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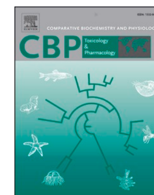

Research article

The interaction between cardiovascular system and thyroid: Atrial natriuretic peptide in the thyroid function of the lizard *Podarcis siculus*: In vivo experiments and immunolocalisation

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ABSTRACT

Although the relationship between thyroid and heart has been extensively studied, especially in cases of heart disease, the hypothesis of a relationship between heart and thyroid is still far from being fully understood. The possible involvement of atrial natriuretic peptide (ANP) in thyroid regulation is supported by the immunohistochemical identification of the peptide in thyroid follicular cells of the lizard *Podarcis siculus*. The aim of this work was to study the action of ANP on the metabolism of thyroid hormones and to analyze the mechanism of ANF action in lizard thyroid follicles. Intraperitoneally administration of ANP (1–4 μg/100 g body weight) to *Podarcis siculus* lizards both after 2 h and after 24 h inhibited circulating plasma levels of T_3 and T_4 resulting in stimulation of TSH (Thyroid-Stimulating Hormone) and decrease of TRH (Thyrotropin Releasing Hormone). The inhibitory effect of ANP on the thyroid cells could be mediated by cGMP, which is one of the main mediators of ANP action. A stimulation of the level of 5-T4 ORD (type II) Monodeiodinase activity at the hepatic level by ANP increased hepatic T_3 levels and decreased hepatic T_4 levels, revealing an alternative mode of signalling by ANP on peripheral biosynthesis of thyroid hormones. In conclusion, our results indicate the ANP role in the regulation of thyroid hormone synthesis and secretion, supported by the immunohistochemical presence of the peptide in the apical region of thyroid follicular cells and in the fibres surrounding lizard follicles, which could underlie an ANP-mediated autocrine and paracrine regulatory pathway.

1. Introduction

Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) are natriuretic peptides (NPs), a family of three structurally related hormonal/paracrine factors (Potter et al., 2009; Grandchamp et al., 2019; Kuwahara, 2021; Juraver-Geslin et al., 2023; Ogawa and Kodama, 2024). Atrial natriuretic peptide (ANP) is released mainly by the cardiac atria and is well conserved between species; in fact, ANP is expressed by the hearts of mammals, amphibians and teleost fish, whereas ventricular NP (VNP) is also found in the ventricle of a some teleost fish species (Takei, 2000; Toop and Donald, 2004; Donald and Toop, 2006). There are few studies on the NP system of the reptiles. Initially, Chapeau et al. (1985), demonstrated that, the atrial myocytes of the snake *Python reticulatus* (Chapeau et al., 1985), and of two lizard species, *Anolis carolinensis* (Reinecke et al., 1985) and *Lacerta veridis* (Reinecke, 1989) showed similar immunoreactivity to mammalian ANP. Bioinformatics and molecular cloning studies have shown that the ANP gene is absent from the hearts of squamates and birds, suggesting that the ANP gene was lost during reptile evolution, after turtles branched off from the amniote lineage (Trajanovska and Donald, 2008). Although it is possible that the ANP gene is present in the genome of squamate reptiles, it has undergone a significant degree of sequence divergence from known vertebrate sequences. Recently, NPs are mainly found in the venoms of two families of venomous snakes: *Elapidae* (elapids) and *Viperidae* (vipers) that share the same 17-residue ring structure as other mammalian NPs and differ in the length and

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sequence of the N-terminal and C-terminal tails (Ang et al., 2022). Dendroaspis natriuretic peptide (DNP), the first NP isolated from snake venom of the green mamba, *Dendroaspis angusticeps*, is a potent natriuretic peptide and diuretic, and it is similar to ANP and BNP (Schweitz et al., 1992). A very recent study (Ogawa and Kodama, 2024) has shown through kinetic analysis that both ANP and DNP bind to NPR-A, a single-pass transmembrane receptor that harbours intrinsic guanylate cyclase (GCase). Cardiac ANP and BNP via the circulatory stream reach their respective receptors in the effector organs to exert their functions in an endocrine manner (Rao et al., 2021). Their main functions are to induce vasodilation, natriuresis and diuresis and to reduce blood pressure (Potter et al., 2009; Nakagawa et al., 2019; Grandchamp et al., 2019; Rao et al., 2021; Ogawa and Kodama, 2024). The physiological role of ANP in regulating the synthesis and secretion of many hormones has been reported mainly in mammals with regard to the kidney, lung, adipose, adrenal, brain, heart, testis and vascular smooth muscle tissue (Lowe et al., 1989; Wilcox et al., 1991; Nagase et al., 1997; Goy et al., 2001; Kimura et al., 2021; Kuwahara, 2021; Datta et al., 2023); whereas little is known about the synthesis and secretion of thyroid hormones and in particular in reptiles. Few studies have revealed the localisation of both the ANP peptide and its membrane receptor in thyroid follicular cells of different mammalian species, thus hypothesising a possible autocrine regulation by ANP on thyroid hormone synthesis and secretion (Sellitti et al., 1989; Sellitti and Hughes, 1990; Ahren, 1990; Hughes and Sellitti, 1991; Sellitti et al., 1991; Sellitti and Doi, 1999; Sellitti et al., 2001; Doi and Sellitti, 1994; Tseng et al., 1990; Costamagna et al., 2002). Although the influence of ANP on the biosynthesis of thyroid hormones has been hypothesised in various mammals, studies have not fully elucidated the mechanism of action of ANP in the thyroid cells. Furthermore, as little or nothing is known about the effect of ANP on the thyroid gland in reptiles, to further investigate the role of ANP in thyroid function, we aimed to examine the effect of ANP on the biosynthesis and release of thyroid hormones and on the morphology of the thyroid gland of the lacertide *Podarcis sicul*us. We also attempted to delineate the mechanism involved in the action of ANP on the thyroid cells involving both central control via the hypothalamic-pituitary-thyroid axis and peripheral control via the target organs as the liver.

