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The role of AT1-receptor blockade on reactive oxygen species and cardiac autonomic drive in experimental hyperthyroidism

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ABSTRACT

The objective of this study was to explore the influence of the renin–angiotensin system on cardiac prooxidants and antioxidants levels and its association to autonomic imbalance induced by hyperthyroidism. Male Wistar rats were divided into four groups: control, losartan (10 mg/kg/day by gavage, 28 day), thyroxine (T4) (12 mg/L in drinking water for 28 days), and T4 + losartan. Spectral analysis (autonomic balance), angiotensin II receptor (AT1R), NADPH oxidase, Nrf2 and heme-oxygenase-1 (HO-1) myocardial protein expression, and hydrogen peroxide (H_2O_2) concentration were quantified. Autonomic imbalance induced by hyperthyroidism (~770%) was attenuated in the T4 + losartan group (~32%) ($P < 0.05$). AT1R, NADPH oxidase, H_2O_2, as well as concentration, Nrf2 and HO-1 protein expression were elevated (~172%, 43%, 40%, 133%, and 154%, respectively) in T_4 group ($P < 0.05$). H_2O_2 and HO-1 levels were returned to control values in the T4 + losartan group ($P < 0.05$). The overall results demonstrate a positive impact of RAS blockade in the autonomic control of heart rate, which was associated with an attenuation of H_2O_2 levels, as well as with a reduced counter-regulatory response of HO-1 in experimental hyperthyroidism.

1. Introduction

Thyroid hormones exert a key role not only on basal metabolism but also on the cardiovascular system (Klein and Danzi, 2007). These hormones are involved in the modulation of oxygen consumption associated with an augmentation in thermogenesis (Danzi et al., 2005). In the same way, the impact of thyroid hormones on cardiac tissue is classically known, provoking increased heart rate, elevation of cardiac output, augmented myocardial contractility, and cardiac hypertrophy (Pantos et al., 2007). In addition, it is important to report the action of the thyroid hormones on the neurohumoral system, such as the autonomic nervous system (Hu et al., 2003).

Thyroid hormones appear to be important for the sympatho-vagal balance control which modulates cardiac function (Rubini et al., 1993; Casu et al., 2005). In hyperthyroidism, the autonomic tone is favorable to sympathetic overactivity and is associated with a reduction of the vagal tone (Burggraaf et al., 2001). Spectral analysis of heart rate variability (HRV) is a relevant method which has been used due to its sensitivity in verifying autonomic imbalance in hyperthyroid patients (Akselrod et al., 1981). Several parameters are verified by spectral analysis, such as low frequency component (LF) and high frequency component (HF) of HRV (Porta et al., 2004). LF component is considered a sympathetic modulation index, and HF component a vagal modulation index. Moreover, the LF/HF ratio characterizes sympatho-vagal tone (Montano et al., 2009). Many studies have associated high sympathetic and low parasympathetic activity with increased cardiovascular mortality (Abboud et al., 2012).

Thyroid hormones effects are also influenced by renin–angiotensin system (RAS) (Carneiro-Ramos et al., 2010). *In vivo* and *in vitro* studies have shown that thyroid hormones provide enhanced plasma renin (Basset et al., 2000), angiotensin II production and similar augment to its receptors (AT1 and AT2) in cardiac tissue (Diniz et al., 2007; Araujo et al., 2011). Angiotensin II – AT1 receptor complex stimulates sequential activation of NADPH oxidase and downstream signaling pathways, such as PI3K/AKT/mTOR, leading to several biological effects in the heart (Garciarena et al., 2008). An association has been established between RAS activation and reactive oxygen species (ROS), since NADPH oxidase, $gp91^{phox}$ subunit (Nox2), is involved in the superoxide anion ($O_2^{\cdot-}$) production (Soccio et al., 2005). The relationship between ROS and thyroid hormones is also well known (Videla et al., 1988). In hyperthyroidism, increased basal metabolism provides augmented ROS production associated with counter-regulatory antioxidant response (Venditti and Di Meo, 2006; Venditti et al., 2008). In this context, nuclear factor (erythroid-derived 2)-like2, also known as Nrf2, is an important transcription factor which may be induced by oxidative stress. In the same way, Nrf2 appears to be influenced by thyroid

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hormone. Previous studies suggested hyperthyroidism-induced enhanced Nrf2 protein expression (Satoh et al., 2009; Araujo et al., 2010). Nrf2 is involved in the control of gene expression, through the antioxidant responsive element, modulating the transcription of many antioxidant proteins, such as heme-oxygenase-1 (HO-1) (Itoh et al., 2004). Such protein exerts a key role on the heme group metabolism and redox control. In addition, studies demonstrated that HO-1 is a central molecule in cardioprotection and cardiac remodeling (Brunt et al., 2009).

