

# Age-related Influence of Hypothyroidism on Caveolins and Nitric Oxide Modulation

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## ABSTRACT

### Background

Hypothyroidism and aging influence cardiac and renal nitric oxide (NO) synthesis. Caveolins, which are negative modulators of NO synthase (NOS) activity, are affected by both factors.

### Objectives

The aim of this study was to evaluate caveolin (cav) participation in the modulation of renal and cardiac NOS activity in adult hypothyroid animals.

### Methods

Euthyroid and hypothyroid [methimazole 0.02% (v/v) in the drinking water during 28 days] male Sprague-Dawley rats were used. Animals were sacrificed to remove the heart and kidneys.

### Results

Right atrial NOS activity decreased with aging and hypothyroidism. Cav-1 expression increased with aging and hypothyroidism. Conversely, left ventricular NOS activity increased with aging and hypothyroidism while the expression of both caveolin isoforms decreased in adult and hypothyroid groups. In the renal medulla, hypothyroidism reduced NOS activity in young and raised it in adult animals and cav-1 expression decreased with aging and in hypothyroid young animals. Cav-3 protein levels decreased in adult hypothyroid animals.

### Conclusions

Hypothyroidism impacts on NOS activity and on that of its modulators, caveolins, in the cardiovascular and renal systems. Hypothyroidism enhances the effects of aging in both systems.

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**Key words** > Caveolins - Nitric oxide - Hypothyroidism - Aging.

## Abbreviations >

<b>cav-1</b>	Caveolin-1	<b>nNOS</b>	Neuronal nitric oxide synthase
<b>cav-2</b>	Caveolin-2	<b>NO</b>	Nitric oxide
<b>cav-3</b>	Caveolin-3	<b>NOS</b>	Nitric oxide synthase
<b>eNOS</b>	Endothelial nitric oxide synthase	<b>T4</b>	Total thyroxine
<b>iNOS</b>	Inducible nitric oxide synthase	<b>TSH</b>	Thyroid stimulating hormone

## INTRODUCTION

Thyroid hormone deficit impacts on all physiological systems, especially on the cardiovascular and renal systems, (1) inducing hemodynamic changes and altering renal function and sodium and water management. (2-4) Over the course of the last years a functional interaction, involving thyroid hormones, endothelial cells and nitric oxide (NO), has been described. Nitric oxide is synthesized from L-arginine and molecular oxygen by a family of enzymes called NO synthases (NOS). There are three known NOS isoforms in cardiac and

renal tissue: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) isoforms. The nNOS and eNOS isoforms are constitutive and activated by the Ca<sup>2+</sup>/calmodulin complex. Endogenous proinflammatory cytokine and/or endotoxin release stimulate iNOS expression. (5) We have previously shown that thyroid hormones regulate the intrinsic pacemaker activity of the heart through a mechanism that partly involves NO. (6-8) Other studies suggest that hypothyroidism is associated with reduced NO availability and, consequently, with deficient endothelium-dependent vaso-

dilation. (9) This lower vascular response would be ascribed to a dysfunctional signaling NO pathway or altered NOS activity by the regulatory proteins called caveolins. (10, 11) Caveolins are associated to invaginations of the plasmatic membrane called caveolae. Three caveolin isoforms have been described: caveolin 1 (cav-1) and caveolin 2 (cav-2), mainly found in endothelial cells, and caveolin 3 (cav-3), confined to skeletal and cardiac muscle cells. (12). Cav-1 and cav-3 negatively regulate NOS catalytic activity both in the heart and kidney. (13, 14) In the renal tissue, hypothyroidism has been associated with marked changes in renal hemodynamics and tubular function. (15, 16) However, the mechanism through which the altered thyroid state induces these changes has not been clearly established. Madrid et al. have observed that blood flow in the renal medulla is mainly regulated by NO and that NOS activity is markedly higher in the renal medulla than in the cortex. (17)

Moreover, numerous studies have shown that aging would be an essential factor in the development of endocrine disorders, such as thyroid axis variations. (18-20). In addition, changes in caveolin abundance and/or functionality, as well as in caveolin-NOS interaction could be related with NOS activity modifications observed with aging and in altered thyroid state conditions. (21, 22) Therefore, the purpose of this study was to evaluate cardiac and renal caveolin participation in the age-related NO system modulation in hypothyroid animals.