Therefore, the objective of this study is to analyze the role of ANP in thyroid function. We examined the effect of ANP on the components of the thyroid axis (hypothalamus: TRH; pituitary: TSH; liver: DIO2) and measured total circulating serum levels of thyroid hormones (T4 and T3) and hepatic contents of thyroid hormones (T_3 and T_4). In addition, we demonstrated the presence of ANP in the thyroid gland suggests that, in reptiles as in other vertebrates, this peptide can modulate thyroid gland activity, probably in an autocrine and paracrine manner.

Podarcis siculus has been chosen as biological model because it is the most abundant reptile species in Southern Italy, living in both cultivated fields and open country. Furthermore, a sophisticated and complex mechanism of regulation of the hypothalamic-pituitary-thyroid axis similar to that of mammals has been identified in lizard *Podarcis siculus* (Sciarrillo et al., 2000), fuelling further interest in exploring the regulation of their functions especially in thyroid function.

ANP has been localised in the adrenal gland of the lizard *Podarcis siculus*, demonstrating its presence in chromaffin tissue, but not in steroidogenic tissue. Moreover, the role of ANP in modulating the activity of the pituitary-adrenal axis in vivo by means of ANP administration was also studied (De Falco et al., 2002; Capaldo et al., 2004). The presence of ANP in the adrenal gland suggests that, in reptiles as in other vertebrates, this peptide, released locally by chromaffin cells, can modulate adrenal gland activity, probably in a paracrine manner. The effects of ANP on the adrenal gland suggest that this peptide may influence salt and fluid homeostasis in reptiles (Capaldo et al., 2004).

Therefore, the Authors in this study have: (1) determined the presence of immunoreactivity to ANP in the thyroid gland of a lizard, *Podarcis siculus* and (2) evaluated the in vivo systemic effects of ANP on the hypothalamic-pituitary-thyroid axis and on the liver, the peripheral organ of thyroid metabolism, by determining morphological alterations in glandular tissue, as well as plasma levels of TRH, TSH and T_3 and T_4 and hepatic monodeiodinase type II contents.

2. Materials and methods

2.1. Animals and experimental procedures

Adult specimens of *P. siculus* of both sexes (average weight 14 g) were captured around Naples (Italy) in June, when the thyroids show signs of high activity. The thyroid gland activity of *P. siculus* increases in the spring-summer period, then decreases from October until the end of winter (Sciarrillo et al., 2000). After capture, the animals were housed outdoors, under a canopy, in large terrariums filled with soil and containing heather, exposed to natural temperature and photoperiod. There were water dishes in the terraria and the animals were fed daily with live larvae. Captivity lasted 15 days to cancel out the stress associated with capture (Di Lorenzo et al., 2021). All animals were captured with the authorisation of the National Committee of the Italian Ministry of Health for in vivo experimentation. All animal experiments were conducted in accordance with accepted standards for human animal care.

The animals were divided into eight groups, each consisting of 10 animals (five males and five females) (De Falco et al., 2014; Di Lorenzo et al., 2020; Sciarrillo et al., 2021; Sciarrillo et al., 2024) as follows:

Group A: Animals injected with a single intraperitoneally (ip) injection of atrial natriuretic peptide (ANP, rat ANP, 28 amino acids, Peninsula Laboratories Europe, Merseyside, England) (1 μg/100 g body weight), dissolved in 0.75 % NaCl, with an injection volume of 0.1 ml. The lizards were sacrificed 2 h after injection.
Group B: Animals treated like group A but sacrificed 24 h later.
Group C: Animals injected with a single intraperitoneally injection of atrial natriuretic peptide (ANP) (2 μg/100 g body weight), dissolved in 0.75 % NaCl, with an injection volume of 0.1 ml. The animals were sacrificed 2 h after injection.
Group D: Animals treated like group C but sacrificed 24 h later.
Group E: Animals injected with a single intraperitoneally injection of atrial natriuretic peptide (ANP) (4 μg/100 g body weight), dissolved in 0.75 % NaCl, with an injection volume of 0.1 ml. The animals were sacrificed 2 h after injection.
Group F: Animals treated like group E but sacrificed 24 h later.
Group G: untreated control animals.
Group H: Animals that received a single ip injection of carrier solution and sacrificed 2 h after the injection.
Group I: Animals treated like group H but sacrificed 24 h later.

The injections were carried between 8.00 and 8.30 a.m., and the animals were anaesthetised by hypothermia. Immediately after collection of blood samples, the animals were killed by decapitation.

2.2. Biochemical analysis

2.2.1. Plasma TRH (thyrotropin-releasing hormone), TSH (thyroid stimulating hormone) and thyroid hormones assays

TRH and TSH levels were determined by immunoradiometric assay (IRMA) as previously reported in Sciarrillo et al. (2024). Sample serum and standards were added to anti-ligand coated tubes. The tracer/capture reagent, a blend of ligand-tagged TSH-rabbit antibody and ^{125}I labeled (10 pCi), was added to each tube. A cubic spline function with the zero standard as one of the standard points was used for calculations. The minimum detectable dose was 0.01 μIU/ml, with an accuracy close to 100 % and a mean intra-assay and inter-assay variance of 5.0 % and 7.5 %, respectively. Cross-reactivity studies were performed using substances, which could theoretically interfere with the performance of the assay. The cross-reactivity for FSH, hCG and LH in TSH IRMA was $<$0.001 and therefore was not considered for data calculations

(Sciarrillo et al., 2024).