Investigations were conducted in this study, as to observe whether the RAS blockade using losartan, has an impact on HRV autonomic control in experimental hyperthyroidism. It was also the objective of the current study to evaluate if there is an association between changes in ROS concentrations, the Nrf2/HO-1 system, and autonomic imbalance induced by hyperthyroidism.

2. Material and methods

2.1. Animals

Male Wistar rats (200 ± 50 g) were divided into four groups (n = 4–10/group) as follows: **1) control,** receiving water *ad libitum*; **2) losartan,** receiving losartan (10 mg/kg/day) by gavage (Crespo et al., 2008), **3) T_4,** receiving L-thyroxine (T_4) (12 mg/L in drinking water) (Araujo et al., 2007); **4) T_4 + losartan,** receiving T_4 and losartan, according to the conditions described in the items 2 and 3, during the experimental protocol (28 days). Each animal ingested approximately 0.45 mL of water or a solution containing 12 mg/L of T4 per day, receiving, at the end of protocol, 12.6 mg of T4. All the animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and Brazilian College of Animal Experimentation (COBEA) and the experimental protocol was approved by UFRGS Animal Care Committee (protocol # 18158).

2.2. Hemodynamic measurements and cardiac hypertrophy

Cardiac hemodynamics was assessed at fourth week of thyroxine treatment. In brief, the rats were anesthetized (ketamine 90 mg/kg; xylazine 10 mg/kg, i.p.) and the right carotid artery was cannulated with a PE 50 catheter connected to a strain gauge transducer (Narco Biosystem Pulse Transducer RP-155, Houston, Texas, USA) linked to a pressure amplifier (HP 8805C, Hewlett Packard, USA). Pressure readings were taken in a microcomputer equipped with an analogue-to-digital conversion board (Windaq 1 kHz sampling frequency, Dataq Instruments, Inc., Akron, Ohio, USA. The catheter was inserted into left ventricle (LV) for recording the left ventricular pressure (these data were used for heart rate and spectral analysis determine), around 5 min. After, the catheter was advanced into aorta for recording the aortic systolic (SAP) and diastolic pressure (DAP) (in mm Hg) around 2 min. In this situation, many times there is loss of accuracy in the measurements due to the manipulation of catheter inside the vessel. For this reason we have 10 animals for heart rate and spectral analysis and only 4 animals for blood pressure data. The cardiac hypertrophy was evaluated by heart weight (in mg) to body weight (in g) ratio (Araujo et al., 2006).

2.3. Autonomic evaluation by spectral analysis

Power spectral analysis was applied to pulse interval (PI) series, or tachogram, created from BP signals through the beat-to-beat PI. Frequency domain analysis of HRV was obtained with an autoregressive algorithm (Rubini et al., 1993; Dias da Silva et al., 2006; Quagliotto et al., 2008) on stationary sequences of 200 beats that were randomly chosen using the stationary test (Porta et al., 2004).

The LF (0.2–0.75 Hz) and HF (0.75–3.0 Hz) spectral components of PI were expressed in both absolute (ms^2) and normalized units (NU). These NU were obtained by calculating the power of LF and HF and correlating them to the total power without the very LF component. This method estimates the center frequency and power of each relevant oscillatory component, which indicates the involvement of the central control of the sympathetic/parasympathetic systems in the cardiovascular responses. The ratio between LF and HF components (LF/HF) express the sympathovagal balance (Rubini et al., 1993; Dias da Silva et al., 2006; Quagliotto et al., 2008).

2.4. Thyroxine concentration

Blood samples were collected from the PE 50 catheter connected to the right carotid artery and immediately centrifuged at 1000 ×*g* for 20 min. Serum thyroxine concentration (in ng/mL) was evaluated by chemiluminescence using the Immunolite 2000 kit (Biomedical Technologies, Inc., Stroughton, MA, USA) (Araujo et al., 2006).