## METHODS

### Animals

All protocol animals were treated according to the National Administration of Drugs, Food and Medical Technology guidelines and rules of the National Ministry of Health and Environment (Provision N° 6344-96). Male Sprague-Dawley rats [2-month old (young) or 18-month old (adult)] from the School of Pharmacy and Biochemistry (Universidad de Buenos Aires) were used. Animals were kept in a humidity and temperature-controlled environment with a 12/12 hour light/darkness cycle. They were fed standard rat chow from Nutrimentos Purina, Buenos Aires, Argentina, and had free access to water until the day of the experiments. Rats were randomly divided into two groups: euthyroid and hypothyroid animals.

### Treatment

Hypothyroidism was induced through treatment with methimazole 0.02% (v/v) in the drinking water during 28 days. (23)

### Treatment efficacy assessment

Change in the thyroid state was assessed with radioimmunoassay measurement of thyroid stimulating hormone (TSH) and total thyroxine (T4) plasmatic levels in samples obtained at the end of the experiment. (24) The kit used to measure TSH was provided by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (Bethesda, EEUU). Results were referred to standard TSH for the rat (rTSH-RP-2). Intra- and inter-assay variation coefficients for TSH changes were 8.7% and 13.4%, respectively.

### Experimental protocol

Following animal euthanasia with an overdose of anesthesia, the heart and both kidneys were removed to separate the right atrium, left ventricle and renal medulla. In these tissues, NOS activity through the conversion of [<sup>14</sup>C (U)]-L-arginine to [<sup>14</sup>C (U)]-L-citruline, (25) NOS isoform protein levels and cav-1 and cav-3 regulatory proteins (Western blot) were assessed.

### NOS activity

Right ventricular, left ventricular and renal medulla tissue homogenates (approximately 50 µg protein) were incubated with Tris-HCl buffer (ph 7.4) containing 1 µg/ml L-arginine, [<sup>14</sup>C (U)]-L-arginine (346 µCi/mL), L-valine (67 mmol/L), NADPH (1 mmol/L), calmoduline (30 nmol/L), tetrahydrobiopterin (5 µmol/L) and CaCl<sub>2</sub> (2 mmol/L) during 60 minutes at room temperature. At the end of the incubation period, NOS activity was interrupted with the addition of a buffer solution containing 20 mmol/L HEPES and 20 mmol/L EDTA at pH 5.5. The reaction mixture was loaded in a cation exchange column (Dowex AG 50W-X8, Na<sup>+</sup> form; Bio-Rad) and [<sup>14</sup>C (U)]-L-citruline was eluted with 0–50 ml ddH<sub>2</sub>O. The amount of [<sup>14</sup>C (U)]-L-citruline eluted was quantified using a liquid scintillation counter (Wallac 1414 WinSpectral; EG&G, Turku, Finland). (25) All components, except [U-<sup>14</sup>C]-L-arginine monochlorhydrate (346 mCi/mmol, Amersham Life Science) were purchased from Sigma Chemie. Protein measurements were performed using the Lowry method, with bovine seroalbumin as standard.

