 (2003) 637–644.
- [12] M.H. Krieger, K.F. Santos, S.M. Shishido, A.C. Wanschel, H.F. Estrela, L. Santos, M.G. De Oliveira, K.G. Franchini, R.C. Spadari-Bratfisch, F.R. Laurindo, Antiatherogenic effects of S-nitroso-N-acetylcysteine in hypercholesterolemic LDL receptor knockout mice, *Nitric Oxide* 14 (2006) 12–20.
- [13] J.A. Garcia, L. dos Santos, A.L. Moura, K.F. Ricardo, A.C. Wanschel, S.M. Shishido, R.C. Spadari-Bratfisch, H.P. de Souza, M.H. Krieger, S-nitroso-N-acetylcysteine (SNAC) prevents myocardial alterations in hypercholesterolemic LDL receptor knockout mice by antiinflammatory action, *J. Cardiovasc. Pharmacol.* 51 (2008) 78–85.
- [14] D.T. Hess, A. Matsumoto, S.O. Kim, H.E. Marshall, J.S. Stamler, Protein S-nitrosylation: purview and parameters, *Nat. Rev. Mol. Cell Biol.* 6 (2005) 150–166.
- [15] E.J. Whalen, M.W. Foster, A. Matsumoto, K. Ozawa, J.D. Violin, L.G. Que, C.D. Nelson, M. Benhar, J.R. Keys, H.A. Rockman, W.J. Koch, Y. Daaka, R.J. Lefkowitz, J.S. Stamler, Regulation of beta-adrenergic receptor signaling by S-nitrosylation of G-protein-coupled receptor kinase 2, *Cell* 129 (2007) 511–522.
- [16] T. Kokkola, J.R. Savinainen, K.S. Monkkonen, M.D. Retamal, J.T. Laitinen, S-nitrosothiols modulate G protein-coupled receptor signaling in a reversible and highly receptor-specific manner, *BMC Cell Biol.* 6 (2005) 21.
- [17] E.J. Whalen, A.K. Johnson, S.J. Lewis, Beta-adrenoceptor dysfunction after inhibition of NO synthesis, *Hypertension* 36 (2000) 376–382.
- [18] K.F. Ricardo, S.M. Shishido, M.G. de Oliveira, M.H. Krieger, Characterization of the hypotensive effect of S-nitroso-N-acetylcysteine in normotensive and hypertensive conscious rats, *Nitric Oxide* 7 (2002) 57–66.
- [19] I.N. Santos, M. Sumitame, V.M. Caceres, M.F. Moreira, M.H. Krieger, R.C. Spadari-Bratfisch, Evidence for two atypical conformations of beta-adrenoceptors and their interaction with Gi proteins, *Eur. J. Pharmacol.* 513 (2005) 109–118.
- [20] R.F. Furchgott, The pharmacological differentiation of adrenergic receptors, *Ann. N.Y. Acad. Sci.* 139 (1967) 553–570.
- [21] A.J. Bilski, S.E. Halliday, J.D. Fitzgerald, J.L. Wale, The pharmacology of a beta 2-selective adrenoceptor antagonist (ICI 118,551), *J. Cardiovasc. Pharmacol.* 5 (1983) 430–437.
- [22] E.J. Danson, Y.H. Zhang, C.E. Sears, A.R. Edwards, B. Casadei, D.J. Paterson, Disruption of inhibitory G-proteins mediates a reduction in atrial beta-adrenergic signaling by enhancing eNOS expression, *Cardiovasc. Res.* 67 (2005) 613–623.
- [23] M.P. Caulfield, N.J. Birdsall, International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors, *Pharmacol. Rev.* 50 (1998) 279–290.
- [24] J.F. Heubach, T. Rau, T. Eschenhagen, U. Ravens, A.J. Kaumann, Physiological antagonism between ventricular beta 1-adrenoceptors and alpha 1-adrenoceptors but no evidence for beta 2- and beta 3-adrenoceptor function in murine heart, *Br. J. Pharmacol.* 136 (2002) 217–229.
- [25] S.R. Jaffrey, S.H. Snyder, The biotin switch method for the detection of S-nitrosylated proteins, *Sci. STKE* 2001 (2001). pl1.
- [26] M.T. Forrester, M.W. Foster, M. Benhar, J.S. Stamler, Detection of protein S-nitrosylation with the biotin-switch technique, *Free Radical Biol. Med.* 46 (2009) 119–126.