2.5. Tissue preparation

Four weeks after treatment, the rats were decapitated, the hearts were rapidly excised, LV was removed, weighed and frozen at −80 °C, for the evaluation of hydrogen peroxide. Part of LV was homogenized (1.15% w/v KCl and phenyl methyl sulphonyl fluoride PMSF 20 mmol/L) in Ultra-Turrax. The suspension was centrifuged at 1000 ×*g* for 20 min at 0–4 °C to remove the nuclei and cell debris (Llesuy et al., 1985) and supernatants were used for the assay the protein expression (AT1 receptor, gp91phox, Nrf2, and HO-1).

2.6. ROS evaluation

Hydrogen peroxide was measured via its horseradish peroxidase (HRPO)-mediated oxidation of phenol red. Slices of LV tissue were incubated for 30 min. at 37 °C in phosphate buffer 10 mmol/L (NaCl 140 mmol/L and dextrose 5 mmol/L). The supernatants were transferred to tubes with phenol red 0.28 mmol/L and 8.5 U/mL HRPO. After 25 min incubation, 1 mol/L NaOH was added and the solution's absorbance values measured at 610 nm. The results were expressed in nmoles H_2O_2 / g tissue (Pick and Keisari, 1980). NAPDH oxidase subunit (gp 91phox) was measured by western blot.

2.7. Western blot analysis

Thirty micrograms of protein were subjected to one-dimensional sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous system using 12% (w/v) separating gel and stacking gel (Laemmli, 1970). The separated proteins were transferred to nitrocellulose membranes electrophoretically using a buffer containing 20 mmol/L Tris, 150 mmol/L glycine, methanol 20%(V/V) SDS 0.1% (w/v), pH 8.2 in a cooled Bio-Rad TransBlot unit. Then, non-specific protein-binding sites were blocked with 1 h incubation with non-fat milk in Tris-buffer (Araujo et al., 2006). The membranes were processed for immunodetection using rabbit anti-Nfr2 (57 kDa) (1:250), rabbit anti-HO-1 (32 kDa) (1:250), rabbit anti-AT1 receptor (43 kDa) (1:500), and rabbit anti-gp91phox (91 kDa) (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) incubated at 4 °C. The bound primary antibodies were detected using rabbit anti-goat, rabbit anti-mouse or goat anti-rabbit HRPO-conjugate secondary antibodies (1:3000 – 1:5000) and membranes were revealed for chemiluminescence. The autoradiographies generated were quantitatively analyzed for the protein levels with an image densitometer (Image master VDS CI, Amersham Biosciences Europe, IT). The molecular weights of the bands were determined by reference to a standard molecular weight marker (RPN 800 rainbow full range Bio-Rad, CA, USA). The results from each membrane were normalized through Ponceau red method (Klein et al., 1995).

Table 1
Thyroid hormone levels and morphometric parameters after 4-week treatment with thyroxine and/or losartan.

	Control	Losartan	T_4	T_4+ Losartan
T_3 (ng/dL)	69 ± 23	54 ± 8	463 ± 119 *	405 ± 157 *
T_4 (μg/dL)	3.2 ± 1.7	2.3 ± 0.1	9.0 ± 2.0 *	8.9 ± 1.7 *
Heart/body weight (mg/g)	2.5 ± 0.1	2.4 ± 0.2	3.8 ± 0.5*	3.2 ± 0.5 *#

Values are expressed as mean ± SD of 10 animals per group.
* significantly different from control ($P < 0.05$). # significantly different from T_4 ($P < 0.05$).

2.8. Determination of protein concentration

Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as the standard (Lowry et al., 1951).

2.9. Statistical analysis

Data were expressed as mean ± SD. To compare multiple groups, one way ANOVA with Student–Newmann–Keuls post hoc test was used. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Hormone and morphometric evaluation

To confirm the establishment of hyperthyroidism model, two classical parameters were measured: thyroid hormones T_3 (in ng/dL) and T_4 (in μg/dL) and cardiac hypertrophy (in mg/g) (Table 1). Serum levels of T_3 were significantly increased in T_4 and T_4 + losartan groups (by ~570% and ~650%, respectively) as compared to their controls. In addition, serum levels of T_4 were also increased in T_4 and T_4 + losartan (by ~181% and ~286%, respectively) as compared to their controls. T_4 group demonstrated an elevated (by ~54%) cardiac hypertrophy index compared to control. However, cardiac hypertrophy index of T_4 + losartan group was significantly reduced in (by ~14%) as compared to T_4 group ($P < 0.05$) (Table 1).

