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Nitric oxide and AQP2 in hypothyroid rats: A link between aging and water homeostasis

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ABSTRACT

Objective. Hypothyroid state and aging are associated with impairment in water reabsorption and changes in aquaporin water channel type 2 (AQP2). Nitric oxide (NO) is involved in AQP2 trafficking to the apical plasma membrane in medullary collecting duct cells. The purpose of this study was to investigate whether aging and hypothyroidism alter renal function, and whether medullary NO and AQP2 are implicated in maintaining water homeostasis.

Materials/Methods. Sprague–Dawley rats aged 2 and 18 months old were treated with 0.02% methimazole (w/v) during 28 days. Renal function was examined and NO synthase (NOS) activity ([^{14}C (U)]-L-arginine to [^{14}C (U)]-L-citrulline assays), NOS, caveolin-1 and -3 and AQP2 protein levels were determined in medullary tissue (Western blot). Plasma membrane fraction and intracellular vesicle fraction of AQP2 were evaluated by Western blot and immunohistochemistry.

Results. A divergent response was observed in hypothyroid rats: while young rats exhibited polyuria with decreased medullary NOS activity, adult rats exhibited a decrease in urine output with increased NOS activity. AQP2 was increased with hypothyroidism, but while young rats exhibited increased AQP2 in plasma membrane, adult rats did so in the cytosolic site.

Conclusions. Hypothyroidism contributes in a differential way to aging-induced changes in renal function, and medullary NO and AQP2 would be implicated in maintaining water homeostasis.

1. Introduction

Thyroid hormones regulate basic metabolism in many organs and cells, having relevant effects on the cardiovascular and renal functions [1–3]. It has been demonstrated that thyroid hormones would be one of the factors involved in the modulation of both cardiovascular nitric oxide (NO) production and negative regulators of NO synthase (NOS) such as caveolin (cav) 1 and 3, regardless of age [4,5]. With regard to renal tissue, it is well known that hypothyroidism is associ-

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; MAP, mean arterial pressure; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; T_4, total thyroxine; TSH, thyroid-stimulating hormone; Ip, Intraperitoneal.

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ated with marked changes in renal hemodynamic and tubular function. Some studies have shown that the major cause of impaired water reabsorption in hypothyroidism is an alteration in renal perfusion, glomerular filtration rate (GFR) and sodium handling, secondary to systemic effects of thyroid hormone deficiency on cardiac output and peripheral vascular resistance [6,7]. However, the specific mechanism whereby hypothyroidism induces alterations in water handling is not yet fully understood. Water movement across cellular membranes is in part mediated by aquaporin water channel type 2 (AQP2), which mediates water permeability across the luminal membrane in late distal tubule and collecting duct [8]. A defective urinary dilution in the hypothyroid state due to alterations in water diffusion in the distal nephron has been reported. In this sense, several authors have shown that impaired urinary diluting capacity would be associated with an upregulation of arginine vasopressin (AVP)-mediated AQP2 expression and membrane trafficking in the inner medulla in the hypothyroid state [9–12]. On the other hand, studies conducted in both humans and experimental animals demonstrate that NO decreases renal vascular resistance and increases GFR, natriuresis and water excretion [13,14]. It is well known that AVP increases collecting duct water permeability by enhancing AQP2 channel insertion in the apical membrane of principal cells through the AVP/cAMP pathway [14]. Moreover, it has been shown that NO donors such as sodium nitroprusside and L-arginine, among others, increase membrane insertion of AQP2 in cultured cells and collecting duct *in vitro* [14,15].

An age-related decline in urinary concentrating ability has been documented in both experimental animals and elderly humans. Senescence processes may reduce the key transport proteins as AQP2 induces changes in water homeostasis [16,17].

Considering that hypothyroidism is one of the major endocrine diseases in adulthood and that it is associated with alterations in renal function, the aim of the present study was to examine both the effects of aging on renal function in hypothyroid rats and the involvement of NO and AQP2 in aging-related renal disorders induced by hypothyroidism.

2. Methods

2.1. Animals

All procedures were reviewed and approved by the National Food, Drug and Medical Technology Administration, 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 "Facultad de Farmacia y Bioquimica" (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 hypothyroid (Hypo). Rats were fed standard rat chow from Nutrimentos Purina (Buenos Aires, Argentina) and received water *ad-libitum*.

2.2. Study design

Rats were rendered hypothyroid after 28 days of treatment with 0.02% methimazole (w/v) in the drinking water [18]. In order to confirm hypothyroidism, serum thyroid-stimulating hormone (TSH), total triiodothyronine (T_3) and thyroxin (T_4) (TSH kit, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, USA) were measured by radioimmunoassay at the end of the experiment [19]. Intra and inter-assay coefficients of variation for TSH were 8.7% and 13.4%, respectively. The same rats were used in an earlier study on the cardiovascular system in hypothyroid rats [4].