### Western blot

Right atrial, left ventricular and renal medulla samples were homogenized on ice with 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). Homogenate protein concentration was measured using the Lowry assay. Equivalent amounts of protein (100 mg protein/lane) were separated by electrophoresis in 7.5% SDS-polyacrylamide gels (Bio-Rad, Munich, Germany); then they were transferred to a nitrocellulose membrane (Bio-Rad) and incubated with anti-NOS and anti-cav-1 and cav-3 primary antibodies, all in 1:500 dilution. Primary antibodies were: rabbit polyclonal anti-inducible NOS (iNOS) (epitope at the carboxyl terminus); anti-endothelial NOS (eNOS) (epitope at the amino terminus), anti-neuronal NOS (nNOS) (epitope at the amino terminus), and rabbit polyclonal anti cav-1 (H-97, sc-7875) and anti-cav-3 (H-100, sc-28828). Finally, a secondary immunoreaction was performed using a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5000). Samples were revealed by chemoluminescence using ECL reactant for 2–4 minutes. Density of the respective Western blot bands was quantified by densitometry, using a Hewlett-Packard scanner and Totallab analyzer (Biodynamics, Seattle, WA, USA), and protein quantities were calculated comparing with the densitometry values of the corresponding standards. Protein levels were expressed as mean optical density of caveolin isoforms and of the β-actin band (using anti-beta actin, clon EP1123Y, rabbit monoclonal antibody) to control any type of alterations in protein charge.

### Statistical analysis

Tables and Figures are presented as mean ± standard error of the mean. This information was assessed using a random design univariate or multivariate analysis with a two-factor

structure (age and thyroid hormone). ANOVA and MANOVA analyses were performed for each variable, as appropriate. The Levene and Shapiro-Wilk tests were used to assess variance homogeneity and data normality, respectively. In case of correct normality and homogeneity of assumed variances, the Bonferri test was applied. When variance was not homogeneous, multiple comparisons were performed using the Tamhane test. The SPSS version 16.0 statistical package was used to perform all statistical analyses. A p value < 0.05 was considered as statistically significant.

**RESULTS**

**Treatment efficacy**

Methimazole was able to establish the hypothyroid state. As shown in Table 1 adult rats had lower levels of TSH than young ones while T4 levels were not modified by age. In addition, hypothyroid animals presented higher TSH and lower T4 levels than the age-matched euthyroid animals. Moreover, hormone treatment induced body weight changes.

**NOS activity**

Aging reduced right atrial NOS activity in euthyroid animals. Hormone deficit decreased NOS in both age groups. However, adult hypothyroid rats presented greater enzymatic activity than young animals (Figure 1, panel A). Conversely, in the left ventricle, aging increased enzymatic activity in euthyroid animals. Hypothyroidism increased this parameter in both age

groups. Adult hypothyroid animals had greater NOS activity than young animals (Figure 1, panel B). In the renal medulla, aging did not modify enzymatic activity in euthyroid rats and hypothyroidism decreased enzymatic activity in young animals but increased it in adult ones (Figure 1, panel C).

**NOS and caveolin isoform protein levels**

Figure 2 shows Western blot analysis of right atrial samples. Aging decreased protein levels of the three NOS isoforms in the euthyroid group. Hypothyroidism reduced the expression of the three NOS isoforms in young rats, while in adult animals, it only increased eNOS and iNOS levels (Figure 2, Panels A, B and C). Aging resulted in increased cav-1 levels (Figure 2, Panel D), and hypothyroidism induced their increase only in adult rats. Neither aging nor hypothyroidism changed cav-3 protein levels (Figure 2, Panel E). In the left ventricle, aging decreased constitutive NOS isoform levels. In young animals, hypothyroidism increased iNOS, whereas in adult rats no changes were observed in the different isoforms (Figure 3, panels A, B and C). Figure 3, Panels D and E show that aging decreased protein levels of both caveolins. Hypothyroidism increased these levels only in adult animals.

