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Hypothyroidism: age-related influence on cardiovascular nitric oxide system in rats

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ABSTRACT

This study investigates whether changes in nitric oxide (NO) production participate in the cardiovascular manifestations of hypothyroidism and whether these changes are age-related. Sprague–Dawley rats aged 2 and 18 months old were treated with 0.02% methimazole (wt/vol) during 28 days. Left ventricular function was evaluated by echocardiography. Measurements of arterial blood pressure, heart rate, nitric oxide synthase (NOS) activity and NOS/caveolin-1 and -3 protein levels were performed. Hypothyroidism enhanced the age-related changes in heart function. Hypothyroid state decreased atrial NOS activity in both young and adult rats, associated with a reduction in protein levels of the three NOS isoforms in young animals and increased caveolin (cav) 1 expression in adult rats. Ventricle and aorta NOS activity increased in young and adult hypothyroid animals. In ventricle, changes in NOS activity were accompanied by an increase in inducible NOS isoform in young rats and by an increase in caveolins expression in adult rats. Greater aorta NOS activity level in young and in adult Hypo rats would derive from the inducible and the endothelial NOS isoform, respectively. Thyroid hormones would be one of the factors involved in the modulation of cardiovascular NO production and caveolin-1 and -3 tissue-specific abundance, regardless of age. Hypothyroidism appears to contribute 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. Age-related changes in NO production participate in the cardiovascular manifestations of hypothyroidism.

Abbreviations: cav, caveolins; cav-1, caveolin-1; cav-3, caveolin-3; eNOS, endothelial nitric oxide synthase; Eut, euthyroid; HR, heart rate; Hypo, hypothyroid; iNOS, inducible nitric oxide synthase; LV, left ventricle; MAP, mean arterial pressure; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; T_4, total thyroxine; TSH, thyroid-stimulating hormone; TPVR, total peripheral vascular resistance; Ip, Intraperitoneal; LVID, left ventricle internal diameter; PWT, posterior wall thickness; AWT, anterior wall thickness; s, systole; d, diastole; EF, ejection fraction; FS, fractional shortening; BW, body weigh; CV, coefficient of variation.

Author contributions: All authors contributed extensively to the work presented in this research article. Dr. Andrea Fellet is Dr. Sarati's and Ms. Martinez' thesis director. She designed the study and participated in data analysis and interpretation, manuscript preparation and determination of intellectual content. She is responsible for the integrity of the work. Dr. Lorena Sarati and Carla Martinez, BSc, are doctoral fellows of Dr. Fellet's and participated in conducting the experiments, collection and analysis of data as well as data interpretation and the writing of the manuscript. Student Nicolás Artés, RPH Noelia Arreche and Dr. Juan José López-Costa took part as technical assistants. Dr. Ana Maria Balaszczuk contributed to manuscript correction, reviewing it critically for important intellectual content.

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1. Introduction

Variations in euthyroid status affect all physiological systems, having therefore more pronounced effects on the cardiovascular system [1]. On the basis of studies in humans and animals with hypothyroidism, it is well recognized that thyroid hormones exert profound direct and indirect actions on the heart and the cardiovascular system. The basis for this is the ability of thyroid hormones to exert cellular action on cardiac myocytes, vascular smooth muscle cells and the endothelium [2,3]. The hemodynamic changes that characterize hypothyroidism include a decrease in cardiac contractility, cardiac output, heart rate (HR) and left ventricular (LV) compliance, as well as an increase in total peripheral vascular resistance (TPVR) [4,5]. Evidence from animal and human studies has clearly shown that aging may contribute to the development of endocrine dysfunctions such as thyroid disorders [6-8]. In particular, thyroid function decreases progressively with aging and the symptoms of aging can easily be confused with those of hypothyroidism [8]. However, the relation between thyroid hormones and aging on the cardiovascular function is controversial. A functional relation involving thyroid hormones, endothelial cells and nitric oxide (NO) has been described and it has been demonstrated that thyroid hormones are able to regulate intrinsic HR in a heart without autonomic regulation. According to current results, the NO pathway would be involved in this mechanism [9-11]. An impaired endothelium-dependent vasodilatation resulting from a reduction in NO availability has been demonstrated in hypothyroidism [12].

On the other hand, it is known that normal cardiovascular aging is also associated with decreased endothelium-mediated vasodilatation. In this context, early ventricular relaxation has been proven to become both slowed and delayed. The age-associated decrease in vascular responsiveness suggests an impaired vascular NO endothelium-mediated signaling or alterations in NO synthase (NOS) activity regulation, which could involve caveolin (cav) proteins [13,14]. It is also known that these protein modulators are expressed in endothelial cells and cardiomyocytes [15]. Alterations in cav distribution and/or cav/NOS interaction have been related to NOS dysfunction associated with aging and thyroid disorders [16,17].

Considering that hypothyroidism is one of the major endocrine diseases in adulthood and that it is associated with a significant increase in cardiovascular risk in the middle-aged, the aim of the present study was to analyze whether changes in NO production participate in the cardiovascular manifestations of hypothyroidism, and also whether these changes are age-related. To this end, determination of NOS activity and NOS and cav protein isoforms levels in cardiac and vascular tissues from young and adult hypothyroid (Hypo) rats was performed.

2. Methods

2.1. Animals

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). Male Sprague–Dawley rats aged 2 (referred to as young) and 18 months old (referred to as adult) from the breeding laboratories of the "Facultad de Farmacia y Bioquímica" (Universidad de Buenos Aires, Argentina) were used throughout the study. Rats were housed two per cage under controlled humidity and temperature conditions, with an automatic 12-h light/dark cycle. Rats were randomly assigned to one of the two groups: euthyroid (Eut) and Hypo rats. Animals were fed standard rat chow from Nutrimentos Purina (Buenos Aires, Argentina) and received water *ad libitum*.

2.2. Treatment

Rats were rendered hypothyroid after 28 days of treatment with 0.02% methimazole (wt/vol) in the drinking water [18].