2.3. Surgical procedures

Animals were placed in metabolic cages for adaptation to the environment two days before the beginning of the experiments. After the adaptation period, body weight (BW, g), urine volume ($ml \cdot day^{-1} \cdot 100\ g\ BW^{-1}$), water intake ($ml \cdot day^{-1} \cdot 100\ g\ BW^{-1}$), serum and urinary Na^+ ($mEq \cdot L^{-1}$), creatinine ($g \cdot dl^{-1}$) and osmolarity (osm) were measured by standard methods using an autoanalyzer. Creatinine clearance was assessed in order to determine GFR and fractional excretion of sodium (FENa). GFR, FENa, osmolar clearance (Closm) and tubular reabsorption of water (TcH2O) were calculated according to a standard formula. Each rat was then instrumented with a catheter inserted in the right femoral artery to measure mean arterial pressure (MAP). Cannulae were connected to a pressure transducer (Statham P23 ID, Gould Inst. Cleveland, OH) and MAP was recorded with a polygraph (Physiograph E & M, Houston, TX) during 30 min. Heart rate (HR) was derived from the pulsatile pressor signal via tachographic beat-to-beat conversion with a tachograph preamplifier (Coulbourn Instruments, Inc., tachometer S77-26, PA, USA). The Labtech Notebook program (Laboratory Tech., Wilmington, MD) was used for data acquisition. All surgical procedures were performed under aseptic conditions and urethane anesthesia (1.0 g/kg, ip). Throughout the experiment animals were kept under anesthesia by additional small doses of urethane and 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.

2.4. Experimental procedures

Hemodynamic parameters were recorded for 30 min to allow stabilization of MAP and HR. Rats were sacrificed by overdose of anesthesia and both kidneys were removed. Western blotting was performed on this tissue for AQP2, NOS, and cav-1 and -3 proteins, and NOS activity was measured according to the method of the conversion of [^{14}C (U)]-L-arginine to [^{14}C (U)]-L-citrulline (n = 7 rats from each group). Determination of NOS activity and Western blotting were performed as reported previously [4]. Pooled samples (7 rats from each group) 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). Histograms of the ratio between optical densities of NOS,

cav isoforms as well as AQP2 and β-actin band (using anti-beta actin, clone EP1123Y, rabbit monoclonal antibody) were used to check for any inaccuracies in protein loading. All experiments were performed in triplicate.

2.5. Differential centrifugation for AQP2

Differential centrifugation of pooled inner medulla homogenates (n = 7 rats from each group) was performed before the Western blot analysis. Inner medulla homogenates were initially centrifuged at 5000 *g* for 10 min to isolate the nuclear fraction; the supernatant was separated and centrifuged at 11,000 *g* for 10 min to separate mitochondrial organelles. The resulting supernatant was centrifuged twice at 16,000 *g* for 70 min. The pellet (plasma membrane-enriched fraction) was further suspended in buffer homogenate and both the supernatant (intracellular vesicle-enriched fraction) and the plasma membrane-enriched fraction were stored at −70 °C [20,21]. Protein measurement, sample preparation and Western immunoblotting for AQP2 were performed as described previously [4].

2.6. Immunohistochemistry study

Kidneys of experimental rats (n = 4 rats from each group) were perfused with saline solution through the left ventricle until they were free of blood. Decapsulated kidneys were cut longitudinally and fixed in phosphate-buffered 10% formaldehyde (pH 7.2), and embedded in paraffin. Paraffin sections were cut at 3 μm, deparaffinized and hydrated. Endogenous peroxidase activity was blocked by treatment with 0.5% H_2O_2 in methanol for 30 min. A polyclonal antibody against AQP2 at a dilution of 1:100 was used [22].

2.7. Immunofluorescence staining method

Kidneys of experimental rats (n = 4 rats from each group) were perfused with saline solution through the left ventricle until they were free of blood. Decapsulated kidneys were cut longitudinally, fixed in phosphate-buffered 10% formaldehyde (pH 7.2) and embedded in paraffin. Paraffin sections were cut at 3 μm, deparaffinized and hydrated. After serum blocking, sections were incubated overnight at 4 °C with primary antibody against AQP2 at a dilution of 1:100. Sections were then washed and incubated for 30 min at room temperature with fluorescent secondary antibody rhodamine-labeled goat anti-rabbit IgG (dilution 1:50). The counterstain was made using DAPI for 30 min at room temperature. Finally, the slides were mounted in DAKO fluorescent mounting medium before observation under a fluorescence microscope (Olympus BX 51).

3. Materials

The antibodies for the NOS isoforms (iNOS (610333), eNOS (610298) and nNOS (610311)) were supplied by BD Biosciences; anti β-actin by Millipore (04-1116); antibodies anti-cav-1 (sc-7875) and -3 (sc-28828) and anti-AQP2 (H-40):sc-28629, by Santa Cruz Biotechnology, CA, USA; secondary antibody (170-6515), by Bio-Rad Laboratories; Western Blot Detection System and Hybond-ECL membranes by Amersham Pharmacia Biotech; biochemicals by Sigma Chemical (Saint Louis, MO, USA); L-[14C (U)] arginine substrate by Perkin Elmer Life and Analytical Sciences, Boston, MA, USA. A Wallac 1414 WinSpectral (EG&G, Turku, Finland) liquid scintillation counter was used and AG 50 W-X8 cation exchange resin was