Sample serum and standards were added to anti-ligand coated tubes. The tracer/capture reagent, a blend of ligand-tagged TRH-rabbit antibody and ^{125}I labeled (10 pCi), was added to each tube. A cubic spline function with the zero standard as one of the standard points was used for calculations. The minimum detectable dose was 0.01 μIU/ml, with an accuracy close to 100 % and a mean intra-assay and inter-assay variance of 5.0 % and 7.5 %, respectively. Cross-reactivity studies were performed using substances, which could theoretically interfere with the performance of the assay. The cross-reactivity for FSH, hCG and LH in TRH IRMA was <0.001 and therefore was not considered for data calculations (Sciarrillo et al., 2024).

T_3 and T_4 levels were determined using radioimmunoassay (RIA) (Byk-Sangtec Diagnostica, Dietzenbach, Germany) (Sciarrillo et al., 2024).

In the T_3 assay, a measured amount of sample serum and standards was added to a tube coated with anti-T_3 rabbit antibody, along with a trace amount of radioactively labeled T_3 ([^{125}I]-T^3, 165 kBq; Byk-Sangtec Diagnostica, Dietzenbach, Germany) and a blocking agent (Tris buffered saline, 4 mM ANS, 6 mM sodium salicylate with 0.2 % sodium azide as a preservative; Sigma Chemical Co., St. Louis, USA) to release T_3 from serum binding proteins. The sensitivity was 0.1 ng/ml with an accuracy of about 97 %. The range of intra-assay variance in 20 assays was 1.0–2.6 %, while the inter-assay variance ranged between 3.9 and 5.7 % in 12 assays (Sciarrillo et al., 2024).

For T_4, a measured amount of sample serum and standards was added to a tube coated with anti-T4 rabbit antibody, along with a trace amount of radioactively labeled T_4 ([^{125}I]-T4, 165 kBq; Byk-Sangtec Diagnostica) and a blocking agent (Tris buffered saline, 4 mM ANS, 6 mM sodium salicylate with 0.2 % sodium azide as a preservative; Sigma Chemical Co.) to release T_4 from serum binding proteins. The sensitivity was 0.45 ng/ml with an accuracy close to 100 %; the mean intra-assay and inter-assay coefficients of variation were 4.6 and 4.3 %, respectively. The cross-reactivity for T_4 in the T_3 RIA (1.3 %) was not considered for data calculations, neither was that for T_3 in the T_4 RIA (0.1 %) (Sciarrillo et al., 2024).

2.2.2. Hepatic thyroid hormones and 5′ORD (type II) monodeiodinase

Livers were removed and flushed in a buffer composed of MOPS and EDTA at pH 7.4. The contents of T_3 and T_4 in hepatic tissue were determined by radioimmunoassay RIA and were expressed as ng/mg of tissue (fresh weight) (Sciarrillo et al., 2024). The activity of the enzyme is expressed as pM T_3/g (of liver/h) (Sciarrillo et al., 2024).

2.3. Histological analysis

Thyroid glands were removed, weighed and immediately fixed in Bouin's fixative and processed for light microscopy (LM). Serially cut paraffin sections (7 μm) were stained with Galgano stain and observation was performed using a Zeiss Axioskop microscope (Milano, Italy). The height of the follicular cells (μm) and the diameter of the follicles (μm) were measured in 30 cells every 3 slides and always on the second section of both normal and treated samples using a digital system of image (KS 300) (Zeiss, Milano, Italy) (Sciarrillo et al., 2010, 2021, 2022, 2024).

2.4. Immunocytochemistry

The sections of thyroid gland were treated by the avidin-biotinylated peroxidase complex (ABC) method (Sciarrillo et al., 2001, 2005, 2009). Briefly, the sections of thyroid mounted on glass slides and dried overnight at 37 °C. They were then deparaffinized in xylene, rehydrated through a graded ethanol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antisera dilutions. Next, the sections were quenched sequentially in 3 % hydrogen peroxide for 15 min and blocked with 6 % milk protein in PBS for 1 h at room temperature; they were after incubated with anti-atrial natriuretic peptide (ANP), raised in rabbit against synthetic human ANP-BSA (Biogenesis, Poole, England, UK) 1:100 in phosphate-buffered saline, pH 7.4 (PBS), overnight at 4 °C in a moist chamber. After several washes to remove excess antibody, the sections were incubated with 1:200 goat antirabbit biotinylated antibody (Vector Laboratories, Burlingame, CA) for 1 h. They were then processed by the ABC method (Vector Laboratories) for 30 min at room temperature. Diaminobenzidine (DAB; Sigma, St. Louis, MO) and 0.01 % hydrogen peroxide in 0.05 M Tris–HCl buffer, pH 7.2, for 10 min to reveal the brown immunoreactive cells. After the DAB reaction, the sections were counterstained with hematoxylin. The specificity of immunostaining was performed by means of the following control procedures: (1) replacement of the specific antiserum with normal rabbit serum, (2) omission of the primary antibody and (3) preabsorption of the primary antiserum with 10 nmol of antigen per ml of optimally diluted serum.

2.5. Statistical analysis

The obtained data have been averaged prior to calculating the experimental group mean and the standard error of the mean. As revealed by the χ^2 test (chi-square test), data were not different from the normal distribution. The control and experimental data of all the groups were tested together for significance using two-way ANOVA, followed by Bonferroni's for multi-group comparison using GraphPad Prism version 8.00 for Windows, GraphPad Software (La Jolla, CA, USA). Differences were considered significant at $^{****}p < 0.001$.

