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THYROID DISORDERS AND NITRIC OXIDE IN CARDIOVASCULAR ADAPTATION TO HYPOVOLEMIA

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Abstract

Objective: To investigate whether nitric oxide participate in the cardiovascular function and haemodynamic adaptation to acute hemorrhage in animals with thyroid disorders. **Materials/Methods**: Sprague-Dawley rats aged 2 months old treated with T3 (Hyper, 20ug/100g body weight) or 0.02% methimazole (hypo, w/v) during 28 days were pretreated with N^G nitro-L-arginine methyl ester (L-NAME) and submitted to 20% blood loss. Heart function was evaluated by echocardiography. Measurements of arterial blood pressure, heart rate, nitric oxide synthase activity and protein levels were performed. **Results**: Hypo decreased fractional shortening and ejection fraction and increased left ventricle internal diameter. Hyper decreased ventricle diameter and no changes in cardiac contractility. Hemorrhage elicited a hypotension of similar magnitude within the 10 min. Then, this parameter was stabilized at about 30-40 min and maintained until finalized 120 min. L-NAME rats showed that the immediate hypotension would be independent of nitric oxide. Nitric oxide synthase inhibition blunted the changes of heart rate induced by blood loss. Hyper and hypo had lower atrial enzyme activity associated with a decreased enzyme isoforms in hypo. In ventricle, Hyper and hypo had a higher enzyme activity which was not correlated with changes in protein levels. Hemorrhage induced an increase heart nitric oxide production. **Conclusions:** Thyroid disorders were associated with hypertrophic remodelling which was and impacted differently on cardiac function and its adaptation to a hypovolemia. Hypovolemia triggered a nitric oxide synthase activation modulating the heart function to maintain haemodynamic homeostasis. This involvement depends on a specific enzyme isoform, cardiac chamber and thyroid state.

Introduction

Hypovolemia, secondary to major blood loss, frequently precedes multiple organ dysfunctions (De Santis and Singer 2015). Activation of several neurohormonal factors (nitric oxide [NO], catecholamines, endothelins, vasopressin, renin-angiotensin system) is involved in the restoration of vascular volume and blood pressure following bleeding (Fujisawa *et al*. 1999; Paczwa and Ganten 1999). This adaptive response to the decrease of the total blood volume implies a peripheral vasoconstriction which induces a redistribution of blood flow to the vital organs within which highlights the heart (Schadt and Hasser 2004). Previously, we demonstrated that hypovolemic state induced by acute hemorrhage provoked a heterogeneous and dynamic NO synthase (NOS) activation modulating the cardiovascular response in young rats. Increased cardiac endothelial NOS expression is an early molecular response to regulate cardiac function after blood loss. Inducible NOS becomes a major source of cardiac NO production in later stages, which could be determinant of heart dysfunction after 120 min of sustained hemorrhagic shock (Balaszczuk *et al.* 2006).

On the other hand, it is well known that cardiovascular function is also influenced by the autonomic nervous system and numerous endocrine hormones in which thyroid hormones have relevance. Thyroid hormone deficiencies, as well as excesses, result in profound changes in cardiac function regulation and cardiovascular haemodynamia mediated by genomic and non-genomic effects (Vargas-Uricoechea *et al.* 2014). A functional relation involving thyroid hormones, endothelial cells and NO has been extensively described in the past last years. There are several studies that showed that thyroid hormones and NO are involved in many different signaling pathways related with normal postnatal cardiac development, maturation and function (Lepic *et al.* 2006). We have previously demonstrated that thyroid hormones are able to regulate intrinsic

heart rate (HR) in a heart without autonomic regulation. According to our results, NO pathway would be involved in this mechanism. Thyroid hormones modulate NO steady-state level which may act as a messenger to modulate the mitochondrial bioenergetic function, resulting in an NO-mediated regulation of the heart pacemaker activity (Fellet *et al.* 2004, 2006, 2008). Additionally, we demonstrated that hypothyroidism contributes in a differential way to aging-induced changes in the myocardium and aorta tissues. Low thyroid hormones levels would enhance the aging effect on the heart related to cardiac NO production (Sarati *et al.* 2012). We also revealed that the heart of male and female rats undergoes distinct adaptive responses to hyperthyroidism that confer to the latter a relatively stronger adaptation profile that appears to be related to the ability to regulate NO production (Rodriguez *et al.* 2015).

Considering that thyroid status alterations are one of the major endocrine diseases in adulthood and its association with a significant increase of cardiovascular risk in the middle-aged, the aim of the present work was to analyze whether changes in NO signalling participate in the cardiovascular manifestations of thyroid disorders and whether these changes are involved in haemodynamic adaptation to acute hemorrhage in animals with thyroid disorders.

Materials and methods

Animals

Male Sprague–Dawley rats 2 months old from the breeding laboratories of the "Facultad de Farmacia y Bioquímica" (Universidad de Buenos Aires, Argentina) were used throughout the study. Rats were housed in humidity- and temperature- controlled environment with an automatic 12/12-h light/dark cycle. Rats were fed standard rat chow from Ganave (Buenos Aires, Argentina) and received tap water ad libitum up to

the day of the experiments. All procedures were reviewed and approved by the National Food, Drug and Medical Technology Administration (ANMAT), National Department of Health and Environment, Argentina (No. 6344/96).

Rats were randomly assigned to one of the three groups

Control rats (Eut, n=15): euthyroid animals who received s.c. injections of 0.9% NaCl (0.1 ml/100 g body weight (BW) every 2nd day during 28 days.

T_3-treated rats (Hyper, n=15): animals received s.c. injections of T_3 (Sigma, 20ug/100g body weight) every 2nd day during 28 days (Heron and Rakusan, 1996).