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_3 (ng/dl)	87.3 ± 7.9	65.3 ± 6.1*	95.2 ± 6.3	71.2 ± 6.9*
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†
KW/BW (mg/g)	4.40 ± 0.18	3.26 ± 0.19*	3.70 ± 0.12†	3.32 ± 0.09
MAP (mmHg)	90 ± 1	58 ± 1*	65 ± 1†	48 ± 1*,†
HR (bpm)	346 ± 1	211 ± 1*	303 ± 1†	200 ± 1*,†
Posm (mEq/l)	313 ± 9.84	324 ± 20.4	327 ± 10.3	334 ± 16.2
Serum Na^+ (mEq/l)	144 ± 0.78	142 ± 0.40	141 ± 0.63	141 ± 0.37
GFR (ml/min·g BW)	0.0019 ± 5.6^{-5}	0.0025 ± 8.3^{-5}*	0.0020 ± 4.3^{-5}	0.0012 ± 7.3^{-5}*,†
Urine volume (ml/100 g·day)	3.10 ± 0.26	4.87 ± 0.28*	2.91 ± 0.21	2.14 ± 0.07*,†
Water intake (ml/100 g·day)	12.13 ± 0.63	12.09 ± 0.65	7.58 ± 0.48†	4.94 ± 0.25*,†
Uosm (mEq/l)	2110 ± 75	2160 ± 125	1740 ± 90†	2075 ± 48*
Urine Na^+ (meq/l)	155 ± 13	286 ± 21*	147 ± 16	235 ± 14*
FENa %	1.15 ± 0.14	3.17 ± 0.35*	1.13 ± 0.21	2.59 ± 0.01*
Closm (ml/min·100 g BW)	0.015 ± 0.009	0.018 ± 0.002	0.010 ± 0.006†	0.009 ± 0.007†
TcH2O (ml/min·100 g BW)	0.013 ± 0.001	0.014 ± 0.002	0.0085 ± 0.001†	0.0072 ± 0.001†

Eut (euthyroid) rats; Hypo (hypothyroid) rats; TSH (thyroid-stimulating hormone); T3 (total triiodothyronine); T4 (total thyroxin); BW (body weight); KW/BW (kidney weight/body weight), MAP (mean arterial pressure); HR (heart rate); Posm (plasma osmolarity), Serum Na + (Serum sodium), GFR (glomerular filtration rate), Uosm (urine osmolarity), urine Na + (urine sodium), FENa% (fractional sodium excretion), Closm (osmolar clearance), TcH2O (tubular reabsorption of water). Data are mean ± SEM; n = 15.

* $P < 0.05$ vs. age-matched Eut rats.

† $P < 0.05$ vs. young rats.

supplied by BIO-RAD Laboratories. Immunostaining was performed with an avidin–biotin–peroxidase complex technique (Vectastain ABC kit; Universal Elite, Vector Laboratories, CA, USA). The fluorescent secondary antibody used in the immunofluorescence staining was rhodamine-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology, San Diego, CA, USA). DAKO fluorescent mounting medium was supplied by North American.

3.1. *Ethical approval for animal experimentation*

Animals were cared for according to regulation 6344/96 of Argentina's National Drug, Food and Medical Technology Administration 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.

3.2. *Statistical analysis*

Data are mean values ± SEM. ANOVA was performed for each variable. Levene's and Shapiro–Wilk's tests were used to evaluate homogeneity of variances and normality of data, respectively. When assumptions of normality and homogeneity of variances 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. All statistical procedures were performed using the SPSS statistical software package, version 16.0. Statistical significance was set at $P < 0.05$.

4. Results

4.1. *Study design efficacy*

Methimazole was effective in inducing a hypothyroid state. Table 1 shows that TSH levels were lower in adult rats compared with young animals, while serum T_3 and T_4 levels remained unchanged with aging. Additionally, Hypo animals had higher serum TSH levels and lower serum T_3 and T_4 levels than age-matched Eut rats.

4.2. *Measurements*

Table 1 shows the effects of hypothyroidism on different parameters measured. Adult Eut animals exhibited a reduction in the kidney weight (KW)/ body weight (BW) ratio compared with young Eut rats and young Hypo rats showed a reduction compared with age-matched Eut animals, but no changes were observed in adult Hypo rats which showed a similar ratio as young Hypo animals. In addition, MAP and HR