2.3. Determination of treatment efficacy

Establishment of altered thyroid status was confirmed by measurement of thyroid-stimulating hormone (TSH) and total thyroxine (T_4) by radioimmunoassay in serum samples obtained at the experimental end-point [19]. The TSH kit was provided by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (Bethesda, USA). Results were expressed in terms of rat TSH standard (rTSH-RP-2). Intra- and inter-assay coefficients of variation for TSH were 8.7% and 13.4%, respectively.

2.4. Surgical procedures

After 28 days of treatment, or control period, animals 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 °C with heating lamps to avoid the influence of temperature on cardiovascular parameters during the experiment. To ensure an open airway, a tracheotomy was performed using polyethylene tubing (3.5 or 4 mm ID, Portex). Mean arterial pressure (MAP) and systolic blood pressure were 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) for the duration of the experiment. HR was derived from the pulsatile pressor signal

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via tachographic beat-to-beat conversion with a tachograph preamplifier (Coulbourn Instruments, tachometer S77-26, PA, USA). The Labtech Notebook program (Laboratory Tech., Wilmington, MD, USA) was used for data acquisition.

2.5. Experimental protocol

Hemodynamic parameters were recorded over a 30-min period to allow stabilization of MAP and HR before the start of the experimental protocol. After a 30-min stabilization period, basal values were measured over a 5-min period. Thereafter, hemodynamic parameters were continuously recorded over a 120-min period ($n=15$ each group).

At the end of the experimental protocol, rats were sacrificed by overdose of anesthesia and the heart and thoracic aorta segments were removed. Western blot analysis for NOS, cav-1 and -3 proteins was performed in these tissues, and NOS activity was measured according to the method of the conversion of [^{14}C (U)]-L-arginine to [^{14}C (U)]-L-citrulline [20].

2.6. NOS activity

Capacity for cardiac and vascular NO formation was assessed determining NOS activity in right atria, LV and thoracic aorta segments from Eut and Hypo animals by measuring the conversion of [^{14}C (U)]-L-arginine to [^{14}C (U)]-L-citrulline. Tissue homogenates (approximately 50 μg protein) were incubated in Tris–HCl buffer (pH 7.4) containing 1 μg/mL L-arginine, [^{14}C (U)]-L-arginine (346 μCi/mL), L-valine (67 mmol/L), NADPH (1 mmol/L), calmodulin (30 nmol/L), tetrahydrobiopterin (5 μmol/L) and $CaCL_2$ (2 mmol/L) for 60 min at room temperature. At the end of the incubation period, the NOS reaction was arrested by addition of a buffer solution containing 20 mmol/L HEPES buffer and 20 mmol/L EDTA, pH 5.5. Reaction mixtures were loaded onto cation exchange columns (Dowex AG 50W-X8, Na+ form; Bio-Rad) and [^{14}C (U)]-L-citrulline was eluted from columns with 0–50 mL ddH_2O. The amount of [^{14}C (U)]-L-citrulline eluted was quantified using a liquid scintillation counter (Wallac 1414 WinSpectral; EG&G, Turku, Finland) as described previously [20]. All compounds, except [U-^{14}C]-L-arginine monohydrochloride (346 mCi/mmol, Amersham Life Science), were purchased from Sigma Chemie. Protein determination was made using the Lowry method, with bovine serum albumin as a standard.

2.7. Western blot analysis

The heart and aortic 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 and anti-cav antibodies, both 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), anti-neuronal NOS (nNOS) (epitope at the amino terminus), anti-cav-1 (H-97, sc-7875) and anti-cav-3 (H-100, sc-28828). 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 ECL 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 [21]. Protein levels were expressed as a ratio of the optical densities of NOS and cav isoforms and β-actin band (using anti-beta actin, clone EP1123Y, rabbit monoclonal antibody) to check for any inaccuracies in protein loading.

2.8. Materials

The antibodies for the three NOS isoforms (iNOS (610333), eNOS (610298) and nNOS (610311)) were supplied by BD Biosciences and anti β-actin by Millipore (04-1116). Antibodies anti-cav-1 (sc-7875) and -3 (sc-28828) were supplied by Santa Cruz Biotechnology and the secondary antibody (170-6515) by Bio-Rad laboratories. The Western Blot Detection System and Hybond-ECL membranes were supplied by Amersham Pharmacia Biotech. Biochemicals were supplied by Sigma Chemical (Saint Louis, MO, USA). L-[^{14}C (U)] arginine substrate was supplied by Perkin Elmer Life and Analytical Sciences, Boston, MA, USA. A Wallac 1414 WinSpectral (EG&G, Turku, Finland) liquid scintillation counter was used. AG 50W-X8 cation exchange resin was supplied by BIO-RAD Laboratories.

2.9. 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) and the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Experimental procedures were approved by the ethics committee of the School of Biochemistry and Pharmacy (CEFFB), Universidad de Buenos Aires, Argentina.

2.10. 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 (age 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. The coefficient variability of each echocardiographic measurement is

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Table 1 – Biological variables.

	Young Eut	Young Hypo	Adult Eut	Adult Hypo
TSH (ng/mL)	14.75±0.83	35.57±4.35*	2.47±0.25†	7.75±0.13†*
T_4 (μg/mL)	2.46±0.030	1.03±0.036*	2.25±0.04	0.94±0.038*
BW (g)	337±12	301±11	562±8†	543±10†
MAP (mmHg)	90±1	58±1*	65±1†	48±1†*
HR (bpm)	346±1	211±1*	303±1†	200±1†*
TPVR (mmHg/mL/min)	2.49±0.20	3.43±0.40*	0.44±0.02†	1.31±0.01*†

Eut (euthyroid rats); Hypo (hypothyroid rats); TSH (thyroid-stimulating hormone); T_4 (total thyroxine); BW (body weight); MAP (mean arterial pressure); HR (heart rate); and TPVR (total peripheral vascular resistance). Data are mean±SEM; $n=15$; *$P<.05$ vs age-matched Eut rats; †$P<.05$ vs young rats.

expressed as coefficient of variation (CV). CV was calculated as SD of the differences divided by the mean of the variable under consideration. All statistical procedures were performed using the SPSS statistical software package version 16.0. Statistical significance was set at $P<.05$.