3.2. Hemodynamic parameters and autonomic balance evaluation

An illustrative blood pressure record, a tachogram, and an example of power spectrum for each group are shown in Fig. 1.

No significant changes in aortic pressure values were apparent among the groups (Table 2). Heart rate of T_4 group was augmented by ~52% as compared to control, which was reduced in T_4 + losartan group (by ~19%). HRV was reduced significantly in T_4 group (by ~60%) as compared to control. However, this factor was recovered in T_4 + losartan group. nuLF component was increased in T_4 group (by ~213%) as compared to control. When hyperthyroid group was treated with losartan, nuLF component significantly reduced (by ~14%), as compared to T_4 group ($P < 0.05$). nuLF component significantly also reduced losartan group (by ~14%) (Table 2). On the other hand, nuHF component was reduced T_4 group (by ~55%), as compared to control. However, nuHF component was significantly augmented in T_4 + losartan group (by ~88%), as compared to T_4 group ($P < 0.05$) (Table 2). LF/HF index was elevated in T_4 group (by ~770%) as compared to control, being reduced in T_4 + losartan group as compared to T4 group ($P < 0.05$). LF/HF index was also significantly reduced in the losartan group (by ~70%) (Table 2).

3.3. AT1 receptor protein expression

In the T_4 and T_4 + losartan groups, an elevation of AT1 receptor protein levels (by ~172%) was observed as compared to their controls ($P < 0.05$) (Fig. 2).

3.4. ROS evaluation

Protein levels of gp91phox were augmented in T_4 group ($P < 0.05$) (Fig. 3A). There was no difference between T4 and T4 + losartan group for gp91phox expression. H_2O_2 levels increased (40%) in T_4

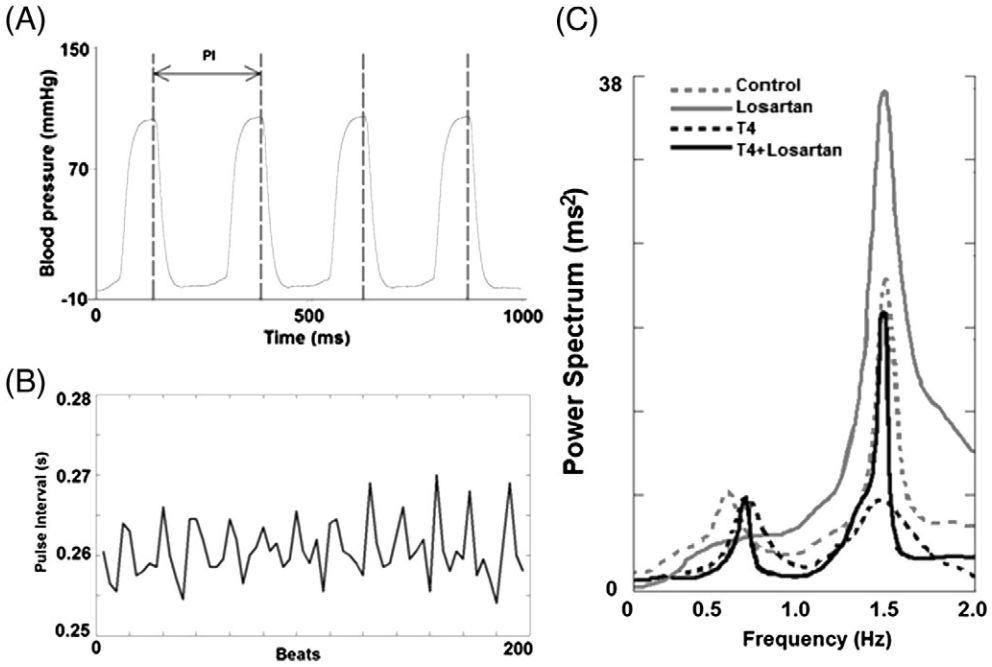

Fig. 1. An example of left ventricular pressure record (Panel A), tachogram (Panel B). Panel C shows an example of power spectrum for each group. The gray lines are examples of a control (C - dotted lines) and a treated (CL - continuous lines) group animals. Black lines are examples of a T4 (dotted lines) and a T4 + Losartan (T4 + L - continuous lines) animals.

Table 2
Hemodynamic and spectral analysis parameters after 4-week treatment with thyroxine and/or losartan.