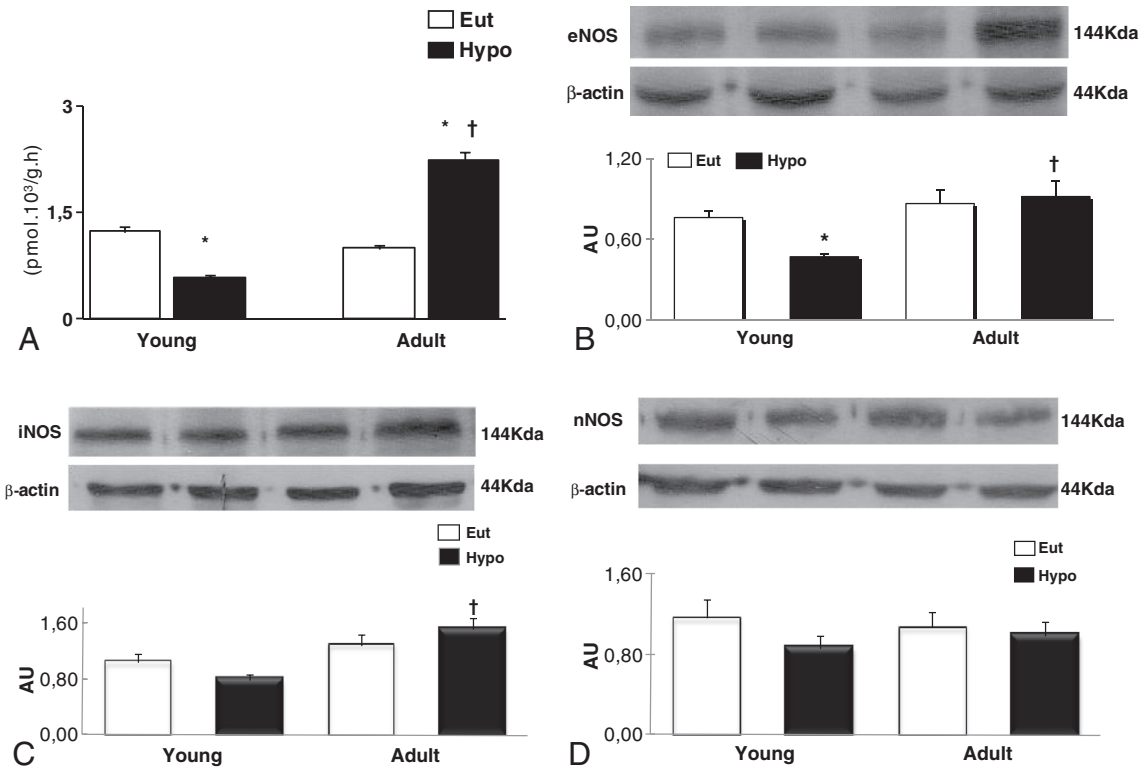

Fig. 1 – Total nitric oxide synthase (NOS) activity in kidney medulla (A) from euthyroid (Eut) and hypothyroid (Hypo) rats. Representative Western Blots of eNOS (B), iNOS (C) and nNOS (D) carried out on proteins from kidney medulla of Eut and Hypo rats. Histograms illustrate mean NOS protein values for each group. All experiments were performed in triplicate. Each blot was normalized to the expression of β-actin from the same gels. Data are mean ± SEM.; n = 7; *P < 0.05 vs. age-matched Eut rats; †P < 0.05 vs. young rats.

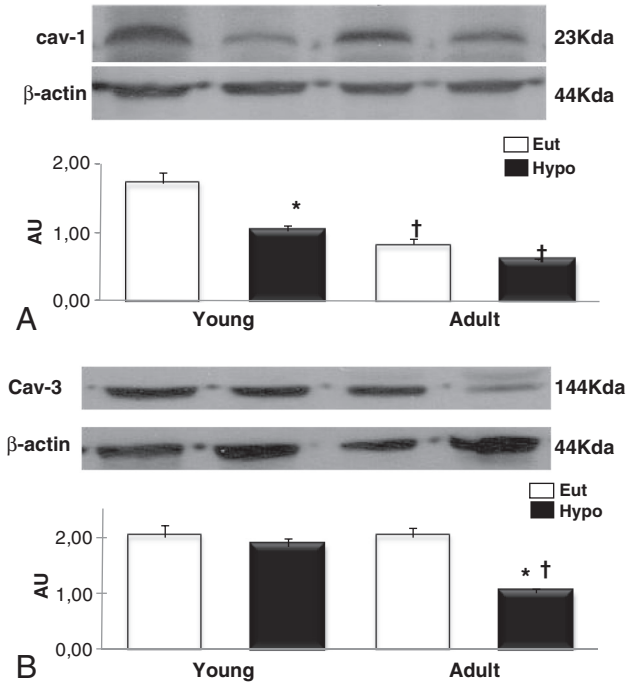

Fig. 2 – Representative Western Blots of caveolin-1 (A) and caveolin-3 (B) carried out on proteins from kidney medulla of Eut and Hypo rats. Histograms illustrate mean caveolin protein values for each group. All experiments were performed in triplicate. Each blot was normalized to the expression of β-actin from the same gels. Data are mean ± SEM; n = 7; *P < 0.05 vs. age-matched Eut rats; †P < 0.05 vs. young rats.

were lower in adult animals compared with young ones, and both groups of Hypo rats exhibited lower MAP than age-matched Eut rats. With regard to the chronotropic response, young and adult Hypo animals showed a decrease in HR compared with Eut rats. Thus, these findings were typical of adult animals and hypothyroidism, and they were consistent with the alterations in cardiovascular function previously reported [4]. Given that cardiac and renal functions are closely linked, the effects of aging on renal function in hypothyroid rats were also studied. No changes in plasma osmolarity (Posm) or serum Na^+ were observed in any experimental group and these results would suggest that animals were in a normal euvolemic state. GFR did not change in adult Eut rats compared with young Eut rats but, while young Hypo rats showed higher GRF than young Eut animals, adult Hypo animals showed decreased GFR when compared with both adult Eut rats and young Hypo ones. Table 1 further shows that urine volume did not change in adult Eut rats when compared with young ones, although they exhibited a lower water intake, but it increased in young Hypo animals although there were no differences in water intake. Additionally, adult Hypo rats showed a reduction in urine volume and water intake compared with both age-matched Eut animals and young Hypo rats. Urine osmolarity (Uosm) decreased in adult Eut animals compared with young Eut rats, no changes were observed in young Hypo rats compared with young Eut ones and it increased in adult Hypo rats compared with age-matched Eut animals. No differences between Hypo animals were detected. Urine Na^+ excretion and FENa did not change with aging, but young and adult Hypo rats showed increased values compared with age-matched Eut animals. Adult Eut and Hypo rats exhibited lower Closm than young ones but low thyroid hormones levels did not change Closm when compared with age-matched Eut animals. Decreased TcH2O was observed with aging in both Eut and Hypo rats compared with young ones, but no differences were detected between Hypo and age-matched Eut animals. In sum, these results would indicate impaired urinary concentration in adult Eut rats. Hypothyroidism differentially affects renal function depending on the age of the animals studied.