3. Results

3.1. Histological analysis and immunocytochemistry before ANP treatments

The lizard *Podarcis siculus* has a thyroid gland positioned transversely about halfway down the trachea; it has a ribbon-like structure formed by follicles that are connected by connective tissue containing blood vessels. Each follicle is formed by an epithelium, composed of thyrocytes that surround a cavity containing a medium-sized colloidal mass. (Sciarrillo et al., 2000). The thyroid gland of lizard exhibits a marked annual cycle, characterised by a functional stasis that begins in autumn and becomes complete stasis in December–January and a resumption of activity in spring with a maximum activity in May–June (Sciarrillo et al., 2000). During the experimental period, control samples (**Group G, H** and **I**) showed maximum thyroid stimulation, revealed by a very high follicular epithelium (10.1 ± 0.04 μm) and a reduced follicular diameter

Table 1
Epithelial height (μm) and Follicular diameter (μm) change in *Podarcis siculus* thyroid gland subjected to 1 intraperitoneally injection of 1–4 1 μg/100 g body weight of ANP and sacrificed 2 h after the last injection (Groups A, C, E) and sacrificed 24 h after the last injection (Groups B, D, F). (****p < 0.001, in the comparison with the control). Since there were no significant differences between the values in the three control groups (Group G. H and I), group G was considered as the control group. A more detailed description is in the text (see Materials and methods section).

Doses ANP (μg/100 g body weight)	Epithelial height (μm)		Follicular diameter (μm)	
	2 h after injection	24 h after injection	2 h after injection	24 h after injection
Control group	10.1 ± 0.04	10.5 ± 0.05	165 ± 26	169 ± 26
1	6.66 ± 0.51****	6.20 ± 0.25****	260 ± 25****	271 ± 10****
2	5.32 ± 0.25****	5.25 ± 0.10****	275 ± 30****	295 ± 20****
4	4.12 ± 0.15****	4.07 ± 0.05****	312 ± 20****	382 ± 15****

of 165 ± 26 μm (Table 1). Cylindrical thyrocytes have a prominent continuous epithelium; chromophobe droplets are visible in the colloid (Fig. 1A).

Control sections of *P. siculus* thyroid gland showed no signs of immunoreactivity (Fig. 1B). ANP immunopositivity was observed in the apical cytoplasm of the follicular cells (arrowhead) (Fig. 1C). ANP fibres were found around the follicles (Fig. 1C, D) (arrows). Immunoreactivity for ANP was also observed in the nerve fibres surrounding the blood vessels and running between and along the thyroid follicles (Fig. 1E).

3.2. Histological analysis after ANP treatments

The main action of ANP on the thyroid gland analysed at different concentrations (1–4 μg/100 g body weight) for different periods of time (2–24 h) was the increase in vascularisation of the gland; in fact, in all histological preparations, intra-parenchymal hypervascularisation of the thyroid gland was evident (Fig. 2). We examined the effect of ANP on follicular epithelium height (μm), follicle diameter (μm) and the presence of colloid resorption vacuoles, important markers of morphological alteration of the thyroid gland. As shown in Fig. 2A, the thyroid gland of lizards treated with 1 injection of ANP at the minimum dose (1 μg/100 g body weight) and sacrificed 2 h after the injection (**Group A**), appeared vascularized with medium follicular epithelium height (6.66 ± 0.51 μm) and a follicular diameter of 260 ± 25 μm (Table 1). Thyrocytes had still a cubic shape and colloid showed rare reabsorption vacuoles compared to controls. The inhibitory effect of ANP on follicle epithelial height and on follicular diameter was also observed 24 h after treatment by

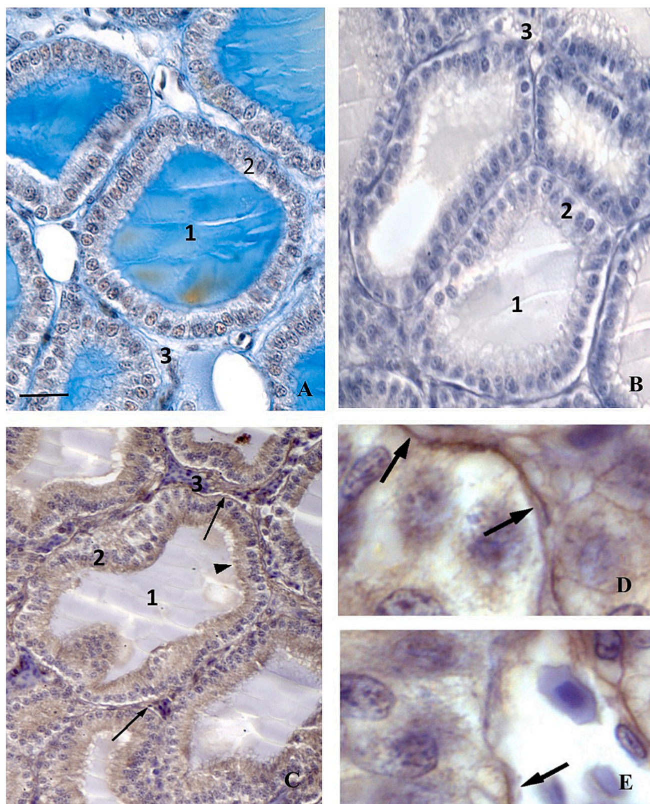

Fig. 1. Thyroid gland of lizard *Podarcis siculus* (stain Galgano I): scale bar: 20 μm. (A) Control lizard: the thyroid follicles showed a high follicular epithelium and the colloid present numerous reabsorbing vacuoles. (B) Control lizard: note the absence of immunoreactivity (C) Control lizard: Immunopositive apical cells for ANP (arrowheads) and ANF nerve fibres (arrows). (D, E) Control lizard: At higher magnification (100×), note the ANF nerve fibres (arrows). 1Follicular cavities containing colloid. 1 Follicular cavities containing colloid, 2 Follicular cells (thyrocytes), 3 Endothelial cells (blood capillaries).