	Control	Losartan	T_4	T_4+ losartan
SAP (mm Hg)	107 ± 4	128 ± 11	109 ± 14	116 ± 12
DAP (mm Hg)	83 ± 7	103 ± 8	84 ± 14	88 ± 14
Heart rate (bpm)	254 ± 47	236 ± 41	388 ± 73*	314 ± 87 *#
Heart rate variability	10.5 ± 1.5	11.9 ± 4.0	4.6 ± 1.8 *	12.1 ± 4.2#
nuLF	0.67 ± 0.28	0.42 ± 0.16*	2.14 ± 0.41 *	1.82 ± 0.52 *#
nuHF	90 ± 2	95 ± 3	43 ± 6 *	77 ± 3 *#
LF/HF	0.10 ± 0.01	0.030 ± 0.001*	0.87 ± 0.34 *	0.28 ± 0.03 *#

Values are expressed as mean ± SD.
Aortic pressure - n = 4/group; SAP = systolic arterial pressure; DAP = diastolic arterial pressure.
Spectral analysis - n = 10/group; LF (nu) = low frequency component in normalized units; HF (nu) = high frequency component in normalized units; LF/HF = sympatho-vagal balance.
* significantly different from control ($P < 0.05$). # significantly different fromT4 ($P < 0.05$).

group as compared to control ($P < 0.05$) (Fig. 3B). These values returned to baseline when hyperthyroid animals were treated with losartan.

3.5. Analysis of Nrf2 and HO-1 by Western Blot

T_4 group demonstrated an augmentation in Nrf2 protein expression (by ~133%) (Fig. 4A) as compared to control ($P < 0.05$). However, hyperthyroid rats treated with losartan did not show reduction in the Nrf2 protein levels as compared to T_4 group. L-Thyroxine treatment resulted in elevated HO-1 protein levels (by ~154%) as compared to control, which were reduced in T4 + losartan group as compared to T_4 ($P < 0.05$) (Fig. 4B).

4. Discussion

In the current study, it was focused on the relationship between RAS and ROS in the modulation of cardiac autonomic drive. Primarily, it was observed an autonomic imbalance, indicating a sympathetic overactivity (given specially by increased LF/HF ratio) in hyperthyroid group. This effect was blunted under RAS blockade. In fact, the inhibition of RAS with losartan, in hyperthyroid rats, provided a partially recovery of spectral analysis parameters, which were associated with reduced H_2O_2 levels and a decreased counter-regulatory response of HO-1.

The development of hyperthyroidism model, in this study, was confirmed by increased thyroid hormones levels. In addition, other relevant aspects as hemodynamic parameters and cardiac mass were analyzed. Actually, hyperthyroid rats developed not only increased heart rate, but also cardiac hypertrophy. Although the increased heart rate and contractility in the hyperthyroidism could lead to cardiac output augment, changes in arterial pressure were not observed in this model. Perhaps, the known vasodilator effect of thyroid hormones could be responsible for arterial pressure maintenance (Klein and Ojamaa, 2001; Bussemaker et al., 2003). Such data denote important effects of thyroid hormones on cardiac tissue (Klein and Danzi, 2007). The hyperthyroidism-induced tachycardia is a classical symptom clinically observed in hyperthyroid patients (Burggraaf et al., 2001). In fact, T_4 group showed increased heart rate, being the increased sympathetic drive probably a key reason for this augment. Thus, to test the hypothesis that thyroid hormones may be responsible by elevation of sympathetic tone, spectral analysis was performed to explore HRV, LF component (index of sympathetic modulation), HF component (index of parasympathetic modulation), and LF/HF ratio (sympatho-vagal balance) (Casu et al., 2005). There was an elevation in sympathetic and reduction in vagal activity on hyperthyroid rat hearts, verified by augmented LF and diminished HF component in this group. When sympatho-vagal balance was analyzed, T_4 group showed an imbalance in this ratio toward the sympathetic system. Therefore, these data suggest that hyperthyroidism leads to an overactivity of sympathetic drive on cardiac tissue. The sympatho-vagal imbalance, assessed by spectral analysis, has been reported in hyperthyroid patients (Burggraaf et al., 2001). In parallel, in experimental hyperthyroidism, it has been also seen an autonomic imbalance to the blood pressure control in hyperthyroid rats, which was prevented by the use of an AT1 receptor antagonist (Basset et al., 2000).