3. Results

3.1. *Treatment efficacy*

Treatment with methimazole was effective in establishing a hypothyroid state. Table 1 shows that TSH levels were lower in adult rats compared with young animals, while serum T_4 levels remained unchanged with aging. Additionally, Hypo animals had higher TSH serum levels and lower T_4 serum levels than age-matched Eut rats.

3.2. *Biological variables*

Table 1 shows the effects of methimazole on biological variables. Interestingly, MAP and HR were lower in adult animals compared with young rats, and young and adult Hypo animals exhibited reduced MAP and HR compared with age-matched Eut animals. Additionally, TPVR was lower in adult rats compared with young animals. Hypo rats showed increased vascular resistance compared with age-matched Eut animals.

Table 2 shows echocardiographic data for all groups. LV systolic and diastolic chamber diameters were increased both with aging and with hypothyroidism. LV anterior and posterior wall thickness in both systole and diastole remained unchanged between young and adult animals; however, a reduction in these parameters was observed in both Hypo groups. LV ejection fraction and fractional shortening were also reduced both with aging and with hypothyroid state. Fig. 1 shows representative images of M-mode echocardiographic tracing.

3.3. *Nitric oxide synthase in young and adult rats*

Fig. 2A illustrates total NOS activity from right atria in young and adult Eut and Hypo rats (Fig. 2A). The results show that adult Eut rats exhibited lower NOS activity than young Eut ones. However, adult Hypo rats exhibited higher NOS activity than young Hypo ones. In the hypothyroid group, atrial NOS activity was reduced in both young and adult rats compared with age-matched Eut animals. Figs. 2B, C and D illustrate representative Western blot analysis in this tissue from all groups of animals. The accompanying histograms illustrate the ratio between the mean values of NOS protein levels and β-actin marker for the different groups. Atrial eNOS, iNOS and

Table 2 – Echocardiographic data.

	Young Eut		Young Hypo		Adult Eut		Adult Hypo	
	Mean±SEM	CV	Mean±SEM	CV	Mean±SEM	CV	Mean±SEM	CV
LVIDs (mm)	1.77±0,06	0.131	2.87±0,09*	0.122	6.7±0,07†	0.040	10.02±0,07*†	0.027
LVIDd (mm)	5.20±0,16	0.119	5.98±0,17*	0.110	9.97±0,14†	0.054	12.06±0,05*†	0.016
AWTs (mm)	2.87±0,09	0.122	2.1±0,09*	0.166	2.93±0,07	0.092	2.4±0,04*	0.065
AWTd (mm)	1.70±0,08	0.182	1.37±0,04*	0.113	1.73±0,12	0.269	1.65±0,09*	0.212
PWTs (mm)	2.70±0,07	0.100	2.43±0,11*	0.175	2.70±0,09	0.129	2.51±0,04*	0.062
PWTd (mm)	1.83±0,16	0.339	1.50±0,10*	0.258	1.87±0,02	0.041	1.60±0,11*	0.266
EF(%)	95±1	0.041	83±2*	0.093	69±2†	0.112	25±1*†	0.156
FS(%)	66±2	0.117	47±3*	0.247	33±1†	0.118	16,9±1*†	0.231

Eut (euthyroid rats); Hypo (hypothyroid rats); LVIDs (LV internal diameter in systole); LVIDd (LV internal diameter in diastole); AWTs (anterior wall thickness in systole); AWTd (anterior wall thickness in diastole); PWWs (posterior wall thickness in systole); PWTd (posterior wall thickness in diastole); EF (ejection fraction); FS (fractional shortening) and CV (coefficient of variation). Data are mean±SEM; $n=15$; *$P<.05$ vs age-matched Eut rats; †$P<.05$ vs young rats.

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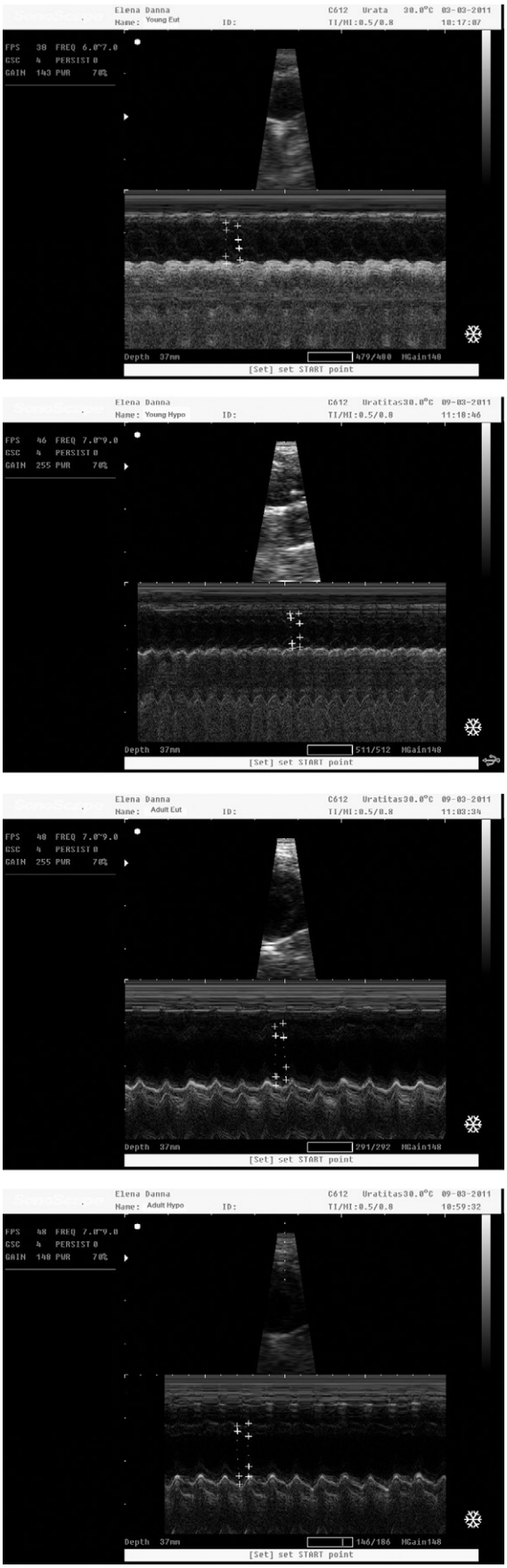

nNOS protein levels were lower in adult animals compared with young ones. Young Hypo rats exhibited decreased protein levels of all three NOS isoforms compared with age-matched Eut rats. By contrast, adult Hypo rats exhibited higher eNOS and iNOS protein levels than adult Eut animals. No differences in nNOS protein levels were observed between adult Eut and Hypo rats. Fig. 2E shows that cav-1 protein levels were higher in adult than in young rats. These protein levels did not change in young Hypo rats but increased in adult Hypo animals compared with age-matched Eut rats. As to cav-3, no differences were observed between young and adult animals. Young and adult Hypo animals showed similar cav-3 protein levels compared with age-matched Eut animals (Fig. 2F).