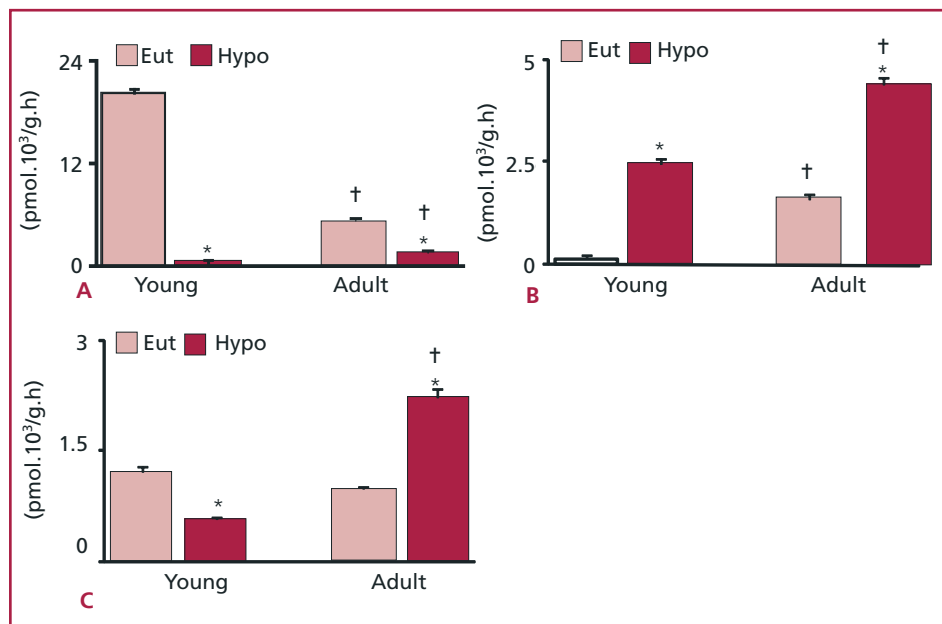
In the renal medulla, aging did not modify the protein levels of any isoform in euthyroid animals.