The inhibition of RAS in our study provided several hemodynamic, morphometric, and molecular alterations in this hyperthyroidism model. There was not only a reduction of heart rate, but also a partial recovery of basal conditions of autonomic system, especially of sympatho-vagal balance (LF/HF ratio) on cardiac system,

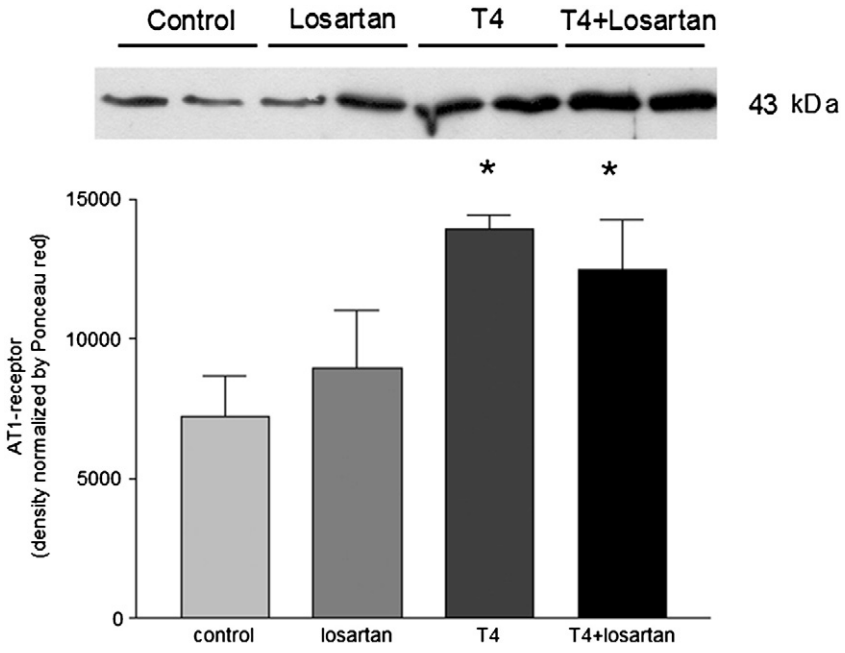

Fig. 2. Western blot analysis of cardiac homogenates using: (A) AT1 receptor (one representative gel of five Western blot experiments, showing two bands for each experimental group). Data as mean ± SD from 4 animals in each group. * significantly different from control ($P < 0.05$).