Fig. 3A illustrates that adult animals exhibited enhanced NOS activity in the LV compared with young rats. Additionally, in the hypothyroid groups, activity of the enzyme was greater than in Eut age-matched rats (Fig. 3A). Figs. 3B and D show that ventricular eNOS and nNOS protein levels were lower in adult animals compared with young animals. iNOS protein levels did not differ between Eut groups of animals but were reduced in adult Hypo rats compared with young animals (Fig. 3C). Hypothyroidism increased iNOS protein levels only in young rats (Figs. 3B, C and D). Ventricular cav-1 and cav-3 protein levels were decreased in adult animals compared with young rats. No significant changes in cav-1 and -3 protein levels were observed in young Hypo animals. However, these protein levels were enhanced in adult Hypo animals compared with age-matched Eut animals (Figs. 3E and F).

In aorta tissue, results show that adult animals exhibited lower aorta NOS activity than young rats (Fig. 4A). This activity was increased in both young and adult Hypo rats compared with Eut animals (Fig. 4A). Aortic eNOS protein levels were lower in adult Eut animals compared with young animals, but no differences were observed between young and adult Hypo animals. eNOS protein levels were increased only in adult Hypo rats, compared with Eut aged-matched animals (Fig. 4B). As to iNOS protein levels, results show that adult and young animals exhibited similar protein levels. Hypothyroidism increased iNOS protein levels only in young animals (Fig. 4C), compared with aged-matched Eut animals. nNOS protein levels did not show positive reactivity in aorta tissue.

Figs. 4D and E show absence of changes in cav-1 and cav-3 in aorta tissues in all experimental groups.

Fig. 5 shows the correlation data of NO-related parameters in young rats. Atrial NOS activity and thyroid hormones levels (Fig. 5A) exhibit a positive correlation ($r=0.975$, $P<.01$). However, a significant negative correlation was observed between LV (Fig. 5B) and aorta (Fig. 5C) NOS activity and thyroid hormones ($r=-0.958$, $r=-0.675$, $P<.01$, respectively). Additionally, the relationship between ventricular NOS activity and EF (Fig. 5D) exhibited a significant negative correlation ($r=-0.877$, $P<.01$). Fig. 6 presents the correlation data for NO-related parameters in adult rats. Atrial NOS activity and

Fig. 1 – Left ventricular representative images of M-mode echocardiographic tracing from euthyroid (Eut) and hypothyroid (Hypo) rats.

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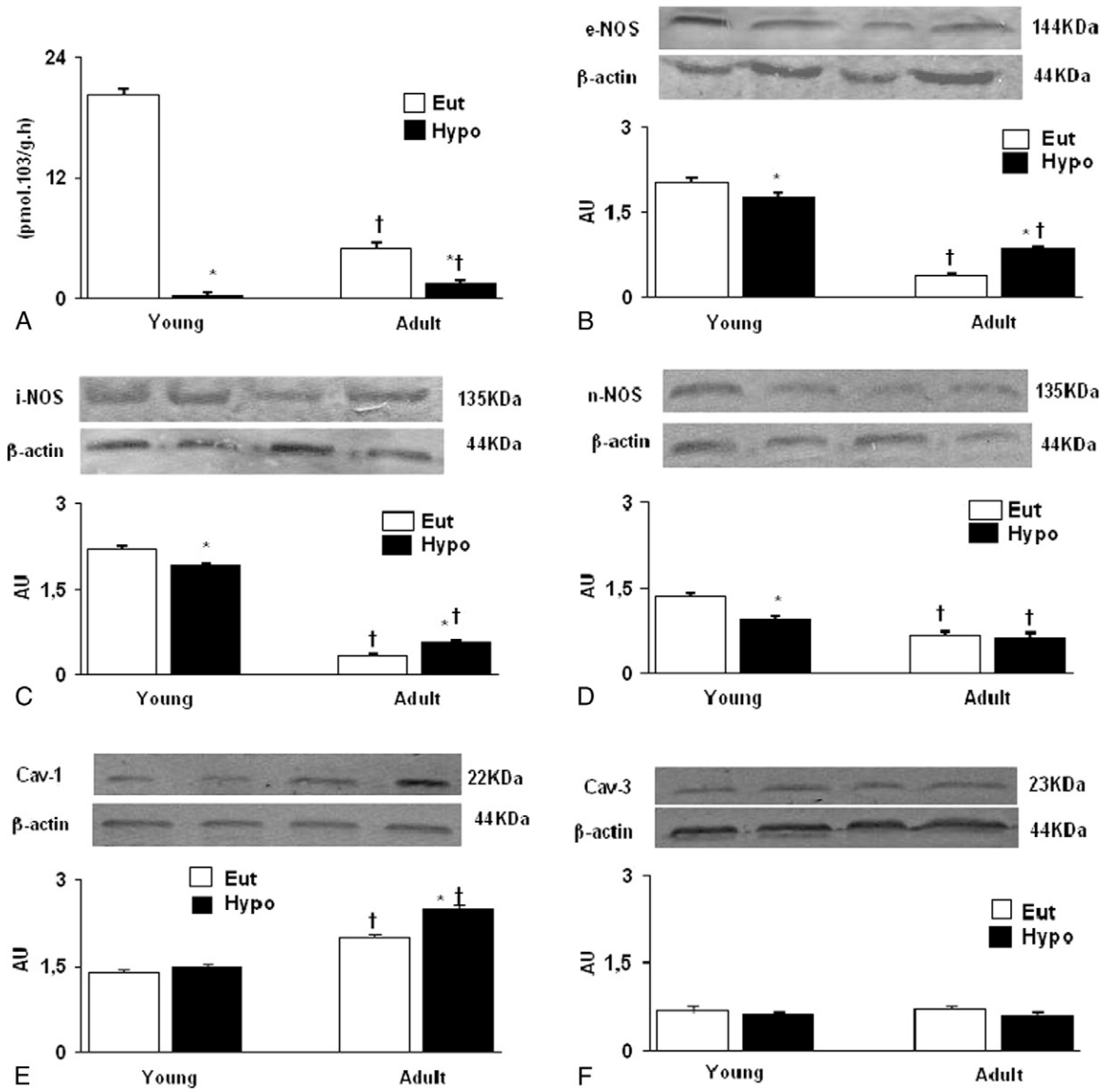