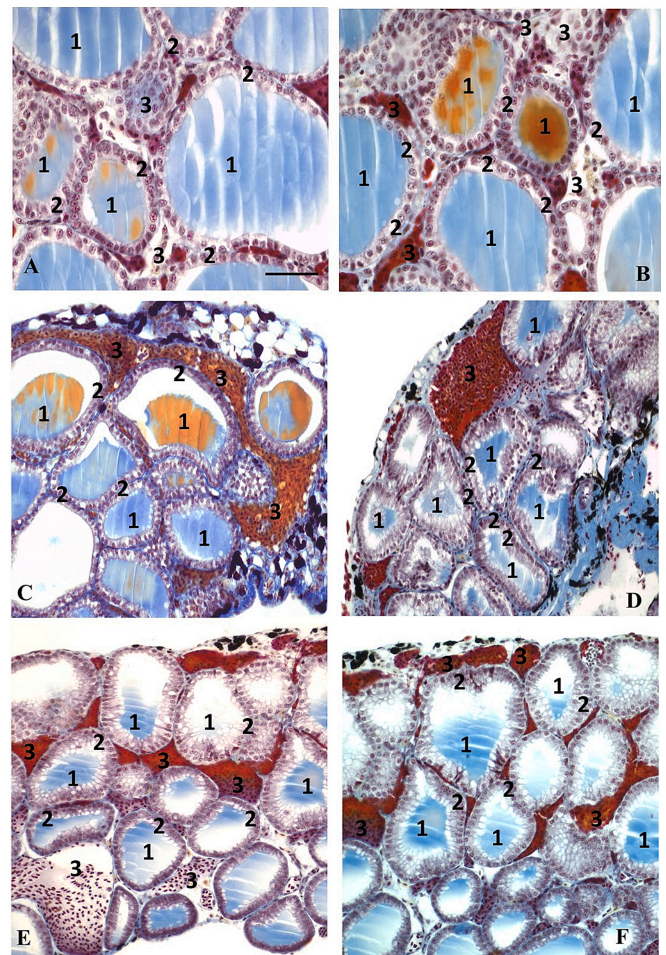

Fig. 2. Thyroid gland of lizard *Podarcis siculus* (stain Galgano I): scale bar: 20 μm. (A) Specimen treated with 1 intraperitoneally injection of 1 μg/100 g body weight of ANP and sacrificed 2 h after the injection (Group A): the cuboidal follicular epithelial cells, the colloid, and rare reabsorption vacuoles are shown. (B) Specimen treated with1 intraperitoneally injection of 1 μg/100 g body weight of ANP and sacrificed 24 h after the last injection (Group B): the follicular epithelium is medium and the colloid is present in the follicles with an evident vascularization of the gland. (C) Specimen treated with1 intraperitoneally injection of 2 μg/100 g body weight of ANP and sacrificed 2 h after the injection (Group C): the thyrocytes assume a cubic shape and the colloid showed many resorption vacuoles. (D) Specimen treated with 1 intraperitoneally injection of 2 μg/100 g body weight of ANP and sacrificed 24 h after the injection (Group D): the follicular epithelium is still lower than normal, the colloid present an evident reabsorbing vacuoles. (E) Specimen treated with 1 intraperitoneally injection of 4 μg/100 g body weight of ANP and sacrificed 2 h after the injection (Group E): the follicular epithelium is LOW, the colloid present an evident reabsorbing vacuoles. (F) Specimen treated with 1 intraperitoneally injection of 4 μg/100 g body weight of ANP and sacrificed 24 h after the injection (Group F): the follicular epithelium is very low and the colloid present numerous reabsorbing vacuoles. 1 Follicular cavities containing colloid, 2 Follicular cells (thyrocytes), 3 Endothelial cells (blood capillaries).

minimum dose (1 μg/100 g body weight) (**Group B**) (Fig. 2B). In fact, lizards showed a thyroid gland with the same morphological aspects (follicular epithelium: 6.20 ± 0.25 μm (Table 1); follicular diameter: 271 ± 10 μm (Table 1)) of lizards treated with the same dose of ANP but sacrificed after 2 h after the injection (**Group B**) (Fig. 2B).

As the dose of ANP administered increases (2 μg/100 g body weight) both after 2 h (**Group C**; Fig. 2C) and 24 h (**Group D**; Fig. 2D), the follicular epithelium thins (5.32 ± 0.25 μm (**Group C**) and 5.25 ± 0.10 μm (**Group D**)), the diameter of the follicle increases (275 ± 30 μm (**Group C**) and 295 ± 20 μm (**Group D**)) (Table 1). At the same time,

numerous resorption vacuoles were observed in the colloid at the periphery and increased vascularisation in the thyroid gland (Fig. 2C, D).

The maximum inhibition of ANP was observed after treatment with the highest dose (4 μg/100 g body weight) after 2 (**Group E**) and 24 h (**Group F**), the follicles had an increased volume due to the accumulation of colloid rich resorption vacuoles while the epithelial cells became smaller and the follicular diameter increased (Table 1) (Fig. 2E, F). The degree of vascularisation observed microscopically on thyroid sections is greatly increased (Fig. 2E, F).

3.3. Effect of ANP on plasma levels of hormones belonging to the HPT axis

The effect of ANP assayed at different concentrations (1–4 μg/100 g body weight) for different periods of time (2–24 h) on plasma thyroid hormones were to be dose - dependent, in fact, the level of circulating T_4 and T_3 were decreased in all treatment groups during the period of the trial. In lizards treated with 1 μg/100 g body weight of ANP for 2 h (**Group A**), plasma T_4 and T_3 were significantly decreased compared to the control (T_4: 5.64 ± 0.04 vs. 6.53 ± 0.05 ng/mL - control group; T_3: 4.15 ± 0.03 vs. 5.84 ± 0.02 ng/mL - control group $p < 0.001$) (Fig. 3A). Same decreasing trend in plasma T_4 and T_3 levels was also found 24 h after the injection of 1 μg/100 g body weight of ANP (**Group B**) (Fig. 3A).