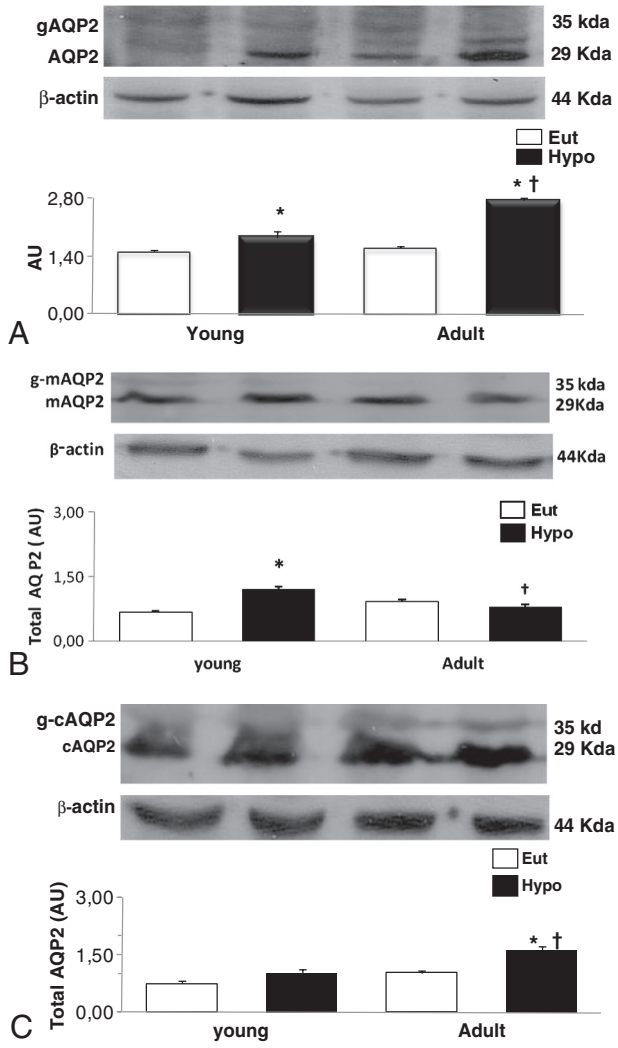

Fig. 3 – Representative Western Blots of AQP2 in total kidney medulla (A), AQP2 in plasma membrane (B) and AQP2 in intracellular vesicle (C) carried out on proteins from kidney medulla of Eut and Hypo rats. Histograms illustrate mean AQP2 protein values for each group. All experiments were performed in triplicate. Each blot was normalized to the expression of β-actin from the same gels. Characteristic form of glycosylated AQP2 (gAQP2) is observed at 35 kDa and of non-glycosylated (AQP2) at 29 kDa. Data are mean ± SEM; n = 7; *P < 0.05 vs. age-matched Eut rats; †P < 0.05 vs. young rats.

4.3. *Nitric oxide synthase*

Fig. 1A illustrates total NOS activity in renal medulla in both young and adult Eut and Hypo rats (Fig. 1A). The results show that aging did not change NOS activity in Eut rats. Hypothyroid state decreased renal medullary NOS activity in young rats and increased it in adult ones. Adult Hypo rats exhibited higher NOS activity than young Hypo ones. Fig. 1 (B, C and D) illustrates representative Western blot analysis of this tissue from all groups. The accompanying histograms illustrate the ratio between mean values of NOS protein levels and β-actin marker for the different groups. No changes were observed in renal medullary NOS protein levels in adult Eut animals. Young Hypo animals showed a decrease in eNOS protein levels, without changes in the other NOS isoforms. Hypothyroidism did not change levels of NOS proteins in the adulthood. Adult Hypo rats showed increased eNOS and iNOS and no changes in nNOS protein levels compared with young Hypo rats (panels B, C and D). Fig. 2 (A and B) illustrates representative Western blot analysis of cav protein levels, known as a negative regulator of NOS enzymes. The accompanying histograms illustrate the ratio between mean values of cav protein levels and β-actin marker for the different groups. Fig. 2A shows that cav-1 protein levels decreased with advanced age. Hypothyroidism decreased cav-1 in young rats but no changes were observed in adult Hypo animals compared with age-matched Eut ones (panel A). Fig. 2B shows that adult Eut animals presented similar cav-3 protein levels as young ones. Hypothyroidism only decreased cav-3 protein levels in adult rats. Adult Hypo rats showed decreased cav-3 protein levels compared with young ones (panel B). Hypothyroidism differentially affected the renal NO system depending on the age of the animals studied.