Fig. 2 – Total nitric oxide synthase (NOS) activity in right atria (A) from euthyroid (Eut) and hypothyroid (Hypo) rats. All values are means ± SEM; $n = 7$; *$P < .05$ vs age-matched Eut rats; †$P < .05$ vs young rats. Representative Western blots of eNOS (B), iNOS (C), nNOS (D), caveolin-1 (E) and caveolin-3 (F) carried out on proteins from right atria of Eut and Hypo rats. Histograms illustrate 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; $n = 7$; *$P < .05$ vs age-matched Eut rats; †$P < .05$ vs young rats.

thyroid hormones levels (Fig. 6A) exhibit a positive correlation ($r = 0.980$, $P < .01$). However, a significant negative correlation was observed between LV (Fig. 6B) and aorta (Fig. 6C) NOS activity and thyroid hormones ($r = –0.963$, $r = –0.971$, $P < .01$, respectively). Additionally, the relationship between ventricular NOS activity and EF (Fig. 6D) exhibited a significant negative correlation ($r = –0.962$, $P < .01$).

4. Discussion

Relevance of our findings lies in the possibility of reshaping the notion about the basic mechanisms responsible for the influence of thyroid hormones on cardiovascular function. The present study demonstrates the importance of NO in cardiac dysfunction associated with hypothyroidism in adulthood. Our data show that thyroid hormones regulate cardiovascular NOS, regardless of age, and hypothyroidism would enhance the aging effect in the heart.

In our research, methimazole treatment was effective to establish the hypothyroid state on the basis of T_4 and TSH levels. TSH measurements were lower in adulthood and these findings are in agreement with Moreira et al who showed a clear age-dependent decline in plasma TSH [22]. Although the mechanism governing this decrease is still unknown, the reduction in TSH secretion observed in adult rats may be due to an increased sensitivity of the thyrotrophs to the negative feedback by T_4. Additionally, aging-related occurrence of other

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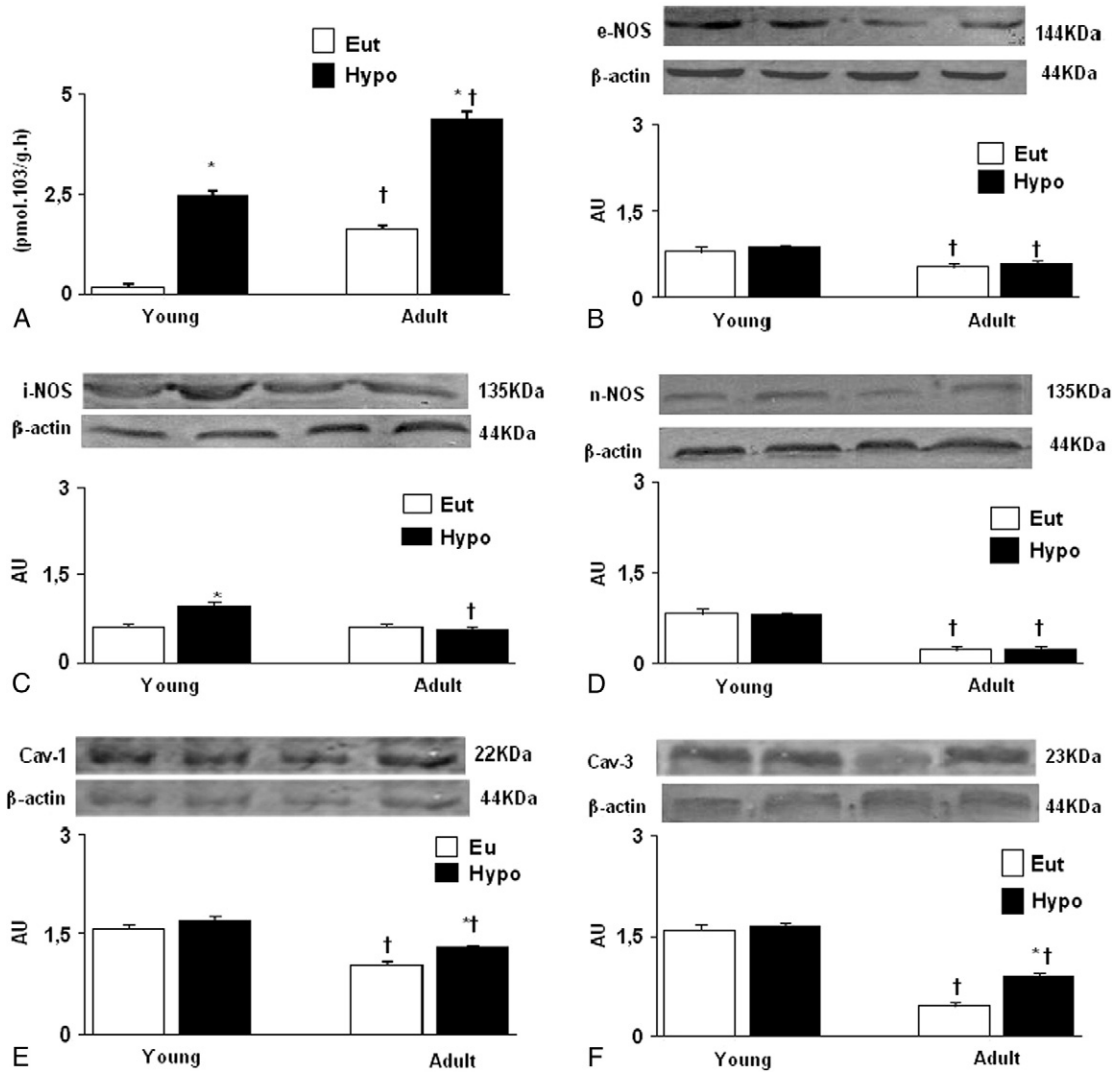