Methimazole-treated rats (hypo, n=15): animals were rendered hypothyroid after 28 days of treatment with 0.02% methimazole (w/v) in the drinking water (Franco *et al.* 2006).

Determination of treatment efficacy

In order to confirm the hypo and hyperthyroid states, serum thyroid-stimulating hormone (TSH), total triiodothyronine (T3) and thyroxin (T4) (TSH kit, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, USA) were measured by radioimmunoassay at the beginning and the end of the experimental period. Intra- and inter-assay coefficients of variation for TSH were 8.7% and 13.4%, respectively (Greeley *et al.* 1982). T3, inter- and intra-assay coefficients of variation varied from 4.2 to 6.0 and 5 to 6. 5% respectively; T4 inter- and intra-assay coefficients of variation varied from 7.1 to7.4 and 2.9 to 5.1% respectively).

Echocardiographic measurements

After 28 days of treatment, or control period, rats were anesthetized with urethane (1.0 g/kg, ip), their chests were shaved under aseptic conditions and echocardiographic

measurements were performed in the left lateral decubitus position. Two-dimensional directed M-mode images were obtained using a Sonoscape (A6 Vet) system with a 9-4 MHz transducer. Measurements were taken in the right parasternal short axis plane at the level of the mitral valve leaflets. LV internal diameter (LVID), LV posterior wall thickness (PWT) and anterior wall thickness (AWT) were measured in both systole (s) and diastole (d). Ejection fraction (EF), fractional shortening (FS) and systolic volume were measured from ventricular internal diameters by the echocardiography system. All determinations were made according to the guidelines of the American Society of Echocardiography. Each rat was then instrumented with catheters. Animals were kept under anesthesia by additional small doses of urethane throughout the experiment. Body temperature was monitored with a rectal probe and maintained at 37.0 ± 0.5 ºC with heating lamps to avoid the influence of temperature on cardiovascular parameters during the experiment.

Cardiovascular assessments in thyroid desorders

After 28 days of treatment, the animals were anesthetized with urethane (1,0 g/kg ip). To ensure an open airway, a tracheotomy was performed using polyethylene tubing (3.5 or 4 mmID, Portex). Mean arterial pressure (MAP) was measured through a cannula inserted in the right femoral artery and connected to a pressure transducer (Statham P23 ID, Gould Inst., Cleveland, OH, USA); measurements were recorded with a polygraph (Physiograph E & M, Houston, TX, USA) during the whole experiment. Heart rate (HR) was determined from the pulsatile pressure signal by beat-to-beat conversion with a tachograph amplifier (model S77-26 tachometer, Coulbourn Instruments, Allentown, PA, USA). The Labtech Notebook program (Laboratory Technology, Wilmington, MD, USA) was used for data acquisition.

Experimental protocol

Eut, Hyper and hypo animals were subdivided into two experimental groups:

1) Hemorrhaged rats (H). After a 30-min stabilization period, basal values were measured over a 5-min period. Subsequently, the acute hemorrhage was performed. Thereafter, hemodynamic parameters were continuously recorded over a 120-min period after the bleeding (n=15 each group).

2) Hemorrhage + L-NAME (H+LNAME): After a 30-min stabilization period, basal values were measured over a 5-min period. Later, the animals received an infusion of N^G nitro-L-arginine methyl ester (L-NAME, 0,5 mg/kg/h IV = 100 µl/h), which was maintained during the experimental period. Subsequently, the acute hemorrhage was performed. Thereafter, hemodynamic parameters were continuously recorded over a 120-min period after the bleeding (n=15 each group).

The hypovolemic state in *H* and *H+LNAME* groups was induced through an acute hemorrhage using a cannula inserted in the left femoral artery (Riviero, PR10). The bleeding was done by a loss of 20% of the blood volume during 2 minutes, at constant flux. The volume was calculated individually for every animal, from the total blood volume (7% of the body mass).

The L-NAME, an unspecific inhibitor of the NOS, was administrated as a continual infusion through a cannula inserted in the right femoral vein (Riviero PR 10, 0,5 mg/kg/h IV = 100 µl/h).

At the end of each experimental protocol, rats were sacrificed by pneumothorax and heart was removed. Western blot analysis for NOS was performed in this tissue, and NOS activity was measured according to the method of the conversion of [14C (U)]-L-arginine to [14C (U)]-L-citrulline.

Nitric oxide synthase activity

Capacity for cardiac NO formation was assessed determining NOS activity in right atria and left ventricle from Eut, Hyper and hypo animals by measuring the conversion of [14C (U)]-L-arginine to [14C (U)]-L-citrulline. Tissue homogenates (approximately 50 μg protein) were incubated in Tris-HCl buffer (pH 7.4) containing 1 μg/mL L-arginine, [14C (U)]-L-arginine (346 μCi/mL), L-valine (67 mM), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and CaCL2 (2 mM) for 60 minutes at room temperature. At the end of the incubation period, the NOS reaction was arrested by addition of a buffer solution containing 20 mM HEPES buffer and 20 mM EDTA, pH 5.5. Reaction mixtures were loaded onto cation exchange columns (Dowex AG 50W-X8, Na+ form; Bio-Rad) and [14C (U)]-L-citrulline was eluted from columns with 0-50 ml ddH2O. The amount of [14C (U)]-L-citrulline eluted was quantified using a liquid scintillation counter (Wallac 1414 WinSpectral; EG&G Company, Turku, Finland) as described previously (Sarati et al. 2012). All compounds, except [U-14C]-L-arginine monohydrochloride (346mCi/mmol, Amersham Life Science), were purchased from Sigma Chemic. Protein determination was made using the Lowry method, with bovine serum albumin as a standard.