4.4. *Renal water channel AQP2 in young and adult rats*

Fig. 3 shows total inner medullary AQP2 protein levels (panel A), plasma membrane fraction (panel B) and intracellular vesicle fraction (panel C) of AQP2. Analysis of total AQP2 showed no changes in the level of this protein between young and adult Eut animals but hypothyroidism increased the level of this protein (panel A) and adult Hypo rats showed an increase of this channel compared with young Hypo rats. Fig. 3B shows that aging did not modify levels of this protein.

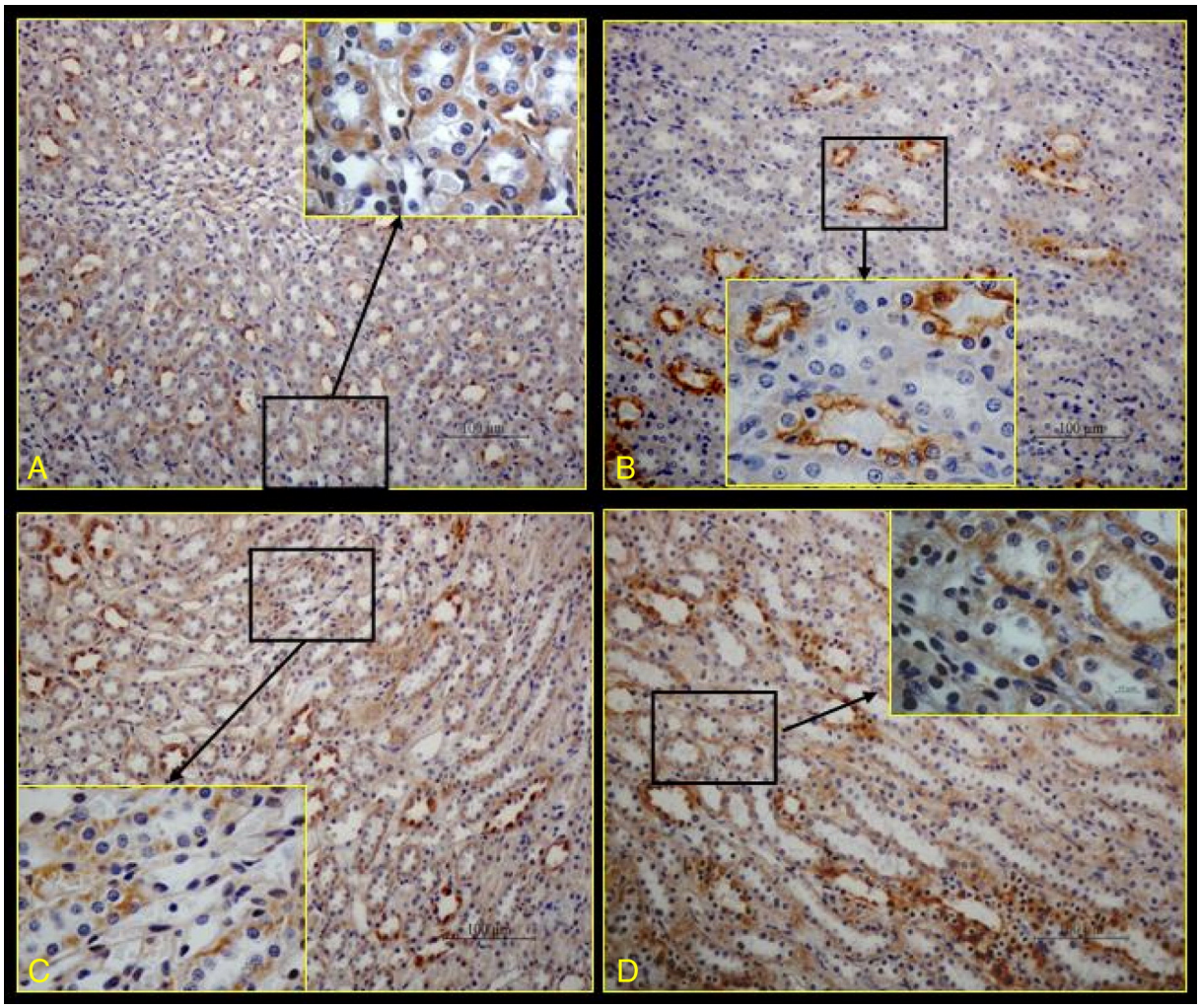

Fig. 4 – Representative micrographs showing immunohistochemical localization of AQP2 in collecting duct cells in sections of paraffin-embedded kidneys from young Eut (A), young Hypo (B), adult Eut (C) and adult Hypo (D) rats. n = 4, original magnification × 200, high magnification pictures × 1000.