Fig. 3 – Total nitric oxide synthase (NOS) activity in left ventricle (A) from euthyroid (Eut) and hypothyroid (Hypo) rats. All values are means ± SEM; $n = 7$; *$P < .05$ vs age-matched Eut rats; †$P < .05$ vs young rats. Representative Western blots of eNOS (B), iNOS (C), nNOS (D), caveolin-1 (E) and caveolin-3 (F) carried out on proteins from left ventricle of Eut and Hypo rats. Histograms illustrate 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; $n = 7$; *$P < .05$ vs age-matched Eut rats; †$P < .05$ vs young rats.

factors, such as reduced hypothalamic TRH secretion, cannot be excluded.

Hemodynamic changes observed in this study were typical of adult animals and hypothyroidism. These results were consistent with alterations of the cardiovascular function. Our findings show that hypothyroidism enhanced age-related changes in the heart. Additionally, when echocardiographic parameters of both the Eut and Hypo groups were evaluated, our results confirmed that thyroid hormones have an effect on heart contraction and relaxation. In this context, hypothyroidism could be associated with increased prevalence of cardiac heart failure in adulthood. It is well known that, with aging, the cardiovascular system undergoes characteristic changes that involve both the heart and the vasculature, including endothelial dysfunction [23]. Aging is associated with diminished contraction and relaxation response of the heart to β-adrenergic or catecholamine stimulation, increasing myocardial working load [24]. Additionally, several authors have shown that aging induced a downregulation of myocardial thyroid hormone receptor α_1, β_1 signaling-mediated transcription of α myosin heavy chain and sarcoplasmic reticulum Ca^{2+}-ATPase genes, contributing to impairment of cardiac contraction and relaxation [25,26]. On the other hand, although over the last decade NO has been implicated in physiological and pathophysiological processes that control many aspects of myocardial function [27], little is still understood about several aspects of NO signaling in the myocardium. In this study, a statistical correlation between cardiac and aorta NOS activity and thyroid hormone levels has been determined.

With regard to atria tissue, our results show that the reduction of atrial NOS activity associated with aging was

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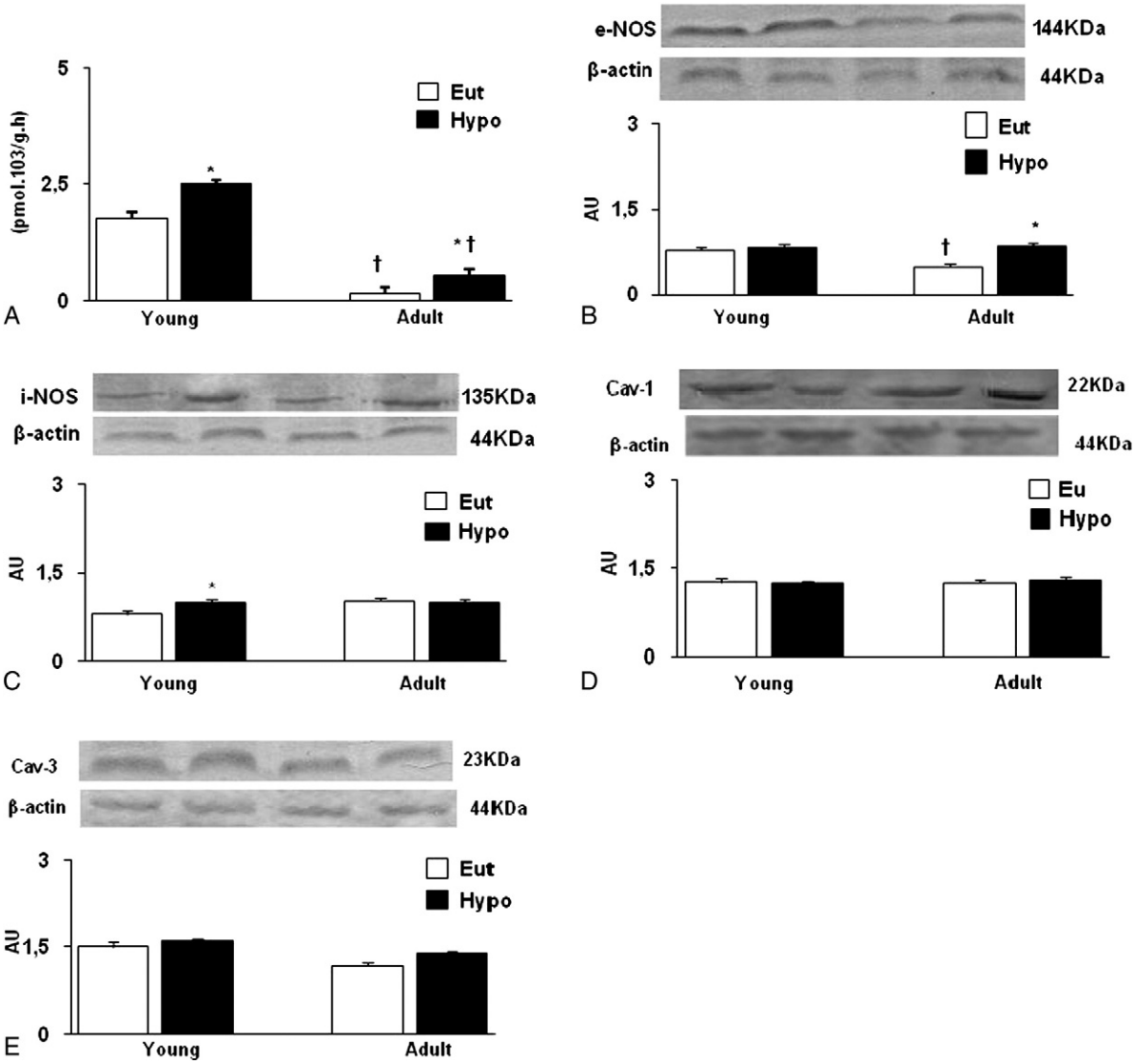

