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MELATONIN IN THE THYROID GLAND: REGULATION BY THYROID-STIMULATING HORMONE AND ROLE IN THYROGLOBULIN GENE EXPRESSION

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Melatonin is an indoleamine with multiple functions in both plant and animal species. In addition to data in literature describing many other important roles for melatonin, such as antioxidant, circadian rhythm controlling, anti-aging, antiproliferative or immunomodulatory activities, our group recently reported that thyroid C-cells synthesize melatonin and suggested a paracrine role for this molecule in the regulation of thyroid activity. To discern the role played by melatonin at thyroid level and its involvement in the hypothalamic-pituitary-thyroid axis, in the present study we have analyzed the effect of thyrotropin in the regulation of the enzymatic machinery for melatonin biosynthesis in C cells as well as the effect of melatonin in the regulation of thyroid hormone biosynthesis in thyrocytes. Our results show that the key enzymes for melatonin biosynthesis (AANAT and ASMT) are regulated by thyroid-stimulating hormone. Furthermore, exogenous melatonin increases thyroglobulin expression at mRNA and protein levels on cultured thyrocytes and this effect is not strictly mediated by the upregulation of TTF1 or, noteworthy, PAX8 transcription factors. The present data show that thyroid C-cells synthesize melatonin under thyroid-stimulating hormone control and, consistently with previous data, support the hypothesis of a paracrine role for C-cell-synthesised melatonin within the thyroid gland. Additionally, in the present study we show evidence for the involvement of melatonin in thyroid function by directly-regulating thyroglobulin gene expression in follicular cells.

Key words: *melatonin, thyroglobulin, transcription factors, C cells, thyroid, thyrotropin-releasing hormone, thyroid-stimulating hormone, aralkylamine N-acetyltransferase, acetyl serotonin methyltransferase*

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxy-tryptamine) is an indoleamine with multiple functions in both plant and animal species (1). In mammals, melatonin was primary described to be secreted by the pineal gland following a circadian rhythm (2, 3). In addition to its well known chronobiotic function (4), melatonin has been extensively reported to be involved in many other mechanisms such as regulation of oxidative stress, apoptosis, mitochondrial homeostasis (5) and every day more rigorous and potentially relevant data are accumulating in literature with regard to its endocrine (5), metabolically beneficial (7), anti-aging, oncostatic, and immunomodulatory activities (8).

Although the pineal gland was for long considered the main site for melatonin synthesis, a large amount of evidence has demonstrated extrapineal sources of melatonin in a number of systems, organs, tissues and cells, such as gut, skin, retina, the Harderian gland, the immune system (8, 9) and - last but not least - thyroid (10, 11).

Within the thyroid gland, thyroid hormone synthesis is mainly regulated by the thyroid-stimulating hormone (TSH), whose expression and secretion are primarily controlled by the stimulatory action of the hypothalamic thyrotropin-releasing hormone (TRH) (12). TSH-induced thyroid hormone biosynthesis involves the expression of different thyroid specific genes, such as the sodium iodide symporter (NIS), thyroperoxidase (TPO) and thyroglobulin (13, 14). TSH also controls the functional activity of the transcription factors PAX8, TTF1/NKx2.1, and TTF2/FoxE1, necessary for the expression of these genes (15, 16). TSH controls thyroglobulin gene transcription on thyrocytes *via* the interaction with its receptor and cAMP and PAX8 are its main physiological mediators (17, 18).

The potent antioxidant melatonin was first reported to be present in the thyroid gland by Kvetnoy (10). More recently, our research group demonstrated that melatonin is, at rat thyroid level, synthesized by C cells -the minor neuroendocrine thyroid cell population- and, also that MT1 melatonin receptors are present in follicular cells (11). These results extended previous studies describing C cells to be involved in the regulation of thyrocyte function (19-22) and suggested a role for thyroid melatonin. In this regard, T_3 and T_4 biosynthesis includes several oxidative reactions catalyzed by an enzyme complex proposed as the thyroxisome (23) confined at the apical membrane at the microvilli-colloid interface (24). The required continuous synthesis of H_2O_2, and hence the linked generation of free radicals (25, 26), makes the control of

oxidation levels crucial for thyroid homeostasis and self-protection. Many control and thyroid self-protection mechanisms have been described so far (27, 29), and their imbalance has been stated to be responsible for causing thyroid disease (30-32). Therefore, on the basis of its powerful antioxidative properties (33) and in the context of the unique oxidative requirements of the thyroid gland, C-cell secreted melatonin might be a candidate to mediate follicular-cell activity and thyroid function.