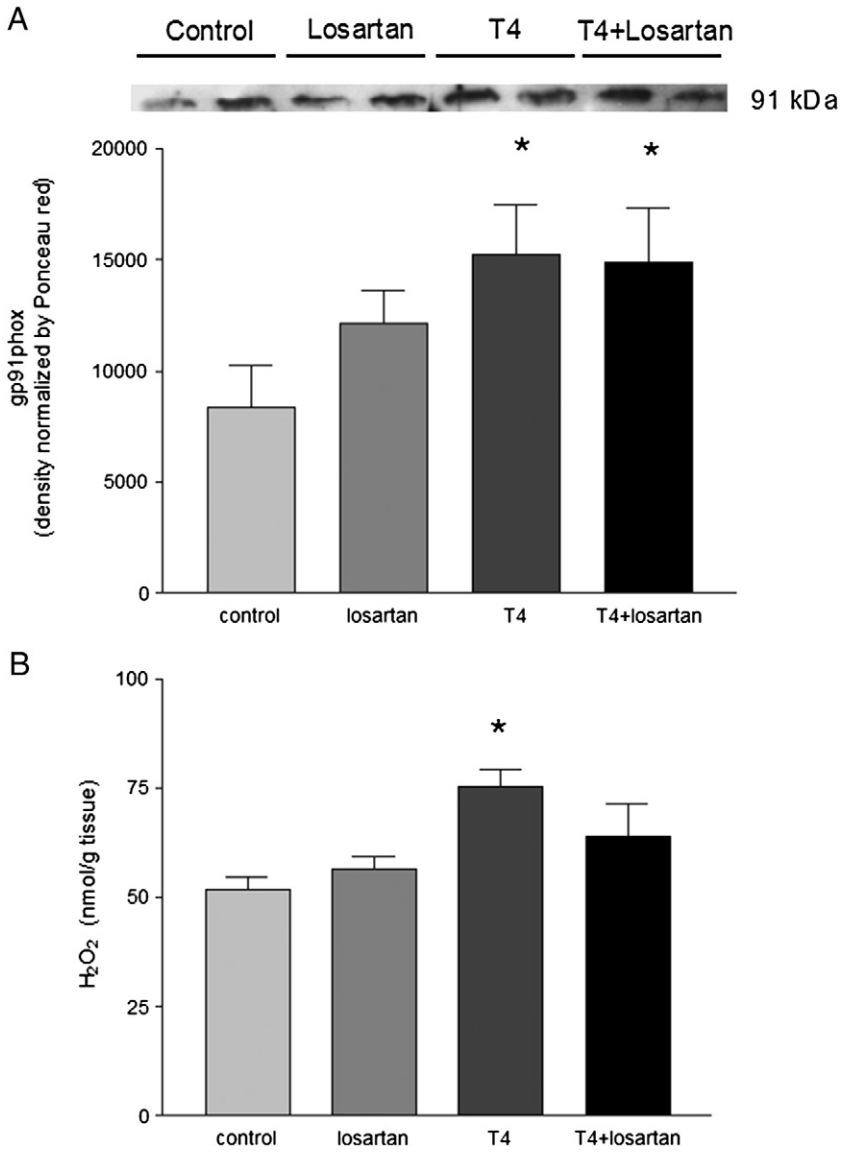

Fig. 3. Western blot analysis of cardiac homogenates using: (A) gp $^{91\ phox}$ (one representative gel of five Western blot experiments, showing two bands for each experimental group). Data as mean ± SD from 4 animals in each group. * significantly different from control ($P < 0.05$) (B) Hydrogen peroxidase levels (H_2O_2). Data as mean ± SD from 6 animals in each group. * significantly different from control ($P < 0.05$).

in hyperthyroid rats treated with losartan. It suggests an important cross-talking between autonomic nervous system and RAS in hyperthyroidism. In addition, RAS inhibition led to a reduction of cardiac hypertrophy index in T_4 + losartan group, corroborating the involvement of RAS as a modulator of cardiac hypertrophy in hyperthyroidism, as already described in literature (Hu et al., 2003). On the other hand, RAS-triggered molecular mechanisms have been explored in hyperthyroid hearts. In this case, it has been reported a key role for NADPH oxidase in RAS-induced signal transduction (Soccio et al., 2005). In addition, this protein is a relevant generator of superoxide anion ($O_2^{\bullet -}$), being this ROS a possible intermediary in RAS-induced signaling (Nakagami et al., 2003). Araujo et al. (2011) demonstrated increased NADPH oxidase gp91^{phox} subunit immunocontent (Araujo et al., 2011) in hearts of hyperthyroid rats.

The relationship between ROS and thyroid hormones has been largely studied (Araujo et al., 2006; Venditti and Di Meo, 2006; Venditti et al., 2008). Classically, thyroid hormones-induced oxygen consumption may promote not only ROS generation, but also counter-regulatory antioxidant responses (Venditti and Di Meo, 2006). NADPH oxidase-derived $O_2^{\bullet -}$ may be detoxified by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) (Halliwell, 2007). In fact, there is an augment not only in myocardial SOD activity, but in its protein expression in hyperthyroidism (Araujo et al., 2006), and H_2O_2 and $O_2^{\bullet -}$ levels (Araujo et al., 2008). Oxidative stress generated by increased H_2O_2 levels may be involved in the activation of the transcription factor Nrf2, which could prepare cells to antioxidant responses (Itoh et al., 2004). Nrf2 appears to be activated by thyroid hormones, increasing not only its nuclear translocation, but also enhancing the transcription of antioxidant proteins, such as thioredoxin and HO-1 (Videla et al., 2012). These effects were inhibited by N-acetylcysteine, suggesting a redox-dependent mechanism (Romanque et al., 2012; Videla et al., 2012). Indeed, protein expression of Nrf2 and HO-1 was increased in hyperthyroid group. In this regard, HO-1 is an enzyme related with antioxidant defense, since products from heme group catabolism, such as carbon monoxide (CO) and bilirubin, can exert key role as antioxidants (Brunt et al., 2009). Therefore, the augment of this protein may be an adaptive response to the elevated H_2O_2 levels provided by hyperthyroid state. On the other hand, HO-1 plays a