Fig. 4 – Total nitric oxide synthase (NOS) activity in aorta tissue (A) from euthyroid (Eut) and hypothyroid (Hypo) rats. All values are means ± SEM; $n = 7$; *$P < .05$ vs age-matched Eut rats; †$P < .05$ vs young rats. Representative Western blots of eNOS (B), iNOS (C), caveolin-1 (D) and caveolin-3 (E) carried out on proteins from aorta tissue of Eut and Hypo rats. Histograms illustrate 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; $n = 7$; *$P < .05$ vs age-matched Eut rats; †$P < .05$ vs young rats.

enhanced by hypothyroidism and the concept of hormonal regulation of NOS activity has been reported in one of our earlier articles [10]. Further, it was also observed that HR presented a similar pattern of changes as NOS activity. This finding provides strong evidence consistent with the hypothesis that NO would act as a messenger to modulate the heart pacemaker activity [11]. These results are in agreement with those obtained by several authors who have shown that a decreased release of another vasodilator substance, atrial natriuretic peptide, occurs during hypothyroidism [28,29]. When protein levels of different NOS isoforms in atrium of adult Eut animals were analyzed, it was determined that the decreased NOS activity might be due to a reduction in protein levels of the three NOS isoforms. Moreover, adult animals showed an increase in protein levels of cav-1 in this tissue. Considering these results as a whole would explain the decrease in enzyme activity observed in adulthood. Additionally, the reduced NOS activity observed in young Hypo rats was associated with decreased protein levels of all three NOS isoforms, without changes in the negative modulators. In contrast, Hypo adult rats had increased eNOS and iNOS protein levels without changes in the nNOS isoform. However, the reduced NOS activity observed in these animals would be due to higher cav-1 protein levels. Thus, hypothyroidism would modify the expression of the enzyme in young animals and cav-1 protein levels in adulthood. Ratajczak et al have shown that a dissociation of cav from caveolae is associated with aging and heart failure, and this process is related to reduced NOS activity [16,17]. It is important to take into account that further experiments are required to specifically determine the involvement of other positive and negative enzyme modulators in all experimental animal groups.

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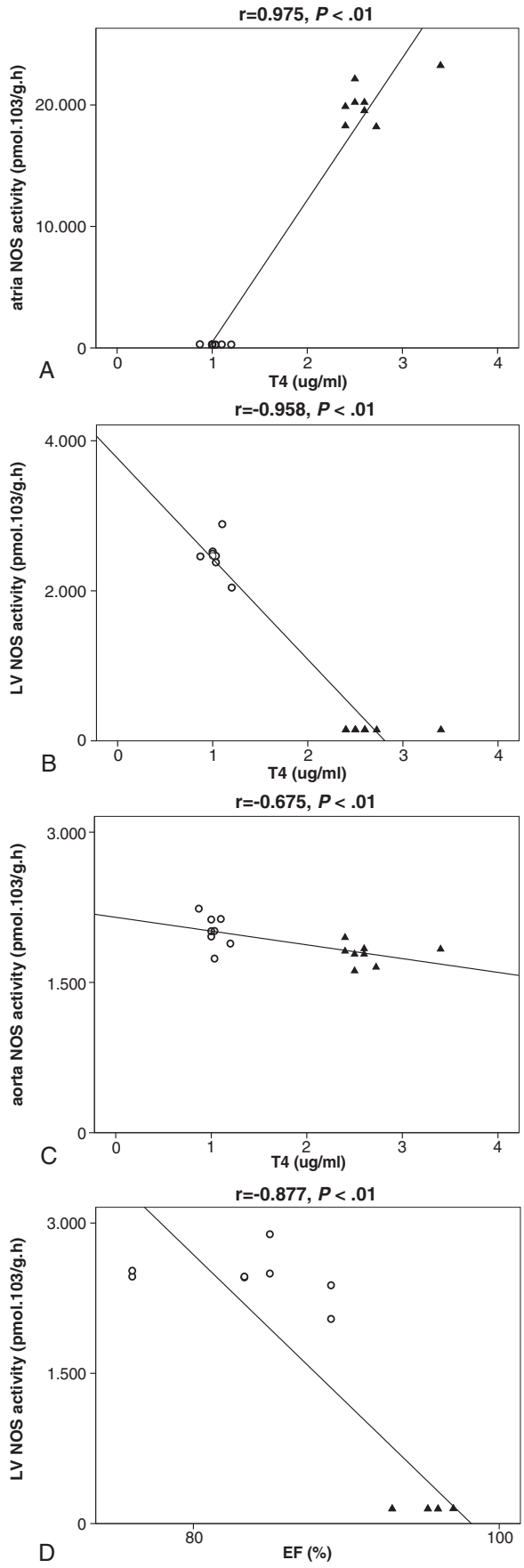

Furthermore, hypothyroidism enhanced the age-related changes in NOS activity in LV tissue. Our findings show that up-regulation of NO production is significantly correlated with increased LV chamber diameter and significantly reduced EF and FS parameters. There are increasing data indicating that NO modulates various components involved in excitation–contraction coupling, including Ca^{2+} influx via the L-type Ca^{2+} channel, Ca^{2+} release from the sarcoplasmic reticulum via the ryanodine receptor Ca^{2+} release channel, and Ca^{2+} reuptake into the sarcoplasmic reticulum via the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) [30]. Therefore, increased NO production would be related to a depression in myocardial contractile function in adulthood, which is enhanced in hypothyroidism. Moreover, higher NOS activity in adult animals would not be due to an alteration in NOS protein levels as this tissue showed a reduction in cav-1 and cav-3 protein levels. Therefore, we posit that age-related changes in ventricle NOS activity would essentially derive from alterations in enzyme activity modulation exerted by both cavs in adulthood. In the hypothyroid groups, the increased activity of the enzyme was accompanied by a rise in inducible NOS isoform protein levels in young Hypo animals. In contrast, although adult animals showed no changes in protein levels of the enzyme they did exhibit an increased expression of both cavs. Once again, modulation of the enzyme activity by cav appears to be more relevant than the alteration of enzyme expression in adulthood.