To date, there is data in literature pointing to the relationship between melatonin and thyroid activity. With regard to the hypothalamic-pituitary-thyroid axis, some effects of melatonin have been reported. Thus, it has been described the decrease of thyroid hormone circulating levels after melatonin administration (34, 35), and the central role played by melatonin on the control of iodothyronine-deiodinases and their influence in seasonal reproductive physiology (36). Moreover, there is also evidence in literature showing melatonin actions on the thyroid gland itself, such as the inhibitory effect of melatonin on cell proliferation and thyroid hormone synthesis (37, 38) or the protective effect of melatonin against oxidative damage in the thyroid gland (39-41). However, to our knowledge, there are no studies at molecular level regarding the implication of melatonin in the activity of normal resting thyrocytes.

In the present study we aimed to analyze the effect of melatonin on the thyroid hormone biosynthetic activity of thyrocytes. In particular, we have analyzed the effect of melatonin on the expression of the thyroid tissue-specific genes: thyroglobulin and two of the main tissue-specific thyroid transcription factors, PAX8 and TTF1 (NKX2-1), regulating thyrocyte function (17). Finally, and based upon previous data from our research group describing the functional expression of the TSH receptor in C cells (20), we have also examined the TSH regulation of the key enzymes for melatonin biosynthesis: aralkylamine N-acetyltransferase (AANAT) and acetyl serotonin methyltransferase (ASMT), formerly known as hydroxy-indole-o-methyl-transferase (HIOMT), previously described by our group (11) to be present in C cells.

MATERIALS AND METHODS

Tissue, cell cultures and treatments

For this study, rat tissues and rat thyroid cell-cultures were used. Animals were fed regular chow and drank water *ad libitum*, and experiments were performed in agreement with the guidelines proposed in The Declaration of Helsinki (http://www.wma.net) involving the use of laboratory animals. TRH treatment was used to induce hyperthyroidism in rats as described by Denereaz *et al.* (42). Studies were performed using 2-month-old male Wistar rats, randomly separated into two groups of 5 rats each. First group received a normal diet for 2 weeks, and was used as control group. Second group received normal diet for 2 weeks supplemented with a solution containing 0.17 mg/mL thyrotropin-releasing hormone (TRH; P1319 Sigma, St. Louis, MO, USA) in the drinking water. At the end of the treatment, rats were sacrificed under anaesthesia (pentobarbital, 15 mg kg^{-1}, intraperitoneal injection), blood samples were taken by aortic puncture and thyroid glands were extracted and immediately frozen in liquid nitrogen. The induced hyperthyroidism state was confirmed by the measurement of thyroid hormone levels (*Table 1*) using an AxSYM analyser. To this end, we used the free T_3 and free T_4 serum microparticle enzyme immunoassay (MEIA) reagent packages (Abbott Laboratories) on the AxSYM System, in accordance with the manufacturer instructions. Free T_3 and T_4 reagent packages, Microparticle Enzyme Immunoassay (MEIA), the AxSYM automated system and Free T_3 and T_4 reagent packages are registered trademarks of Abbott Laboratories, Abbott Park, IL (USA).

The following rat cell lines were used: CA77 (rat neoplastic C-cells, generously provided by Dr. T. Ragot, Institut Gustave Roussy, Paris, France), grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 15% foetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin; and PC-Cl3 (rat non-transformed follicular cells, generously provided by Dr. Massimo Santoro, Centro di Endocrinologia e Oncologia Sperimentale di C.N.R Naples, Italy), which were grown in 6H medium, consisting of Coon's modified F-12 medium supplemented with 5% FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin and a mixture of six hormones: 1 mIU/mL TSH, 10 µg/mL insulin, 10 ng/mL somatostatin, 5 µg/mL transferrin, 10 nM hydrocortisone, and 10 ng/mL glycyl-L-histidyl-L-lysine acetate. 5H-medium consisted of 6H-medium without TSH. Cell cultures were maintained at

Table 1. Serum free T_3 and T_4 in TRH-induced hyperthyroidism rat experimental model. Data are expressed as mean ± S.D. Student's T-test P value: *P < 0.01.

	FreeT_3 (pg/mL)	**FreeT_4** (ng/dL)
Control	3.65 ± 0.2	1.87 ± 0.1
TRH	4.93 ± 0.8*	2.80 ± 0.2*

Table 2. Primer sequences and PCR products.