- [27] I.V. Turko, F. Murad, Protein nitration in cardiovascular diseases, *Pharmacol. Rev.* 54 (2002) 619–634.
- [28] C. Heymes, J.K. Bendall, P. Ratajczak, A.C. Cave, J.L. Samuel, G. Hasenfuss, A.M. Shah, Increased myocardial NADPH oxidase activity in human heart failure, *J. Am. Coll. Cardiol.* 41 (2003) 2164–2171.
- [29] K. Nakamura, K. Kusano, Y. Nakamura, M. Kakishita, K. Ohta, S. Nagase, M. Yamamoto, K. Miyaji, H. Saito, H. Morita, T. Emori, H. Matsubara, S. Toyokuni, T. Ohe, Carvedilol decreases elevated oxidative stress in human failing myocardium, *Circulation* 105 (2002) 2867–2871.
- [30] U. Landmesser, S. Spiekermann, S. Dikalov, H. Tatge, R. Wilke, C. Kohler, D.G. Harrison, B. Hornig, H. Drexler, Vascular oxidative stress and endothelial dysfunction in patients with chronic heart failure: role of xanthine-oxidase and extracellular superoxide dismutase, *Circulation* 106 (2002) 3073–3078.
- [31] C.A. Farquharson, R. Butler, A. Hill, J.J. Belch, A.D. Struthers, Allopurinol improves endothelial dysfunction in chronic heart failure, *Circulation* 106 (2002) 221–226.
- [32] Y. Shizukuda, P.M. Buttrick, Oxygen free radicals and heart failure: new insight into an old question, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 283 (2002) L237–L238.
- [33] I. Papapostolou, N. Patsoukis, C.D. Georgiou, The fluorescence detection of superoxide radical using hydroethidine could be complicated by the presence of heme proteins, *Anal. Biochem.* 332 (2004) 290–298.
- [34] H. Zhao, J. Joseph, H.M. Fales, E.A. Sokoloski, R.L. Levine, J. Vasquez-Vivar, B. Kalyanaraman, Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence, *Proc. Natl. Acad. Sci. USA* 102 (2005) 5727–5732.
- [35] D.J. Chess, W. Xu, R. Khairallah, K.M. O'Shea, W.J. Kop, A.M. Azimzadeh, W.C. Stanley, The antioxidant tempol attenuates pressure overload-induced cardiac hypertrophy and contractile dysfunction in mice fed a high-fructose diet, *Am. J. Physiol. Heart Circ. Physiol.* 295 (2008) H2223–H2230.
- [36] M.O. Date, T. Morita, N. Yamashita, K. Nishida, O. Yamaguchi, Y. Higuchi, S. Hirotsu, Y. Matsumura, M. Hori, M. Tada, K. Otsu, The antioxidant N-2-mercaptopyrionyl glycine attenuates left ventricular hypertrophy in in vivo murine pressure-overload model, *J. Am. Coll. Cardiol.* 39 (2002) 907–912.
- [37] O.I. Aruoma, B. Halliwell, B.M. Hoey, J. Butler, The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid, *Free Radical Biol. Med.* 6 (1989) 593–597.
- [38] S.R. Thomas, K. Chen, J.F. Keaney Jr., Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway, *J. Biol. Chem.* 277 (2002) 6017–6024.
- [39] G.R. Drummond, H. Cai, M.E. Davis, S. Ramasamy, D.G. Harrison, Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide, *Circ. Res.* 86 (2000) 347–354.
- [40] H. Cai, M.E. Davis, G.R. Drummond, D.G. Harrison, Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway, *Arterioscler. Thromb. Vasc. Biol.* 21 (2001) 1571–1576.
- [41] H. Cai, Z. Li, S. Dikalov, S.M. Holland, J. Hwang, H. Jo, S.C. Dudley Jr., D.G. Harrison, NAD(P)H oxidase-derived hydrogen peroxide mediates endothelial nitric oxide production in response to angiotensin II, *J. Biol. Chem.* 277 (2002) 48311–48317.
- [42] D.C. Fernandes, J. Wosniak Jr., L.A. Pescatore, M.A. Bertoline, M. Liberman, F.R. Laurindo, C.X. Santos, Analysis of DHE-derived oxidation products by HPLC in the assessment of superoxide production and NADPH oxidase activity in vascular systems, *Am. J. Physiol. Cell. Physiol.* 292 (2007) C413–C422.