When evaluating aorta tissue, our results show that a down-regulation of NOS activity was accompanied by a reduction in eNOS activity, without changes in the inducible isoform of the enzyme and cav protein levels in adult rats. The findings about protein expression of this enzyme with aging are controversial. Llorens et al have shown that aging upregulates the NO pathway in structures involved in the regulation of blood pressure like the heart and vessels [31]. However, our results are in agreement with Barton et al who have shown that the release of NO is reduced in aorta of aged rats [32]. Our study suggests that diminished vasodilatation and impaired shear stress-induced NO production would be associated with age-related endothelial dysfunction in rat aorta. The decreased NOS activity observed in adult animals could be involved in the changes in TPVR. Moreover, we have shown that hypothyroidism increases NOS activity both in young animals and in adults. We have previously demonstrated that typical vascular changes associated with hypothyroidism would seem to be inversely correlated with the L-arginine–NO system [9]. The greater activity of the enzyme in the Hypo groups derives from the inducible isoform in young rats and from the endothelial isoform in adult ones. Results observed in young Hypo rats are in disagreement with those of Grieve et al who showed by immunohistochemical analysis that labeling for eNOS may be increased after propylthiouracil treatment, compared to control animals [33]. In addition, our data contrast with the findings of Quesada et al indicating that

Fig. 5 – Correlation between (A) atrial NOS activity, (B) LV NOS activity, (C) aorta NOS activity and thyroid hormones. Correlation between (D) LV NOS activity and EF. ▲ Young Eut rats; ○ Young Hypo rats; $n=8$.

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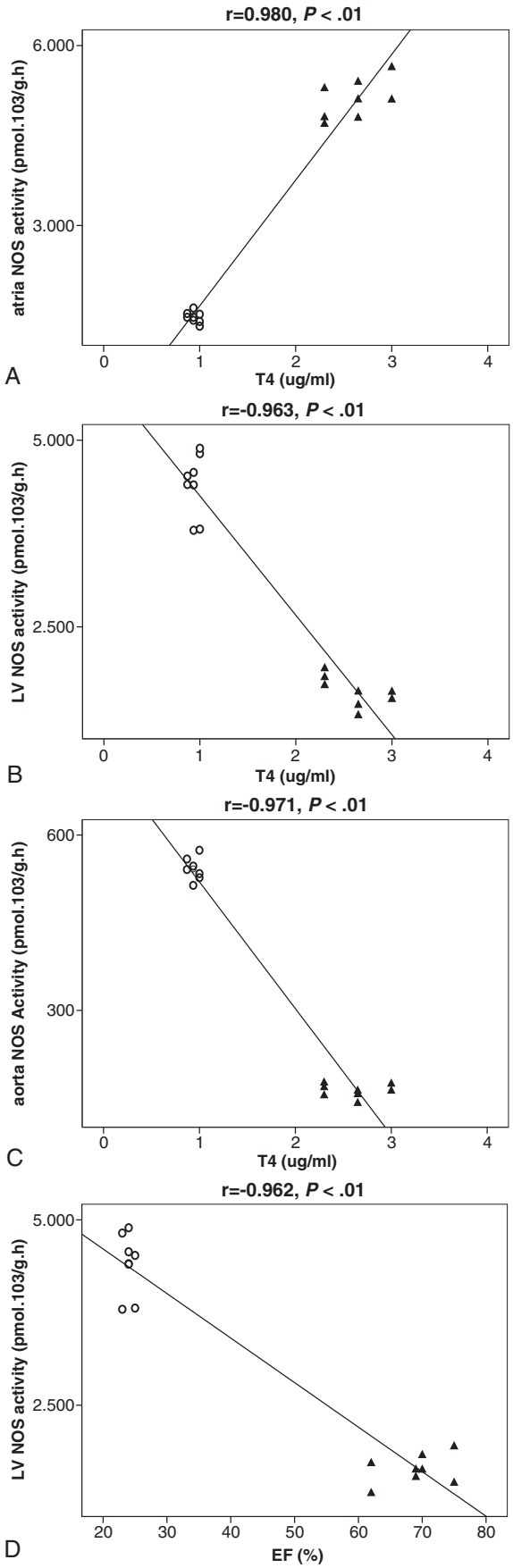

aorta NOS activity is significantly reduced in Hypo animals [34]. This discrepancy could be due to methodological differences between the experiments, as well as to differences in age and strain of animals used. Taking into account NO signaling, we cannot rule out that the cardiovascular dysfunction observed in hypothyroidism would be associated with a reduction in NO bioavailability as a consequence of increased oxidative stress.

Although the mechanism underlying the changes in NOS activity under normal or decreased thyroid levels should be explored, we cannot rule out the possibility that differences observed between tissues may be due to the presence of different thyroid hormone receptors and/or other cofactors of the enzyme [35,36].

5. Conclusions

The present study is, to our knowledge, a novel finding indicating that thyroid hormones would be one of the factors involved in the modulation of cardiovascular NO production and cav-1 and -3 tissue-specific abundance regardless of age. Hypothyroidism appears to impact and/or contribute in a differential way to aging-induced changes in the myocardium and aorta tissues. Although the present work did not allow us to identify the molecular mechanism involved, low thyroid hormones levels would enhance the aging effect in the heart but not in aorta tissues.

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Conflict of interests

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

Fig. 6 – Correlation between (A) atrial NOS activity, (B) LV NOS activity, (C) aorta NOS activity and thyroid hormones. Correlation between (D) LV NOS activity and EF. ▲ Adult Eut rats; ○ Adult Hypo rats; $n = 8$.

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