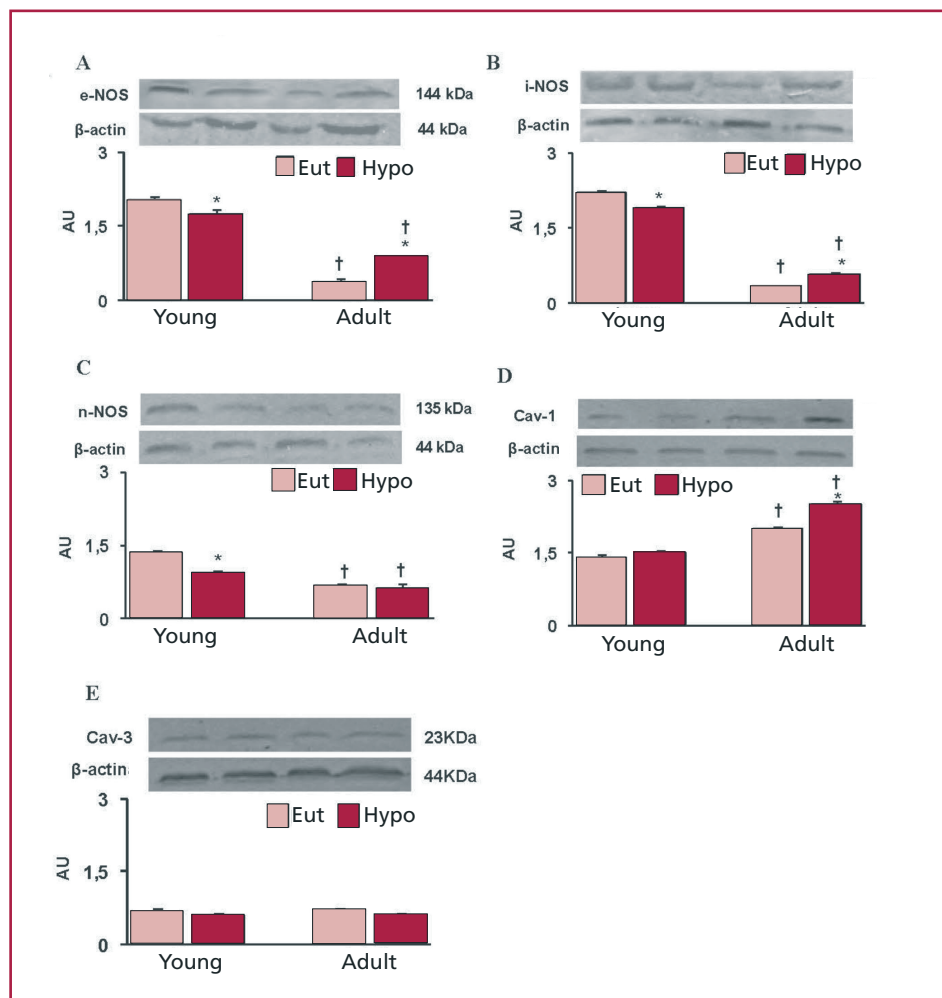
**Table 1.** Biological variables

	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†*
T4 (µg/m)	2.46 ± 0.03	1.03 ± 0.04*	2.25 ± 0.04	0.94 ± 0.04*
BW (g)	337 ± 12	301 ± 11	562 ± 8†	543 ± 10†

Eut: Euthyroid. Hypo: Hypothyroid. TSH: Thyroid stimulating hormone. T4: Thyroxine. BW: Body weight. Results are expressed as mean ± standard error of the mean. n = 15; \* p < 0.05 vs. age-matched euthyroid rats; † p < 0.05 vs. young animals.

**Fig. 1.** Total nitric-oxide synthase (NOS) activity in the right atrium (A), left ventricle (B) and renal medulla (C) of euthyroid (Eut) and hypothyroid (Hypo) rats. Values are expressed as mean ± standard error of the mean; n = 15; \* p < 0.05 vs. age-matched euthyroid rats; † p < 0.05 vs. young animals.





**Fig. 2.** Representative eNOS (A), iNOS (B), nNOS (C), caveolin-1 (D) and caveolin-3 (E) Western blot analyses performed in right atrial tissue samples of euthyroid (Eut) and hypothyroid (Hypo) rats. Histograms illustrate mean values for each group. All experiments were carried out in triplicate. Each measurement was normalized with  $\beta$ -actin expression from the same gel. Values are expressed as mean  $\pm$  standard error of the mean;  $n = 15$ ; \*  $p < 0.05$  vs. age-matched euthyroid rats; †  $p < 0.05$  vs. young animals.

Hypothyroidism decreased eNOS levels in young animals, whereas it did not modify the expression of any NOS isoform in adult ones (Figure 4, panels A, B and C). Aging decreased cav-1 protein levels while hypothyroidism decreased it only in young rats (Figure 4, Panel D). Aging did not change cav-3 expression in euthyroid rats (Figure 4, Panel E), while hypothyroidism only decreased cav-3 levels in adult animals.