Gene	**Primer Sequence**	**Product (bp)**
β-actin	F: 5'-CAGATGTGGATCAGCAAGCAGGAGTACGAT-3'	126
	R: 5'-GCGCAAGTTAGGTTTTGTCAAAGAA-3'	
TG	F: 5'-GGCAAGGAGAACCCGTGGAAATGTCTTCATGT-3'	119
	R: 5'-AGGCAGAGTAGAAGGGCAGTCCAAAAGCATA-3'	
PAX8	F: 5'-TGCCAGGACCTGCGTAAGAGAGCTGCGAGT-3'	127
	R: 5'-TTCACAAAAGCCCCTCCTAACTGATTC-3'	
TTF1 (NKX2-1)	F: 5'-TTCTCCCTTTCCTTTCTCTTTCCTACCTAA-3'	173
	R: 5'-CCTAAGCTTGAGAACCCATTTGAACTACCA-3'	
AANAT	F: 5'-GAGATCCGGCACTTCCTCACCCTGTGTCCAGA-3'	94
	R: 5'-CCCAAAGTGAACCGATGATGAAGGCCACAAGA-3'	
ASMT	F: 5'-AGTGACATCATGGGTGGGAATTTATGACTT-3'	105
	R: 5'-CCCTACCCCACCATTACTGTGACATC-3'	
	R: 5'-ACTGCAAGGCCAATACAGTTGA-3'	

37°C in a humidified atmosphere with 5% CO_2. Cell cultures were always used at 70 – 80% confluence. Phenotypic characteristic features of both PC-Cl3 (expression of thyroglobulin, TSH-R, PAX8 and TTF1) and CA77 (expression of calcitonin, calcitonin gene related peptide and TSH-R) cell lines were confirmed in the cell cultures used for the *in vitro* studies.

qRT-PCR

Thyroid gland tissue samples were homogenized in TRIsure solution (TRIsure, Bioline, USA) using a homogenizer (Tissueruptor Qiagen, Hilden, Germany) and total RNAs were then purified according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically. For cell-line RNA extracts, cells were plated in 6-well plates, and RNA was purified and RNA concentration determined as described above.

One microgram of total RNA from the different cell lines and treatments was used to synthesize cDNA. In order to discard DNA contamination before cDNA synthesis, RNA samples were incubated in gDNA wipeout buffer (Quantitect Reverse Transcription kit, Qiagen. Hilden, Germany) at 42°C for 2 min and then used directly for reverse transcription. cDNA synthesis was carried out using the Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany).

For the detection and quantification of transcripts, quantitative real-time PCR was carried out using the SensiFAST SYBR No-ROX one-step Kit (Bioline, London, UK) in accordance to the comparative Ct method described by Livak *et al.* (43, 44). β-actin mRNA was used as house-keeping gene. The primers used to amplify thyroglobulin, β-actin, AANAT, ASMT, PAX8, TTF1 (NKX2-1) transcripts were designed for two-temperature-PCR cycle conditions: denaturation at 95°C for 15 s followed by alignment at primer-specific temperature and elongation at 70°C for 30 s; for 35 cycles. Specific two-temperature primers were designed with Oligo 7 primer design software (*Table 2*). Every single reaction was performed in duplicate. DNase treated non-retrotranscribed RNA-samples were used as internal negative controls. Quality controls for qPCRs, consisted in the confirmation of correct-size single band amplification analyzed in agarose gels and single peak melting curve, for every set of primers and PCR reaction (data not shown).

Protein extracts and Western blotting

PC-Cl3 and CA77 cells were plated at 10^4 cells/cm^2 and 7 × 10^4 cells/cm^2 on 75 cm^2 flasks, respectively, and allowed to reach 80 – 90% confluence. Cells were lysated in 1 ml of 40 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM PMFS in the presence of protease inhibitor cocktail (Roche Diagnostics Mannheim Germany). Cellular debris were pelleted and discharged by centrifugation at 14,000 × g for 10 min at 4°C and the supernatants were used immediately for assays, or stored at –80°C. Protein concentrations were measured by BCA reagent (Pierce, Rockford, IL, USA).