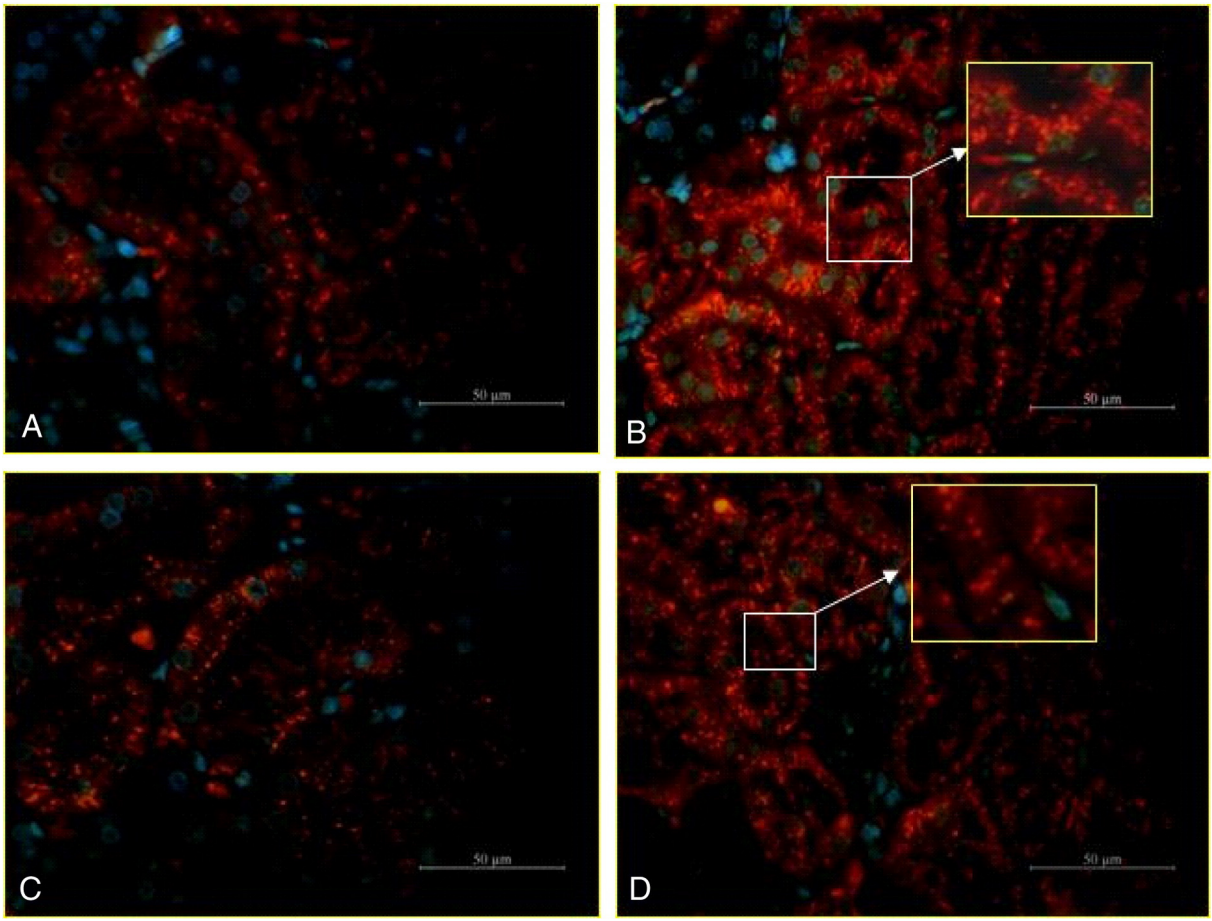

Fig. 5 – Representative micrographs showing immunofluorescence localization of AQP2 in collecting duct cells in sections of paraffin-embedded kidneys from young Eut (A), young Hypo (B), adult Eut (C) and adult Hypo (D) rats. n = 4, original magnification × 400, high magnification pictures × 1000.

Young Hypo rats exhibited increased plasma membrane fraction of AQP2 compared with age-matched Eut animals but no differences were observed in adult Hypo rats. Adult Hypo rats exhibited lower AQP2 protein levels than young Hypo animals (panel B). Fig. 3C shows that the intracellular vesicle fraction of AQP2 protein levels remained unchanged in adult Eut animals. No differences were observed in young Hypo animals compared with young Eut rats. By contrast, adult Hypo rats showed higher protein levels than both adult Eut animals and young Hypo rats (panel C).

Fig. 4 (A–D) shows AQP2 immunohistochemical analysis. No differences were detected between Eut animals. By contrast, labeling differed in the hypothyroid state. Much more intense immunostaining of AQP2 was observed in the apical membrane of principal cells of the renal collecting duct in young Hypo rats when compared with Eut ones. On the other hand, adult Hypo animals displayed intense positive immunostaining for AQP2 in cytoplasm localization. Additionally, immunofluorescence staining (Fig. 5, A–D) results were similar to those of immunohistochemical analysis and showed that AQP2 expression was increased in both hypothyroid groups of rats. Specifically, young rats exhibited increased AQP2 expression in the membrane fractions, whereas adult rats showed increased AQP2 expression in the intracellular vesicle fractions.

5. Discussion

The present study provides new evidences that hypothyroidism contributes in a differential way to aging-induced changes in renal function. Moreover, medullary NO and AQP2 water channel are involved in compensatory mechanisms to maintain body water homeostasis. In this study, methimazole effectively induced hypothyroidism, shown by increased TSH levels, lower T_4 levels and a reduced KW/BW ratio. The lower TSH levels observed with aging in this study are in agreement with Moreira et al.'s findings showing a clear age-dependent decline in plasma TSH [23]. Although the mechanism driving this decrease is still unknown, the reduction in TSH secretion observed in adult rats may be due to increased sensitivity of the thyrotrophes to the negative feedback of T_4. Additionally, aging-related occurrence of other factors, such as reduced hypothalamic TRH secretion, should not be excluded.