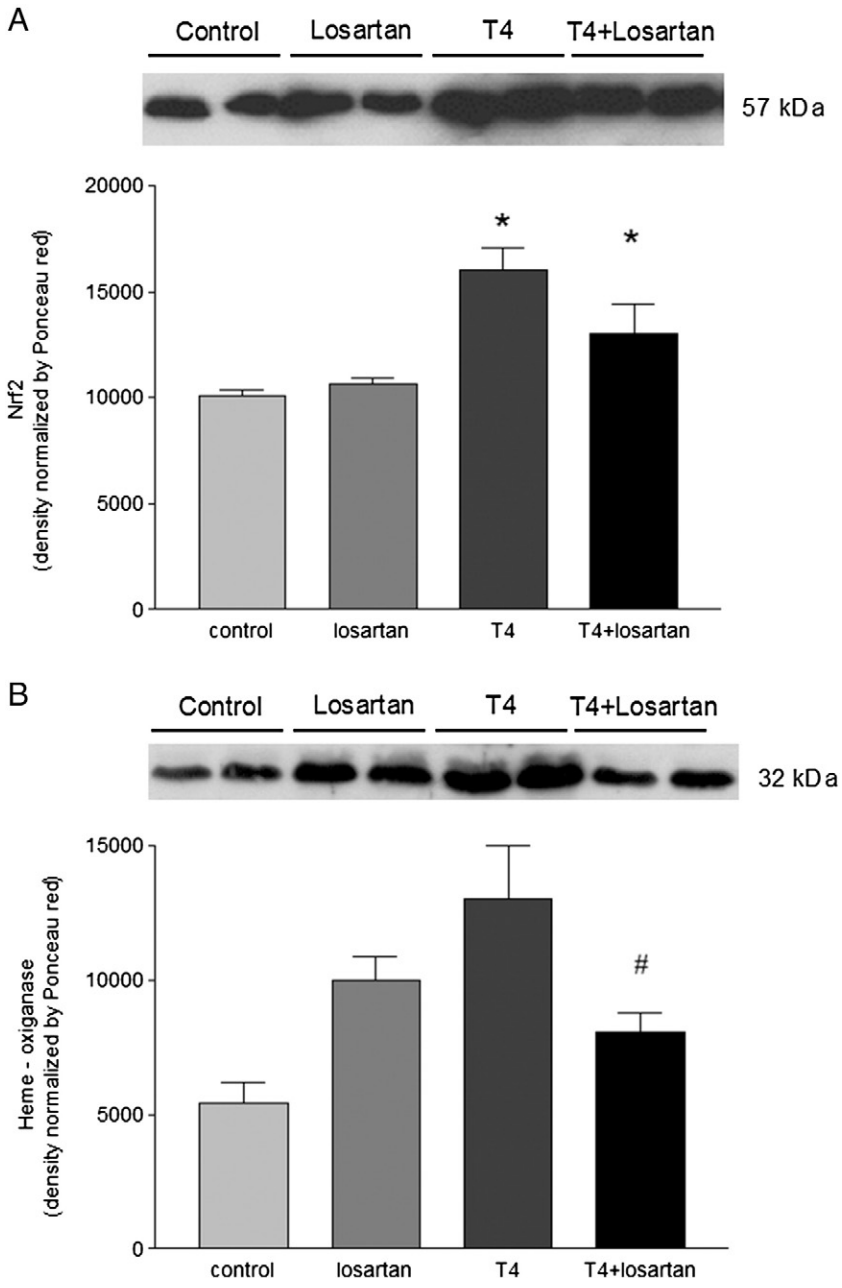

Fig. 4. Western blot analysis of cardiac homogenates using: (A) Nrf2, and (B) Heme-oxygenase-1 (HO-1) (one representative gel of five Western blot experiments, showing two bands for each experimental group). Data as mean ± SD from 4 animals in each group. * significantly different from control and losartan groups ($P < 0.05$). # significantly different from T_4 ($P < 0.05$).

cardioprotective role, influencing favorably the myocardial remodeling (Chan et al., 2011).

In summary, antioxidant adaptation was sensitive to RAS blockade, suggesting that the redox environment plays a relevant role as mediator of functional alterations in hyperthyroidism. In fact, RAS inhibition was important to reduce sympathetic and to increase parasympathetic activity, indicating a remarkable beneficial effect to the control of HRV in hyperthyroidism. These data could bring insights to the management of autonomic dysfunction and contribute to the reduction of cardiovascular morbi-mortality.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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