- [43] L. Adam, M. Bouvier, T.L. Jones, Nitric oxide modulates beta(2)-adrenergic receptor palmitoylation and signaling, *J. Biol. Chem.* 274 (1999) 26337–26343.
- [44] A. Chesley, M.S. Lundberg, T. Asai, R.P. Xiao, S. Ohtani, E.G. Lakatta, M.T. Crow, The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3'-kinase, *Circ. Res.* 87 (2000) 1172–1179.
- [45] W.Z. Zhu, M. Zheng, W.J. Koch, R.J. Lefkowitz, B.K. Kobilka, R.P. Xiao, Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes, *Proc. Natl. Acad. Sci. USA* 98 (2001) 1607–1612.
- [46] C. Communal, K. Singh, D.B. Sawyer, W.S. Colucci, Opposing effects of beta(1)- and beta(2)-adrenergic receptors on cardiac myocyte apoptosis: role of a pertussis toxin-sensitive G protein, *Circulation* 100 (1999) 2210–2212.
- [47] M. Grimm, S. Gsell, C. Mittmann, M. Nose, H. Scholz, J. Weil, T. Eschenhagen, Inactivation of (G)alpha proteins increases arrhythmic effects of beta-adrenergic stimulation in the heart, *J. Mol. Cell. Cardiol.* 30 (1998) 1917–1928.
- [48] R.P. Xiao, P. Avdonin, Y.Y. Zhou, H. Cheng, S.A. Akhter, T. Eschenhagen, R.J. Lefkowitz, W.J. Koch, E.G. Lakatta, Coupling of beta2-adrenoceptor to Gi proteins and its physiological relevance in murine cardiac myocytes, *Circ. Res.* 84 (1999) 43–52.
- [49] A. Sabri, E. Pak, S.A. Alcott, B.A. Wilson, S.F. Steinberg, Coupling function of endogenous alpha(1)- and beta-adrenergic receptors in mouse cardiomyocytes, *Circ. Res.* 86 (2000) 1047–1053.
- [50] O.E. Brodde, H. Bruck, K. Leineweber, Cardiac adrenoceptors: physiological and pathophysiological relevance, *J. Pharmacol. Sci.* 100 (2006) 323–337.
- [51] M. Sun, M. Chen, F. Dawood, U. Zurawska, J.Y. Li, T. Parker, Z. Kassiri, L.A. Kirshenbaum, M. Arnold, R. Khokha, P.P. Liu, Tumor necrosis factor-alpha mediates cardiac remodeling and ventricular dysfunction after pressure overload state, *Circulation* 115 (2007) 1398–1407.
- [52] G. Condorelli, C. Morisco, G. Stassi, A. Notte, F. Farina, G. Sgaramella, A. de Rienzo, R. Roncarati, B. Trimarco, G. Lembo, Increased cardiomyocyte apoptosis and changes in proapoptotic and antiapoptotic genes bax and bcl-2 during left ventricular adaptations to chronic pressure overload in the rat, *Circulation* 99 (1999) 3071–3078.
- [53] M.C. Broillet, S-nitrosylation of proteins, *Cell. Mol. Life Sci.* 55 (1999) 1036–1042.
- [54] J.S. Stamler, S. Lamas, F.C. Fang, Nitrosylation, the prototypic redox-based signaling mechanism, *Cell* 106 (2001) 675–683.
- [55] J. Lin, C. Steenbergen, E. Murphy, J. Sun, Estrogen receptor-beta activation results in S-nitrosylation of proteins involved in cardioprotection, *Circulation* 120 (2009) 245–254.
- [56] J. Sun, E. Picht, K.S. Ginsburg, D.M. Bers, C. Steenbergen, E. Murphy, Hypercontractile female hearts exhibit increased S-nitrosylation of the L-type Ca<sup>2+</sup> channel alpha1 subunit and reduced ischemia/reperfusion injury, *Circ. Res.* 98 (2006) 403–411.
- [57] J. Li, T.R. Billiar, R.V. Talanian, Y.M. Kim, Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation, *Biochem. Biophys. Res. Commun.* 240 (1997) 419–424.
- [58] J.S. Stamler, Redox signaling: nitrosylation and related target interactions of nitric oxide, *Cell* 78 (1994) 931–936.