Kidney is a target tissue for thyroid hormones that may contribute to the progression of age-related thyroid hormones modifications [24]. With regard to renal function, impact of aging on GFR is quite variable in rats and depends on strain, gender and environmental conditions [25]. GFR did not change in adult Eut rats compared with young ones. Additionally, in the present study, urine output and Posm remained un-

changed in adult Eut rats compared with young ones, while Uosm, Closm and water intake decreased. Urine sodium and FENa remained unchanged in adult rats, which indicates an alteration in the urine concentration mechanism of aging rats. This finding is in concordance with Sand et al.'s, who showed that urine concentrating ability is reduced during normal aging in both people and rats [17]. This reduced ability to concentrate urine would not be related to either the medullary NO system or AQP2 water channel. This alteration could be due to the abundance of many other transport proteins that contribute to urine concentrating ability in this experimental age group. Further experiments are required to specifically determine which transport proteins are involved.

Regarding thyroid disorders, it is well known that the hypothyroid state can lead to a decrease in renal blood flow, GFR and solute-free water excretion [6,7]. Impaired urinary dilution leading to water retention and hyponatremia was reported in patients with hypothyroidism [26]. Interestingly, this study shows that Hypo animals exhibited a biphasic response in renal function with aging. Young rats exhibited polyuria without changes in water intake, together with higher GFR. Notably, these alterations were not of sufficient magnitude to lead to hypo/hypernatremia and/or modifications in Posm. However, these animals showed increased urine sodium excretion and FENa, without changes in Uosm and Closm. By contrast, adult Hypo rats showed reduced urine output accompanied by reduced water intake and GFR, demonstrating a decline in renal function. However, these changes were not sufficient to produce alterations in serum parameters, indicating absence of hypovolemia despite the diminished thirst response. Once again, the deleterious effects of hypothyroidism seem to be more pronounced with aging [4]. Increased Uosm observed in adult rats was not of sufficient magnitude to induce substantial modifications in osmolar clearance. This rise could be due to the increase in sodium excretion and FENa observed in these animals. Thus, young and adult Hypo rats did not exhibit alterations in urine concentrating ability, although they did exhibit urinary sodium loss. A potential cause for this sodium loss could be reduced activity of Na^+/K^+-ATPase, with a decline of ion gradient driving forces as reported in proximal tubules and collecting ducts.

On the other hand, it is well recognized that NO is involved in sodium and water homeostasis, and this system would be affected by thyroid hormones as well as by aging [27,28]. The present study's findings suggest that hypothyroidism induces a biphasic response in NOS activity, opposite to urinary output depending on the age of the animal, in order to maintain water homeostasis. Western blot analysis showed that changes in medullary NOS activity could be due to lower eNOS protein levels in young Hypo rats. However, the rise in NOS activity in adult Hypo animals could be due to lower cav-3 proteins levels. As demonstrated previously in heart, the regulation of NOS activity in young and adult Hypo rats would be due to NOS isoforms and cav, respectively [4]. Modulation of the enzyme's activity by cav appears to be more relevant than alteration of enzyme expression in adulthood. However, we cannot rule out the possibility that other cofactors and/or modulators of the enzyme activity could be implicated.

On the other hand, the results show an increase in AQP2 protein levels in both young and adult Hypo rats. These results are in agreement with the findings of several authors who have shown an upregulation of this channel associated with hypothyroidism [9,10,12]. Schrier reported an AQP2 upregulation water channel expression and trafficking to the apical membrane of the principal cells of the collecting duct in hypothyroid patients [29]. However, this protein was differentially located in the principal cells of renal collecting duct depending on age. It is interesting to note that AQP2 water channel is mainly localized in the apical membrane in young Hypo animals. On the other hand, the location of these channels in adult Hypo rats was mainly established in the cytoplasm, suggesting that they would be a non-functional water channel. These findings are unexpected because urine volume was increased in young animals but decreased in adult ones, while AQP2 recruitment to the membrane was greater in young rats than in adult ones. In agreement with our data, Klokkers et al. have reported that, in AVP-treated cells, AQP2 was predominantly localized in the plasma membrane and that, after additional incubation with cGMP-elevating agents like NO, AQP2 was mostly localized in the cytosol, indicating an increased retrieval of AQP2 from the plasma membrane by NO [30]. Moreover, Garcia et al. found that inhibition of AVP-stimulated water permeability by nitric oxide in the collecting duct would be the result of activation of cGMP-dependent protein kinase, which in turn decreases intracellular cAMP [31].

Additionally, a similar relationship between NO and AQP2 water channel has also been found in the inner medulla, but in this case in an experimental model of hypovolemia induced by hemorrhage [32]. Thus, NO levels would be involved into the potential linkage between aging, hypothyroidism and water handling.

In conclusion, the present study shows data relevant to understanding the association between NO-AQP2-hypothyroid damage in the regulation to water homeostasis. The variables analyzed show an integrative view of the potential mechanisms that determine the renal abnormalities in renal water and sodium handling of hypothyroid rats. These findings provide strong evidence consistent with the hypothesis that hypothyroidism impacts renal parameters in different ways with aging, and medullary NO and AQP2 would be implicated in the compensatory mechanisms to maintain water homeostasis.

Authors' contributions

All authors have 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.

Ana Uceda and Mariana Feldman took part as technical assistants.

Dr. Jorge E Toblli and Dr. Ana Maria Balaszczuk contributed to manuscript correction, reviewing it critically for important intellectual content.

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Conflict of interest

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

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