## DISCUSSION

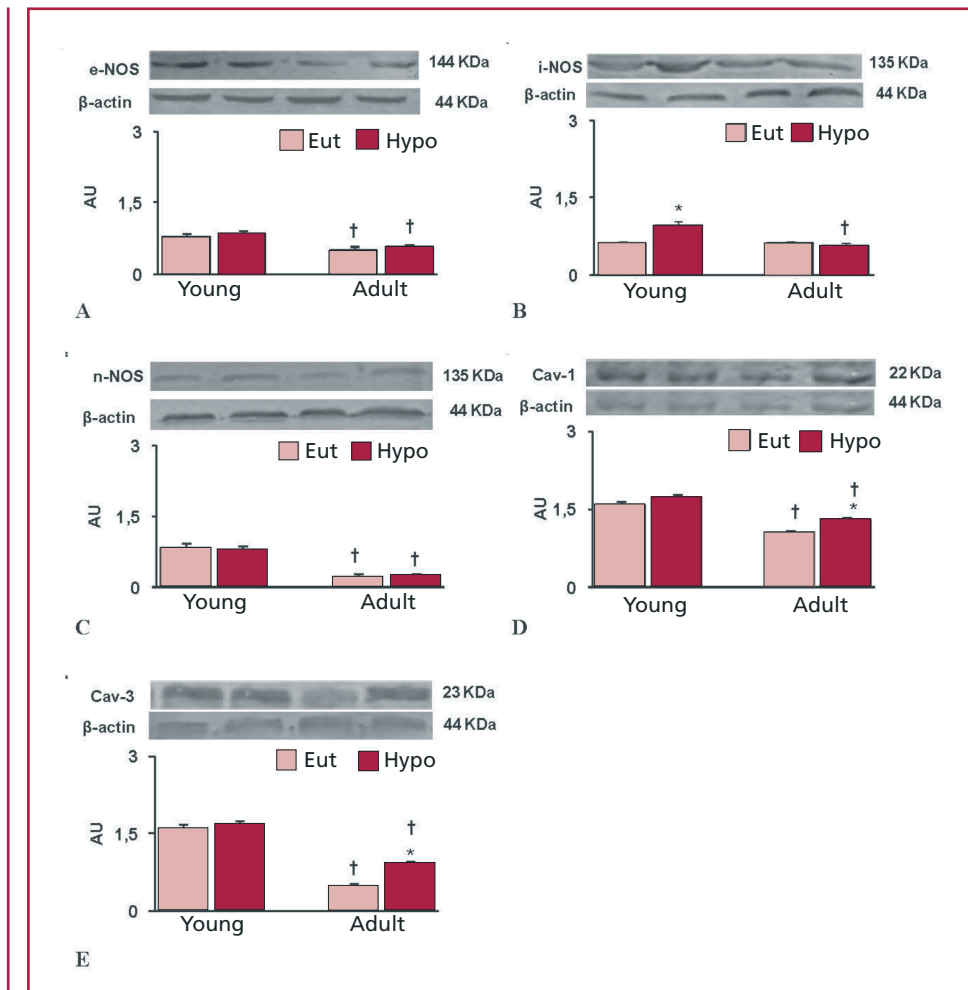
This study shows that NOS system and caveolin involvement in cardiovascular and renal function in hypothyroidism depends on age and type of tissue. The normal aging process occurs with changes at the histological, biochemical and morphological levels in the cardiovascular and renal systems. (26)

Results show that aging decreased TSH plasmatic levels in euthyroid animals. These findings are in agreement with other studies reporting decreased TSH plasmatic levels with aging (27, 28). The decreased TSH secretion in adult animals could be due to increased thyroid cell sensitivity to T4 negative feedback, as well as to lower hypothalamic thyrotropin-releasing hormone (TRH) synthesis.

Moreover, over the last decades NO has been im-

plicated in numerous processes controlling various aspects of cardiovascular and renal function. (29) However, little is known about the mechanism involved in this regulation. In the present work, we showed that aging influences NOS activity. In the right atrium, decreased NOS activity in euthyroid adult animals would be attributed to the reduction in the protein levels of the three NOS isoforms, in accordance with increased cav-1 protein levels. These results are in agreement with those reported by Muñoz et al. showing that aging increases caveolin expression in muscle tissue. (30) In addition, lower NOS activity observed in young animals was associated with decreased protein levels in the three NOS isoforms without changes in the protein levels of negative modulators. Conversely, adult hypothyroid animals presented increased eNOS and iNOS protein levels without nNOS changes. However, reduced enzymatic activity in these animals could be associated to higher cav-1 protein levels. These findings would support hypothyroidism modification of NOS protein levels in young rats and caveolin levels in adult animals. Ratajczak et al. have shown caveolin dissociation from membrane caveolae with aging. (21, 22) In the left ventricle, both aging and hypothyroidism increased NOS activity. This effect is associated