Calcium dependence

In order to determine calcium dependence, atria and ventricle NOS activity was determined using [U-14C] arginine as substrate as described above. Tissue slices (2-3 mm thick) from Eut, hypo and Hyper animals were obtained. Some slices from haemorrhaged groups were preincubated (15 min) with calmidazolium (Cz, 1μM) (Elesgaray et al. 2008) before incubation with [U-14C] L-arginine during 30 minutes at 37 ºC. The amount of [14C] L-citrulline obtained was determined with a liquid

scintillation counter. Nitric oxide production (measured as pmol of [14C] citrulline) was expressed in pmol/g wet weight min.

Western blot analysis

The right atria and left ventricle samples were homogenized on ice with a Tissue Tearor (Biospec Products) in homogenization buffer (50 mmol/L Tris, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1% Triton, 1 mmol/L PMSF, 1 μmol/L pepstatin, 2 μmol/L leupeptin, 1× protease inhibitor cocktail from Roche Diagnostics). Protein concentration in the Triton-soluble supernatant was determined using the Lowry assay. Equal amounts of protein (100 μg protein/lane) were separated by electrophoresis in 7.5% SDS-polyacrylamide gels (Bio-Rad, Munich, Germany), transferred to a nitrocellulose membrane (Bio-Rad) and then incubated with rabbit polyclonal anti-NOS antibodies, diluted at 1:500. The primary antibodies were: polyclonal rabbit anti-inducible NOS (iNOS) (epitope at the carboxy terminus), anti-endothelial NOS (eNOS) (epitope at the amino terminus) and anti-neuronal NOS (nNOS) (epitope at the amino terminus). Finally, a secondary immunoreaction with a goat anti-rabbit antibody conjugated with horseradish peroxidase (dilution 1:5000) was performed. Samples were revealed by chemiluminescence using Kalium reagent for 2–4 min. Density of the respective bands was quantified by densitometric scanning of Western blots using a Hewlett-Packard scanner and Totallab analyzer software (Biodynamics, Seattle, WA, USA), and protein amounts were calculated by comparison to the densitometric values of the corresponding standard. Protein levels were expressed as a ratio of the optical densities of NOS isoforms and β-actin band (using anti-beta actin, clone EP1123Y, rabbit monoclonal antibody) to check for any inaccuracies in protein loading.

Materials

The antibodies against the three isoforms of NOS (iNOS (610333), eNOS (610298) and nNOS (610311) were supplied by BD Biosciences and anti β-actin by Millipore (04-1116). Secondary antibody (170-6515) was by Bio-Rad laboratories. The Western Blot Detection System was supplied by Amersham Pharmacia Biotech. Biochemicals were supplied by Sigma Chemical (Saint Louis, MO, USA).

Ethical approval for animal experimentation

Animals were cared for according to regulation 6344/96 of Argentina's National Food, Drug and Medical Technology Administration (ANMAT). Experiments with animals had been performed in accordance with UK legal requirements. Experimental procedures were approved by the ethics committee of the Facultad de Farmacia y Bioquímica (CICUAL; EXP UBA N° 0054570), Universidad de Buenos Aires, Argentina.

Statistical analysis

Data in tables and figures are mean values ± SEM. Data were evaluated with univariate and multivariate approaches for a completely randomized design, with a structure of two factors (hemorrhage and thyroid hormones). For each variable, ANOVA or MANOVA analysis was performed when appropriate. The Levene's and Shapiro–Wilk's tests were used to evaluate homogeneity of variances and normality of data, respectively.

When normality and homogeneity of variances assumptions were satisfied, the Bonferroni multiple comparison test was run. In the case of non-homogenous variances, a multiple comparison test, such as Tamhane, was run. To detect association among

variables, a correlation analysis was performed and the Pearson coefficient was calculated. All statistical procedures were performed using the SPSS statistical software package version 22.0 statistical significance was set at $P<.05$.

Results

Treatment efficacy

Treatment with methimazole and T_3 was effective in establishing a hypothyroid and hyperthyroid state, respectively. TSH plasmatic levels were higher and lower in hypo and Hyper rats respectively than Eut animals. T_3 and T_4 levels decreased in hypo rats, while T_4 increased in Hyper animals. Body weights were similar in the three groups of animals. Basal MAP values were similar in Eut, hypo and Hyper rats. However, basal HR values were lower and higher in hypo and Hyper rats compared with Eut animals, respectively (Table 1).

Echocardiographic measurements

Table 1 also shows echocardiographic data for all groups. LV systolic and diastolic chamber diameters increased in hypo rats meanwhile these diameters decreased in Hyper animals. LV anterior and posterior wall thickness in both systole and diastole remained unchanged between Eut and Hyper animals; however, a reduction in these parameters was observed in hypo group. LV ejection fraction and fractional shortening did not change between Eut and Hyper animals but they were reduced both in hypothyroid state. Figure 1 shows representative images of M-mode echocardiographic tracing.

Changes in systemic hemodynamic parameters during and after hemorrhagic state

Figure 2 illustrates the time course of MAP and HR during 120 min after bleeding. Baseline MAP (mm Hg) measurements were not different among the three groups of rats. Hemorrhage induced a marked decrease in MAP in Eut group, which reached a value of 44±4 mm Hg at 10 min following the bleeding period ($P<0.001$ versus basal values), with subsequent stabilization at about 55±4 mm Hg at 35 min ($P<0.01$ versus basal values). The pressure response to bleeding was similar in the three experimental groups. However, the magnitude of immediate hypotension after bleeding was greater in animals with thyroid alterations. The pressure remained low during the entire experimental time presenting lower values of animals with thyroid disorders (panel A). L-NAME treatment has not altered basal MAP in the three experimental groups. Blood pressure in L-NAME Eut treated rats group was 37 mm Hg ($P<0.01$ versus basal values) at 10 min hemorrhage, rising to 82 ± 4 (P=ns), 100 ± 4 ($P< 0.01$) and 105 ± 4 mm Hg ($P<0.01$) during 35, 60 and 120 min, respectively following the bleeding. The inhibition of NO system induced a recovery of the MAP after bleeding, registering a stabilization of this parameter to the 60 minutes the hemorrhage, in values higher than hypo and Hyper animals. In relation to the hypothyroid rats, the L-NAME treatment induced a greater hypotensive response after bleeding. However, in the later stages, MAP was gradually increased stabilizing in 53 mmHg at about 65 min. The hyperthyroid animals presented a lower hypotensive response to the euthyroid, but managed to reach MAP values close to the baseline at about 100 minutes (panel B).