The highest inhibition was obtained with 4 μg/100 g body weight of ANP both 2 (**Group E**) and 24 h (**Group F**). Specifically, T_4 levels decreased significantly ($p < 0.001$) in the treated animals by the highest dose of ANP (control group: 6.53 ± 0.05 ng/mL vs. treated group (**Group E**): 3.19 ± 0.05 ng/mL and treated group (**Group F**): 3.37 ± 0.01 ng/mL) (Fig. 3B); and T_3 (control group: 5.84 ± 0.02 ng/mL vs. treated group (**Group E**): 2.51 ± 0.01 ng/mL and treated group (**Group F**): 2.54 ± 0.05 ng/mL) (Fig. 3B).

This ANP-induced inhibition of plasma levels of circulating thyroid hormones paralleled a significant increase in plasma TSH levels and, to a lesser extent, a reduction in plasma TRH production (Fig. 3B). TSH levels significantly increased in animals treated from the lowest dose of ANP (control group: 4.18 ± 0.05 μUI/mL vs. treated group (**Group A**): 5.22 ± 0.05 μUI/mL) to the highest dose of ANP (7.41 ± 0.05 μUI/mL (**Group E**)) (Fig. 3B).

Besides, in the group treated with the highest dose of ANP (4 μg/100 g body weight), the TRH level decreased from 5.89 ± 0.05 to 2.35 ± 0.05 μUI/mL (**Group E**) (Fig. 3B).

For TSH and TRH, the trend is also identical to that found for animals treated with the different doses of ANP but sacrificed after 24 h (Fig. 3B).

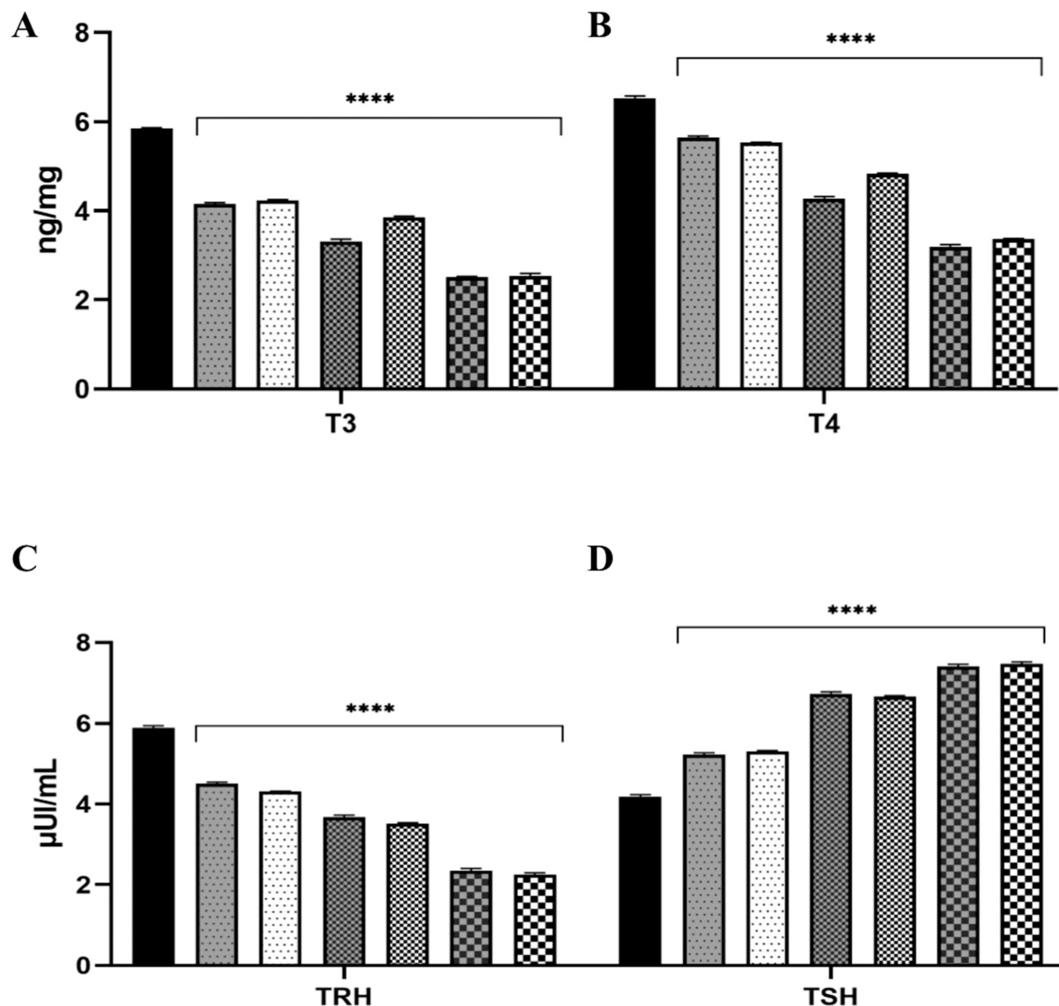

Fig. 3. Plasma T_3 (A), and T_4 (B), TRH (C), TSH (D) levels in *Podarcis siculus* subjected to 1 intraperitoneally injection of 1, 2, 4 μg/100 g body weight of ANP and sacrificed 2 h and 24 h after the injection. (****p < 0.001, in the comparison with the control). Since there were no significant differences between the values found in the three control groups (Group G, H and I), group G was considered as the control group. A more detailed description is in the text (see Materials and methods section).