**Fig. 3.** Representative eNOS (A), iNOS (B), nNOS (C), caveolin-1 (D) and caveolin-3 (E) Western blot analyses performed in left ventricular tissue samples of euthyroid (Eut) and hypothyroid (Hypo) rats. Histograms illustrate mean values for each group. All experiments were carried out in triplicate. Each measurement was normalized with  $\beta$ -actin expression from the same gel. Values are expressed as mean  $\pm$  standard error of the mean;  $n = 15$ ; \*  $p < 0.05$  vs. age-matched euthyroid rats; †  $p < 0.05$  vs. young animals.



with decreased eNOS and nNOS as well as cav-1 and cav-3 protein levels. In the young hypothyroid group, increased NOS activity was accompanied by increased iNOS protein levels. Conversely, in adult hypothyroid animals there was greater expression of both caveolins without modifications in the protein levels of NOS isoforms. The findings of the present study would be in agreement with those reported by Quesada et al. showing increased ventricular NOS activity in hypothyroidism. (31) Moreover, Carreras et al. suggest that greater NO synthesis in the liver and muscle of young hypothyroid rats arises mainly from the enzyme inducible isoform. (32) Once more, caveolin modulation of enzymatic activity seems to be more relevant than altered protein expression in adulthood.

Regarding NO effects on renal function (6-8), results show that the NO system in the renal medulla is regulated both by thyroid hormones and aging. In young hypothyroid animals, lower NOS activity could be due to reduced eNOS levels compared with age-matched euthyroid animals. Increased enzymatic activity observed in adult hypothyroid rats would be ascribed to lower cav-1 and cav-3 protein levels, again suggesting that caveolins would play a central role in adulthood. These findings are consistent with the

hypothesis that hypothyroidism would impact differently on renal homeostasis according to age. However, the mechanisms underlying NOS activity changes are still under study, and thus it cannot be ruled out that the differences found in this study could be due to the presence of different thyroid hormone receptors and/or other cofactors or enzymatic modulators. (33, 34).

## CONCLUSIONS

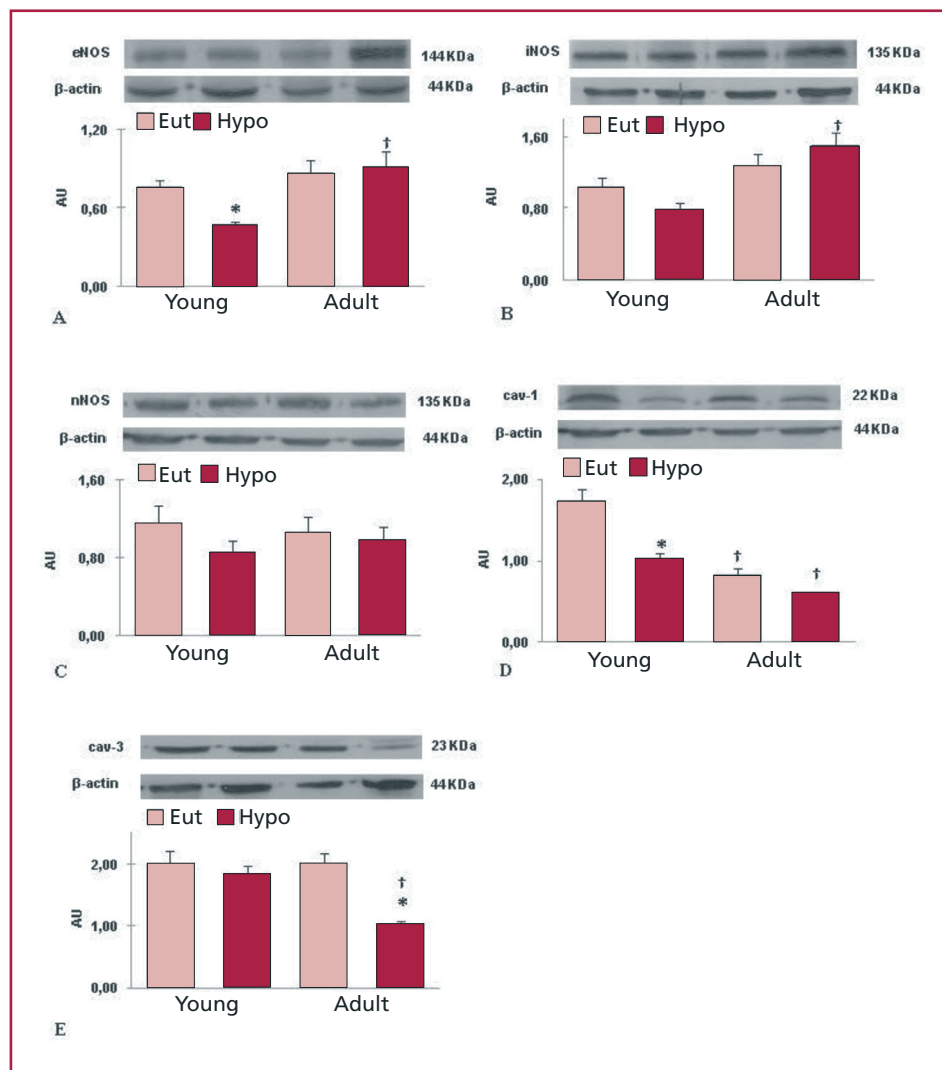
The present study shows relevant results that help understand the NO-hypothyroidism-aging association. We have shown that thyroid hormones regulate the NO and caveolin system, both at cardiac and renal levels and that the effect of hypothyroidism would be age-dependent.