Panel C illustrates the time course of the HR in Eut, hypo and Hyper animals after bleeding. Basal HR of Eut animals was around 352±15 bpm, while that of the hypo and Hyper rats was 214±15 bpm and 424±13 bpm, respectively. The hemorrhage induced a bradycardia of short duration followed by a gradual increase in this parameter in all the

300 groups. Eut animals have attained the stabilization of this parameter at about 30 minutes
301 after hemorrhage, whereas this time was between 10-15 minutes to achieve the
302 stabilization in hypo and Hyper rats (panel C). Treatment with L-NAME annulled the
303 changes of HR to hemorrhage in Eut and hypo rats. L-NAME Hyper treated animals
304 showed a similar HR response after withdrawal (panel D).
305
306 **Nitric oxide synthase activity and western blot**
307 Figure 3 (panel A) shows that hypo and Hyper animals exhibited a decreased atrial NOS
308 activity compared with Eut rats. Bleeding increased NOS activity in all groups of
309 animals. However, the magnitude of change was greater in Eut and Hyper rats
310 compared with hypo animals. Endothelial NOS isoform decreased in hypo rats (panel
311 B). There were no differences between Eut and Hyper groups of animals. Hemorrhage
312 increased eNOS protein levels in Eut and hypo rats. However, Hyper animals showed
313 decreased eNOS protein levels after withdrawal. Inducible and neuronal NOS were
314 lower in hypo rats compared with Eut group. Inducible and neuronal NOS proteins
315 levels did not change in Eut and Hyper rats (panels C and D). Bleeding increased iNOS
316 proteins levels in all groups of animals meanwhile it did not change nNOS protein
317 levels in experimental groups (panel C and D).
318 Figure 4 (panel A) shows that hypo and Hyper rats showed increased left ventricle NOS
319 activity compared with Eut animals. Hemorrhage increased NOS activity in all groups
320 of animals. eNOS, iNOS and nNOS proteins levels did not change with thyroid status.
321 Bleeding increased eNOS protein levels in Eut and hypo animals (panel B) and iNOS
322 proteins levels in Eut rats (panel C). nNOS did not change with hemorrhage (panel D).
323 Figure 5 (panel A) showed that when NOS activity was evaluated on atria slices, we
324 obtained similar results to those obtained using homogenates. Hypo and Hyper animals

exhibited a decreased atrial NOS activity compared with Eut rats and bleeding increased NOS activity in all groups of animals. Calmidazolium treatment attenuated NOS activity in Eut and hypo animals without without modification in hyper group of animals. In ventricle, hypo and Hyper rats showed increased left ventricle NOS activity compared with Eut animals and hemorrhage increased NOS activity in all groups of animals. Pretreatment with calmidazolium attenuated and blunted the increase of NOS activity induced by hemorrhage in Eut and hypo animals, respectively. The NOS activity increase after bleeding was not altered by calmidazolium in Hyper animals.

Discussion

The present study provides new evidences that changes in cardiac function associated with thyroid disorders and hypovolemia not only involve effects on sympathetic nervous system, but may also involve changes in the response of the myocytes to NO bioavailibity. This study investigated the role of NO in the cardiovascular adaptation following acute hemorrhage in rat with thyroid disorders. TSH measurements showed that T_3 and methimazol treatment were effective to establish hyper and hypothyroid state, respectively. In our experimental condition, basal MAP values were similar in the three experimental groups. These findings are surprising. It would be expected that MAP decreases and increases in hypo and hyperthyroid state, respectively. However, our results showed that MAP did not change compared with euthyroid control rats. Maintained pressure values in animals with thyroid disorders may be due to changes induced on diastolic pressure are similar in magnitude to the changes induced on systolic pressure despite having very different HR values. It is important to consider that this discrepancy with others researchers might be due to the different duration and degree of hypo and hyperthyroidism developed in our experimental conditions. Additionally, we have shown that L-NAME infusion did not

alter basal blood pressure values in experimental groups. It is probable that in this condition the inhibition of the constitutive NOS activity is only partial, being the quantity of NO that exceeds sufficient to maintain MAP within the basal range.

When we evaluated cardiac function associated with thyroid status, echocardiographic data confirmed that hypothyroid animals have FS and EF decreased and increased left ventricle internal diameter. This would indicate that myocardial contractility would be altered and the ventricle would not be filling properly especially during diastole in these animals. It is important to note that FS depends primarily afterload. The most common causes of decreased FE in hypothyroidism would be blockages in the coronary arteries, increased blood pressure, heart rhythm disturbances, or weakening of the heart muscle. In this context, hypothyroidism could be associated with increased prevalence of cardiac heart failure associated with thyroid hormones deficiencies (Biondi 2012). On the other hand, although hyperthyroid animals showed a decrease in the diameter of the ventricle, no change was observed in cardiac contractility in our experimental model. Taken together, these results suggest that hypo and Hyper rats would exhibit different thyroid disorder-induced remodeling adaptation. Hypothyroidism would exhibit a greater eccentric hypertrophic response while hyperthyroid animals would exhibit concentric hypertrophic response. These findings agreed with others authors who described the development of the eccentric hypertrophy in hypothyroidism (Wang *et al.* 2010; Sarati *et al.* 2012) and concentric left ventricle hypertrophy associated with thyrotoxicosis (Basset *et al.* 1980; Abergel *et al.* 1995). The mechanisms of the animal model of thyroid disturbances induced cardiac hypertrophy are multifactorial. It is not clear whether thyroid hormone status-induced cardiac hypertrophy results from a direct effect on the heart, alterations of the adrenergic nervous system signaling, or altered cardiac loading conditions. Taking into