3.4. Effect of ANP on hepatic thyroid hormones content and 5-T4 ORD (type II) monodeiodinase activity

ANP was also able to stimulate the activity of 5-T4 ORD (type II) Monodeiodinase in liver cells. As illustrated in Fig. 4A, a significant increase in the level of hepatic 5-T4 ORD (type II) Monodeiodinase activity was found after treatment with all three doses of ANP after both 2 and 24 h. Indeed, an increase in 5′ORD (type II) activity was observed in lizards exposed to an injection of ANP and sacrificed 2 h (**Group A**) at both the lowest (2.94 ± 0.02 pM T_3/g/h) and highest dose of ANP (4.01 ± 0.01 pM T_3/g/h) (**Group E**) compared to control lizards (2.59 ± 0.05 pM T_3/g/h) (Fig. 4A).

In addition, after 24 h the treatment with ANP caused a significant increase in enzyme activity in specimens treated with the highest dose of ANP (4.15 ± 0.01 pM T_3/g/h) compared with control specimens (2.59 ± 0.05 pM T_3/g/h) (Fig. 4A).

Hepatic T_3 content increased reached from 2.15 ± 0.04 ng/mg of tissue fresh weight (control Group) to 2.83 ± 0.01 ng/mg of tissue fresh weight (**Group A**) ($p < 0.001$), 3.49 ± 0.02 ng/mg of tissue fresh weight (**Group C**) ($p < 0.001$) and 5.51 ± 0.02 ng/mg of tissue fresh weight (**Group E**) ($p < 0.001$) (Fig. 4B). On the contrary, T_4 hepatic contents decreased in all treated groups reaching the minimum value of 1.19 ± 0.02 ng/mg of tissue fresh weight in **Group E** ($p < 0.001$) compared with control specimens (1.84 ± 0.05 ng/mg of tissue fresh weight) (Fig. 4C).

In lizards sacrificed 24 h after the injection of the different doses of ANP (**Group B**, **D**, **F**), there was also same trend in the activity of the 5′ORD (type II) with in the hepatic T_4 content, resulting in an increase in hepatic T_3 (Fig. 4B, C).

4. Discussion

Thyroid function is regulated by the expression of specific genes encoding key proteins involved in the biosynthesis of thyroid hormones (Moran et al., 2022; Muhammad et al., 2023). Although thyroid-stimulating hormone (TSH) from the anterior pituitary gland is the main physiological regulator of the thyroid gland (Pirahanchi et al., 2023), other hormones, neurotransmitters and growth factors participate in the physiological and pathological modulation of thyroid function.

Although the relationship between thyroid and heart has been extensively studied, especially in cases of heart disease, such as heart failure, arrhythmias and ischaemic heart disease (Pingitore et al., 2023), thyroid hormones directly modulate ANP and BNP synthesis, as suggested by an increase in cellular mRNA content, which has already been demonstrated in experimental studies (Diekman et al., 2001; Liang et al., 2003; Rodriguez et al., 2003; Schultz et al., 2004). Therefore, thyroid hormones can directly increase ANP levels independently of cardiac haemodynamics (Christ-Crain et al., 2005).

The hypothesis of a relationship between the heart and the thyroid gland is still far from being fully understood, despite being born well over two hundred years ago, in 1813, when Caleb Hillier Parry described in detail eight cases of thyroid gland enlargement associated with heart palpitations (Wicomb et al., 1982).

In this work, we have demonstrated by immunohistochemical assay

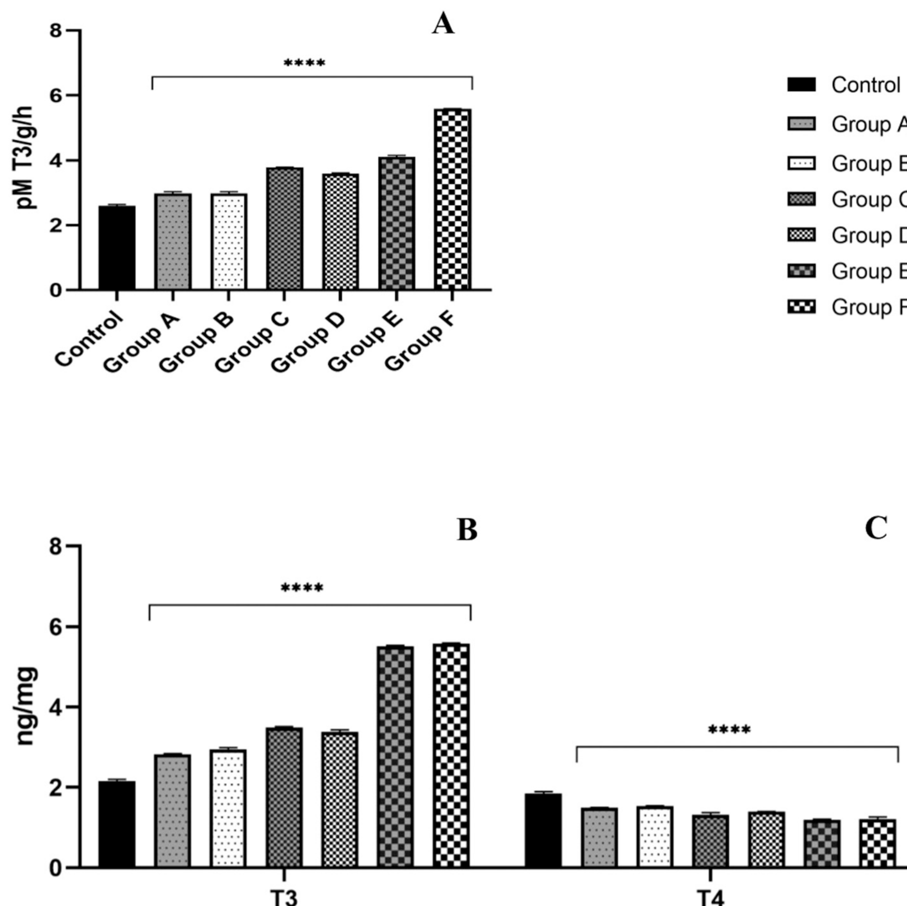

Fig. 4. Hepatic 5′ORD-monodeiodinase (type II) activity(A) and hepatic T_3(B), T_4 (C) content in *Podarcis siculus* subjected to 1 intraperitoneally injection of 1, 2, 4 μg/100 g body weight of ANP and sacrificed 2 h and 24 h after the injection. (****$p < 0.001$, in the comparison with the control). Since there were no significant differences between the values found in the three control groups (Group G, H and I), group G was considered as the control group. A more detailed description is in the text (see Materials and methods section).