## RESUMEN

### Caveolinas y modulación del sistema del óxido nítrico en el hipotiroidismo según avanza la edad

#### Introducción

El hipotiroidismo y la edad impactan sobre la producción de óxido nítrico (NO) cardíaco y renal. Las caveolinas, moduladores negativos de la actividad enzimática de la NO sintetasa (NOS), se afectan con ambos factores.



**Fig. 4.** Representative eNOS (A), iNOS (B), nNOS (C), caveolin-1 (D) and caveolin-3 (E) Western blot analyses performed in renal medulla tissue samples of euthyroid (Eut) and hypothyroid (Hypo) rats. Histograms illustrate mean values for each group. All experiments were carried out in triplicate. Each measurement was normalized with  $\beta$ -actin expression from the same gel. Values are expressed as mean  $\pm$  standard error of the mean; n = 15; \* p < 0.05 vs. age-matched euthyroid rats; † p < 0.05 vs. young animals.

### Objetivos

Evaluar la implicación de las caveolinas (cav) en la modulación de la actividad de la NOS cardíaca y renal en animales hipotiroideos adultos.

### Material y métodos

Se utilizaron ratas macho Sprague-Dawley eutiroides e hipotiroideas [metimazol 0,02% (v/v) en el agua de bebida durante 28 días]. Los animales fueron sacrificados para extraer el corazón y los riñones.

### Resultados

La actividad de la NOS en la aurícula derecha disminuyó con la edad y el hipotiroidismo. La expresión de cav-1 aumentó con la edad y el hipotiroidismo. La actividad de la NOS en el ventrículo izquierdo aumentó con el avance de la edad y el hipotiroidismo. La expresión de ambas caveolinas disminuyó en los grupos adulto e hipotiroideo. En la médula renal, el hipotiroidismo disminuyó la actividad de la NOS en jóvenes y la aumentó en adultos. La expresión de cav-1 disminuyó con la edad y en jóvenes hipotiroideos. Los niveles proteicos de cav-3 disminuyeron en animales adultos hipotiroideos.

### Conclusiones

El hipotiroidismo impacta sobre la actividad de la NOS y de sus moduladores, las caveolinas, en el sistema cardiovascu-

lar y renal. El hipotiroidismo intensifica los efectos del avance de la edad en ambos sistemas.

**Palabras clave** > Caveolinas - Óxido nítrico - Hipotiroidismo - Edad

### Conflicts of interest

None declared.

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