account the latter, we evaluate cardiovascular hemodynamics changes to hemorrhage in animals with thyroid disorders. It is well known that cardiovascular adaptation to this hypovolemic state is under dynamic control of the sympathetic and parasympathetic divisions, the magnitude of hemorrhage, the rate of bleeding, and the species examined (Schadt and Ludbrook, 1991). Different circulating endocrine and local paracrine factors such as NO have been postulated to modulate the cardiovascular response to hypovolemia (Goldstein *et al.* 1999). In this study, we showed that hemorrhage elicited a significant decrease between 50-59% of arterial blood pressure within the 10 min after bleeding from basal values in all experimental groups. The magnitude of this immediate hypotension would seem similar in all rats. After this time, this parameter increased stabilizing its values at about 30-40 min and was maintained until finalized 120 min. Pretreatment with L-NAME before bleeding induced a similar immediate decrease (at about 60%) in all experimental groups. This immediate hypotension was followed by a faster recovery of blood pressure to basal values in euthyroid and hyperthyroid hemorrhaged rats. This parameter did not reach basal values in hypo rats. Taken together, these findings suggest that the immediate hypotension would be independent of NO system, however, after this time (10 min) NO would modulate systemic vascular response probably due a direct vasodilatory action on vascular smooth muscle especially in euthyroid and hyperthyroid animals. This effect of NO seems to be lower in hypothyroidism in which basal values of MAP were not reached. However, we cannot throw away the effects of NO on the integrated mechanisms which become activated in response to hemorrhage as well as the release of several neurohormonal vasoconstrictor factors (catecholamines, endothelins, vasopressin, renin–angiotensin system) (Fujisawa *et al.* 1999; Moreno *et al.* 2002).

Focusing on chronotropic response, our results showed an increase and decrease of basal pacemaker activity in hyper and hypothyroid rats compared with respective euthyroid animal. These changes would confirm the tachycardic action of T_3 (Sun *et al.* 2001).We also showed that NOS inhibition was not modified basal values of HR in the three groups of animals, but blunted the changes induced by blood loss. Hypovolemic state provoked after the expected immediate reflex-induced tachycardia, a bradycardic stage followed by a gradual increase of HR during 120 min (Balaszczuk *et al.* 2006). It is known that blood pressure is maintained in the early stage of hemorrhage by reflex increase in HR, vascular resistance, and peripheral sympathetic nerve activity. The inhibition of NO system would affect the immediate baroreflex response in our experimental model. The bradycardia, observed in the early stages, may result from alterations of the activation of unmyelinated vagal afferents (C fibers) from the left ventricle induced by the loss of 20% in the blood volume. A decrease of HR may seem unreasonable during hemorrhage but it could be a part of a complex reflex in order to reduce an ongoing blood loss by reducing blood pressure by means of peripheral vasodilatation and, at the same time, maintain organ blood flow. The absence of the bradycardia suggests that NO could be involved in the cholinergic modulation of HR in the early stage of hypovolemic state. However, the relationship between the NO system and the absence of the later increase of HR was not well understood. It was reported that systemic inhibition of NOS in vivo in humans, by L-NMMA, significantly reduced renal plasma flow in the absence of alterations in glomerular filtration rate, blood pressure, or pulse rate (Wolzt *et al.* 1997). By contrast, Schmetterer *et al.* (1999) showed a significant decrease in heart rate after infusion of L-NMMA. These findings suggest that nitric oxide, present in the sinoatrial and atrioventricular nodes, seems to play an important role in pacemaker activity control. The action of L-NAME on the

later tachycardia may be a primary action due to inhibition of NO pathway or a secondary effect resulting from the absence of the maintained hypotension after the hemorrhage.

Considering NO system in the heart, our results showed that animals with thyroid alterations have a lower atrial NOS activity than Eut rats. This lower activity of the enzyme is associated with a decrease of the three NOS isoforms protein levels in hypo rats. Meanwhile, hyperthyroid animals showed no changes in protein levels of the three isoforms of the enzyme. These findings allow us to think that negative modulators of the enzyme, like caveolins, are probably increased in hyperthyroidism.

In addition, a contradictory result was found in the left ventricle. Animals with thyroid disorders had a higher enzyme activity than euthyroid and this rise was not correlated with changes in NOS protein levels. Positive modulators of NOS activity would be exacerbated in thyroid disorder.

Focusing on physiological involvement of NO during hypovolemic state, we observed that acute hemorrhage results in an excessive production of NO in right atria as well as in left ventricle at 120 min after blood loss in all experimental groups. In the atrium, increased NOS activity induced by bleeding could be due to increased endothelial and inducible isoform of the enzyme in euthyroid and hypothyroid animals and only iNOS protein levels in hyperthyroidism. Experiments with calmidazolium confirmed these findings. Conversely, withdrawal induced a decrease in protein levels of eNOS in hyperthyroidism.