the presence of ANP in the thyroid gland of *Podarcis siculus*; our results, which showed the presence of ANF-IR fibres around the follicles and in the cell apices, suggest that ANP could be involved in the autocrine and paracrine regulation of the thyroid gland in the *P. siculus* lizard.

ANP administration was able to inhibit the thyroid of specimens of both sexes; in fact, our study provides evidence that sex difference in lizards does not significantly influence the morphophysiology of the adult thyroid gland under ANP treatment. The maximum inhibition of thyroid hormone release was obtained in lizard thyroid gland thyrocytes with 4 μg/100 g body weight of ANP, significant inhibition was also obtained with the lowest dose of ANP (1 μg/100 g body weight).

It is known that ANP plays an essential role in the guanylyl cyclase (GC)/cGMP-signalling pathway; in fact, ANP binds to the natriuretic peptide A receptor, a guanylyl cyclase receptor, converting GTP to cGMP (Rao et al., 2021) and since cGMP is a regulatory pathway in thyroid hormone biosynthesis, it is believed that ANP may participate in the regulation of thyroid function using cGMP. Therefore, the morphological pictures of the thyroid gland showed a gland with reduced epithelial height but with numerous reabsorption vacuoles compared to that of controls indicating a production of thyroid hormones but accompanied in parallel by a decrease in circulating thyroid hormones release.

The inhibitory actions of ANP on thyroid hormones release in the lizard *P. siculus* are in agreement with the few previous mammalian studies that indicated that ANP reduced thyroid hormone release when administered to mice (Ahren, 1990) and also on bovine thyrocytes (Costamagna et al., 2002) and in cultured human thyrocytes (Sellitti et al., 1989; Sellitti and Hughes, 1990; Tseng et al., 1990).

Moreover, since iodide uptake and, thus, the biosynthesis and release of thyroid hormones depend on a variety of factors, a strict parallelism in the action of ANP on thyroid function is not to be expected, but rather a variety of factors may intervene in the mechanism of ANP regulation on the thyroid gland. As is well known, the main stimulation of the thyroid gland is the action of pituitary TSH, whose essential messenger is cAMP. Therefore, ANP, after binding to the guanylyl cyclase receptor NPR-A, could convert GTP to cAMP and produce downstream effects via a signalling cascade; in fact, our treatment with ANP causes a significant increase in circulating plasma levels of TSH. This significant increase in plasma TSH and consequently a decrease of TRH levels could also be a direct consequence of the decrease in thyroid hormone release caused by ANP administration, which would therefore, not activate the negative feedback to TSH.

Interestingly, in contrast to previous observations, a stimulation of the level of 5-T4 ORD (type II) Monodeiodinase activity at the hepatic level by ANP increased hepatic T_3 levels and decreased hepatic T_4 levels, revealing an alternative mode of signalling by ANP on peripheral biosynthesis of thyroid hormones. This underlines the complexity of the intracellular mechanisms of ANP action on thyroid hormone biosynthesis.

In conclusion, the present results indicate that ANP was able to inhibit parameters of thyroid function in lizard thyroid. The possible main mechanism of ANP action on the regulatory pathway of thyroid hormone biosynthesis in this cell type appears to be paracrine and autocrine given the positivity expressed by both the apical region of thyrocytes and the fibres surrounding thyroid follicles. This primary event of ANP action on thyrocytes disrupts normal regulation by thyroid hormones, mainly T_3 with negative feedback on TSH and TRH secretion. Therefore, upon exogenous ANP administration there was a reduction in serum levels of thyroid hormones, accompanied by a concomitant increase in circulating TSH and decrease of TRH levels). Our results are also in agreement with morphological variations of the gland based on morphometric parameters such as the height of the follicular epithelium, follicular diameter and the presence of resorption vacuoles. In addition to the previous observations, ANP played an essential role in the regulation of vascular tone by virtue of its vasodilatory action. Finally, in contrast to the inhibitory effect produced by the administration of ANP, a vasodilator, on the morphophysiology of the thyroid gland, the stimulatory effect produced by the administration of endothelin-1, a vasoconstrictor, on the thyroid gland of this lizard must be considered (Virgilio et al., 2003).

5. Conclusion

ANP and BNP, together with the myocardial enzyme troponin, are the only currently known biomarkers of heart failure. However, in light of the results obtained, it is also possible to take into account other factors unrelated to the heart, such as thyroid function. These results validate the concept that enhancing the effects of natriuretic peptides and in particular, observations of our current study will provide the basis for future studies aimed at ascertaining these complex regulatory pathways of the hypothalamic-pituitary-thyroid axis in patients with primary hyperthyroidism.

CRediT authorship contribution statement

Rosaria Sciarrillo: Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Alessandra Falzarano:** Methodology, Investigation, Formal analysis. **Vito Gallicchio:** Methodology, Investigation, Formal analysis. **Assunta Lallo:** Methodology, Investigation, Formal analysis. **Francesca Carrella:** Methodology, Investigation, Formal analysis. **Aldo Mileo:** Investigation, Formal analysis. **Anna Capaldo:** Formal analysis. **Maria De Falco:** Writing – original draft, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2025.110259.

Data availability

Data will be made available on request.

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