On the other hand, the left ventricle increased NOS activity induced by hemorrhage could be due to a rise in eNOS protein levels in euthyroid and hypothyroid animals. Additionally, hypovolemia increased iNOS protein levels in euthyroid rats. Our results also suggested that the NOS activity changes induced by hypovolemic state

could involve alteration of positive modulators of the enzyme in hyperthyroidism. Experiments with calmidazolium confirmed these findings. Calmodulin antagonist attenuated NOS activity increase in Eut animals meanwhile blunted the rise in hypo rats and did not modify it in Hyper group.

In summary, the key findings of this study are that thyroid hormones deficiencies, as well as excesses result in alterations of cardiac function regulation and cardiovascular haemodynamia. Although hypothyroidism and hyperthyroidism are associated with cardiac remodelling, they affect cardiac function and haemodynamic parameters in a different way. Our results demonstrated that both thyroid disorders were associated with hypertrophic remodelling which was and impacted differently on cardiac function and consequently its adaptation to a hypovolemic state. Additionally, although the effect of the thyroid disorders on NO production depends on the studied cardiac chamber, the impact of bleeding is similar in both chambers. Hypovolemia induced by acute hemorrhage triggered a NOS activation modulating the heart function to maintain haemodynamic homeostasis. The involvement of NO pathway depended on a specific NOS isoform, cardiac chamber and thyroid state.

Declaration of interest

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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561

562 **Legends**

563 Figure 1. Left ventricular representative images of M-mode echocardiographic tracing
564 from euthyroid (panel A), hypothyroid (panel B) and hyperthyroid rats (panel C).
565 Figure 2. Changes in mean arterial pressure (MAP) and heart rate (HR) during and after
566 hemorrhage in euthyroid (Eut), hypothyroid (hypo) and hyperthyroid rats (Hyper)
567 (panels A and C). Changes in MAP and HR during and after hemorrhage in L-NAME
568 treated Eut, Hypo and Hyper rats (panels B and D) ◆ Eut rats ; ■ hypo rats;▲ Hyper
569 rats. Points are mean values (error bars represent SEM); n=15/group; *P<0.05 vs. basal
570 values.

Figure 3. Total NOS activity in right atria from euthyroid (Eut), hypothyroid (hypo) and hyperthyroid (Hyper) rats (panel A). The values are mean ± SEM; n=15/group; *P<0.05 vs Eut rats; † P<0.05 vs rats without hemorrhage. Representative Western Blots of eNOS (panel B), iNOS (panel C), nNOS (panel D), carried out on proteins from Eut, hypo and Hyper right atria. Histograms illustrate the mean NOS protein values for each group. All experiments were performed in triplicate. Each blot was normalized with the expression of the β-actin from the same gels. Data are mean ± SEM.; n=7/group; *P<0.05 vs Eut rats; † P<0.05 vs rats without hemorrhage. EutH: euthyroid hemorrhaged rats; hypoH: hypothyroid hemorrhaged rats; HyperH: hyperthyroid hemorrhaged rats.

Figure 4. Total NOS activity in left ventricle from euthyroid (Eut), hypothyroid (hypo) and hyperthyroid (Hyper) rats (panel A). The values are mean ± SEM; n=15/group; *P<0.05 vs Eut rats; † P<0.05 vs rats without hemorrhage. Representative Western Blots of eNOS (panel B), iNOS (panel C), nNOS (panel D), carried out on proteins from Eut, hypo and Hyper left ventricle. Histograms illustrate the mean NOS protein values for each group. All experiments were performed in triplicate. Each blot was normalized with the expression of the β-actin from the same gels. Data are mean ± SEM; n=7/group; *P<0.05 vs Eut rats; † P<0.05 vs rats without hemorrhage. EutH: euthyroid hemorrhaged rats; hypoH: hypothyroid hemorrhaged rats; HyperH: hyperthyroid hemorrhaged rats.

Figure 5. Total NOS activity in right atria (panel A) and left ventricle (panel B) slices from euthyroid (Eut), hypothyroid (hypo) and hyperthyroid (Hyper) rats. CZ: calmidazolium.EutH: euthyroid hemorrhaged rats; EutHCZ: euthyroid hemorrhaged rats pretreated with CZ; hypoH: hypothyroid hemorrhaged rats; hypoHCZ: hypothyroid hemorrhaged rats pretreated with CZ; HyperH: hyperthyroid hemorrhaged rats;

596 HyperHCZ: hyperthyroid hemorrhaged rats pretreated with CZ. Data are mean ± SEM;

597 n=7/group; *P<0.05 vs Eut rats; † P<0.05 vs rats without hemorrhage.

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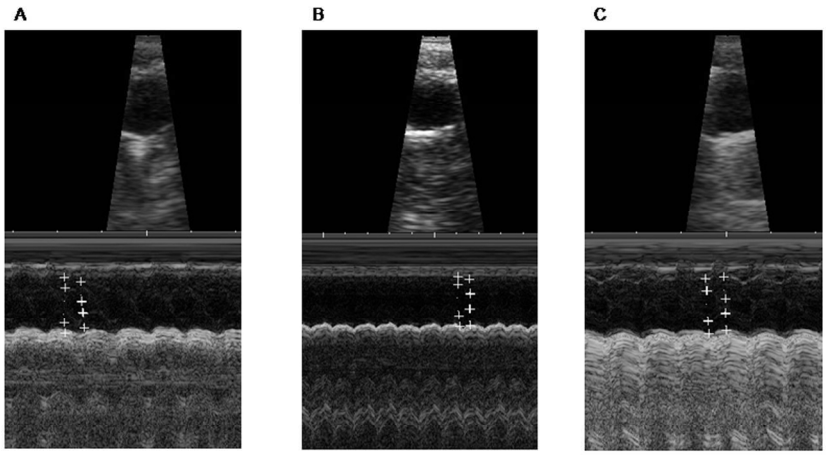

Fig. 1. Ogonowski et al.

Figure 1
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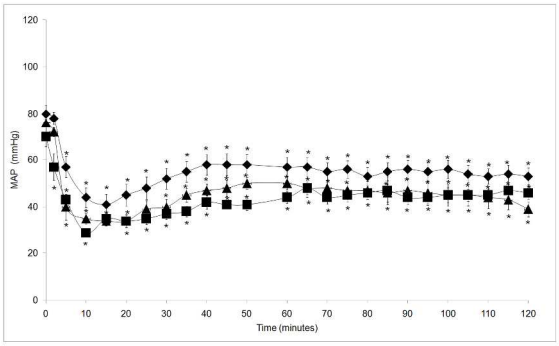
A



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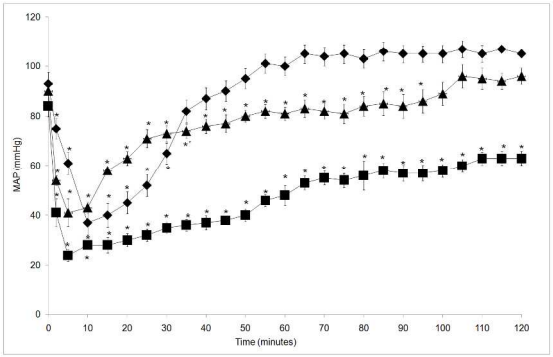

Figure 2, A and B

Figure 2, A and B
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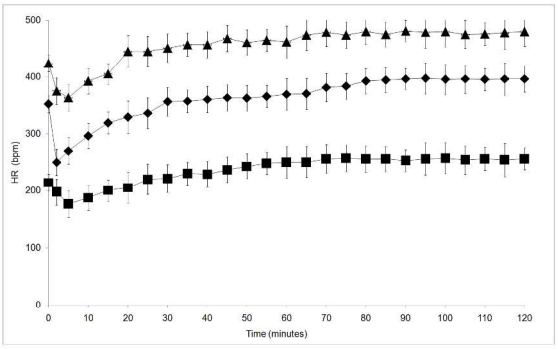
C



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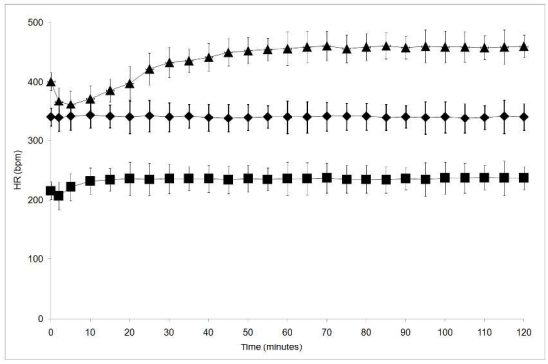

Figure 2, C and D

Figure 2, C and D
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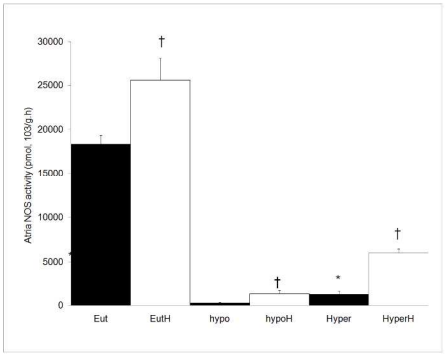
A



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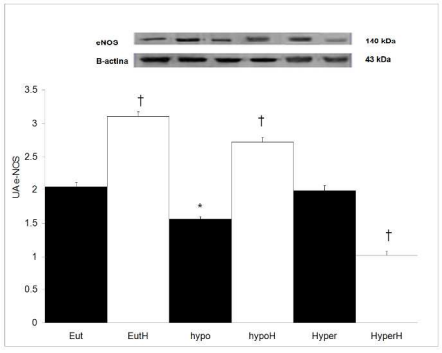
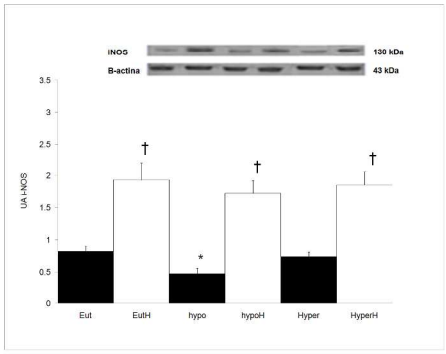


Figure 3, A and B

Figure 3, A and B
209x297mm (300 x 300 DPI)

C



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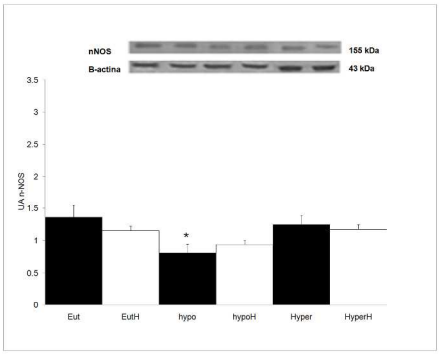

Figure 3, C and D

Figure 3, C and D
209x297mm (300 x 300 DPI)

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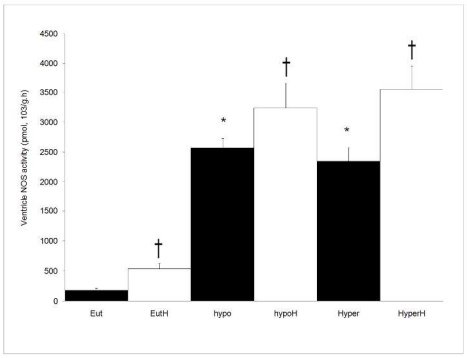

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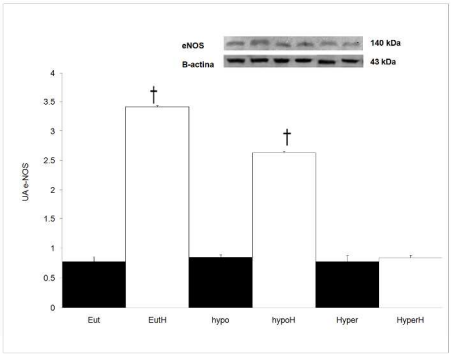
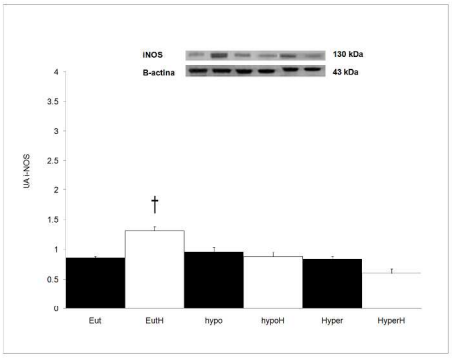


Figure 4, A and B

Figure 4, A and B
209x297mm (300 x 300 DPI)

C



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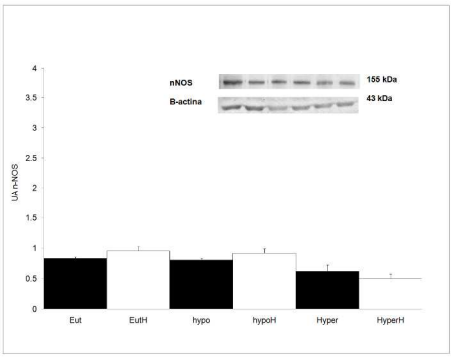

Figure 4, C and D

Figure 4, C and D
209x297mm (300 x 300 DPI)

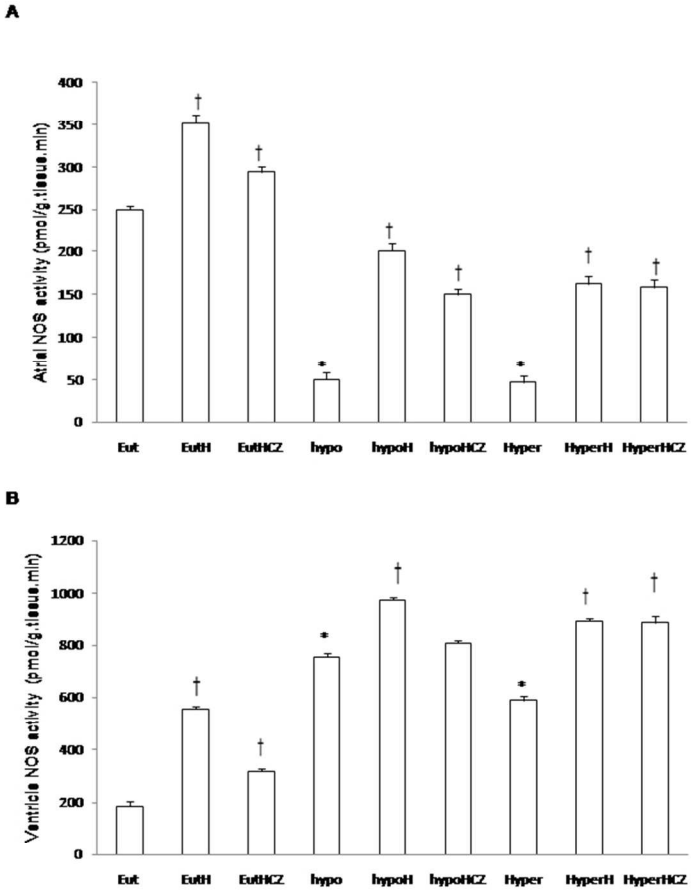

Fig.5 Ogonowski et al.

Fig 5
190x275mm (96 x 96 DPI)

Table 1. Biological variables.

Group of animals	Eut	Hypo	Hyper
TSH (ng/ml)	14.75±0.83	35.57±4.35*	5.57±0.03*
T_3 (ng/dl)	1.131±0.123	0.750±0.036*	1.034±0.036
T_4 (ug/ml)	2.475±0.031	1.034±0.036*	3.775±0.270*
BW (g)	337±12	338±12	298±12
HR (bpm)	352±15	214±13*	424±15*
MAP(mmHg)	80±4	70±4	76±2
LVIDd (mm)	5.40±0.18	5.99±0.19*	4.7±0.10*
LVIDs (mm)	2.80±0.06	3.01±0.10*	2.21±0.07*
AWTd (mm)	1.70±0.02	1.37±0.01*	2.01±0.10
AWTs (mm)	2.87±0.03	2.10±0.02*	2.8±0.10
PWTd (mm)	2.10±0.17	1.50±0.10*	2.03±0.15
PWTs (mm)	2.90±0.08	2.43±0.12*	3.23±0.08
EF (%)	86±3	83±1*	88±3
FS (%)	56±2	46±2*	51±2

Eut (euthyroid rats); Hypo (hypothyroid rats); Hyper (hyperthyroid rats); TSH (thyroid-stimulating hormone); T_3 (triiodothyronine); T_4 (total thyroxine); BW (body weight); HR (heart rate); MAP (mean arterial pressure); LVIDd (LV internal diameter in diastole); LVIDs (LV internal diameter in systole); AWTd (anterior wall thickness in diastole); AWTs (anterior wall thickness in systole); PWTd (posterior wall thickness in diastole); PWTs (posterior wall thickness in systole); EF (ejection fraction); FS (fractional shortening). Data are mean ± SEM; n=15;*P<0.05 vs. Eut rats.