Thirty micrograms of proteins were loaded on 10% and 7% SDS-PAGE for PAX8 and thyroglobulin, respectively, and transferred to Amersham Hybond ECL nitrocellulose membrane (GE Healthcare) for 1 at 3 mA/cm^2. Membranes were then blocked during 2 h in 5% non-fat powdered milk in PBST. Immunodetections were carried out by overnight incubation at 4°C with rabbit anti-thyroglobulin antibody (dilution 1/8000, A 0251 Dako, Denmark) and rabbit anti-PAX8 (dilution 1/2000, Anti-PAX8 PA 0330 BioPat, Italy). Mouse anti-β-tubulin and mouse anti-GAPDH (Sigma, St. Louis, MO, USA) were used to detect the corresponding proteins used as house-keepers for thyroglobulin and PAX8 expression analyses, respectively. The membranes were washed in PBST and then incubated for 1hour with the appropriate secondary antibodies coupled to horseradish peroxidase (anti-mouse 1/1000 or anti-rabbit 1/1000; both Sigma, St. Louis, MO, USA). Finally, bands were visualized by Super Signal West Femto (Pierce, Rockford, IL USA), according to the manufacturer's instructions, and semiquantitative analyses were performed by using the ImageJ software (NIH Bethesda, Maryland, USA).

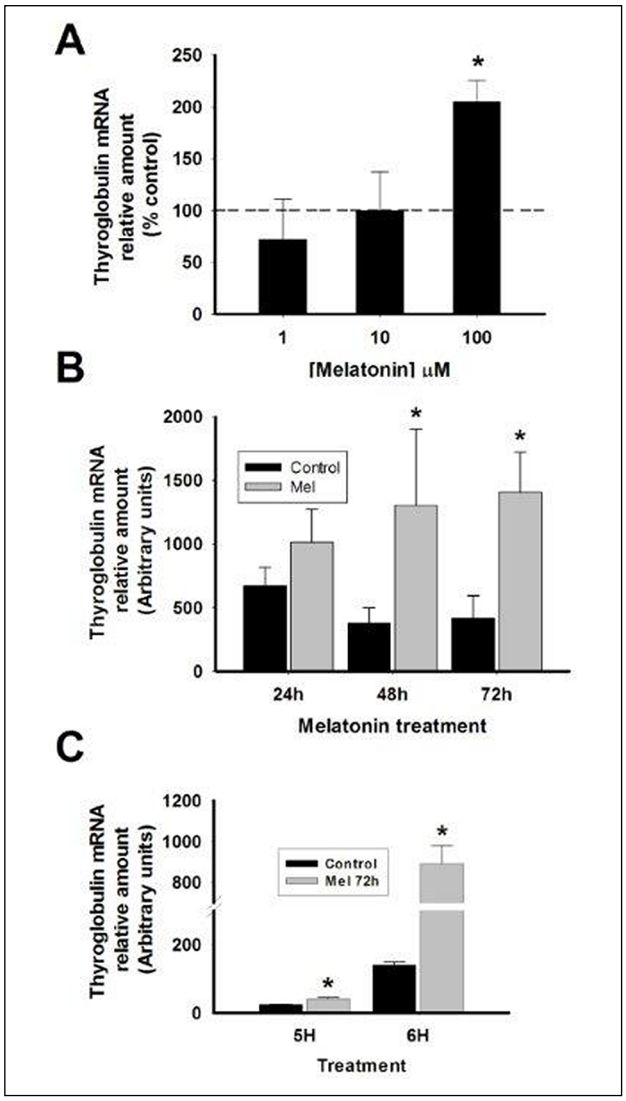

Fig. 1. Effect of melatonin on thyroglobulin mRNA steady state in PC-Cl3 cells. Total RNA was subjected to SYBR® Green RT-qPCR analysis. Graph shows the specific mRNA relative amount using appropriate primers and normalized to the β-actin housekeeping mRNA. Data represent mean ± S.D. (n = 4).
(A): Dose effect of 24-h melatonin treatment on thyroglobulin expression. PC-Cl3 cells were cultured in 6H medium and 24-h treated with 1 µM, 10 µM or 100 µM melatonin. ANOVA one-way multiple comparison vs. control (Holm-Sidak method) * P = 0.008;. (B): Time-course analysis of melatonin effect on thyroglobulin mRNA steady state. PC-Cl3 cells were cultured in 6H medium and treated with 100 µM melatonin for 24, 48 and 72 h. ANOVA two-ways all pairwise multiple comparison procedures (Holm-Sidak method) * P = 0.002;. (C): Effect of 100 µM melatonin for 72 h in the absence (5H) or presence (6H) of TSH. ANOVA two-ways all pairwise multiple comparison procedures (Holm-Sidak method). 5H Cont vs. Mel * P = 0.001, 6H Cont vs. Mel * P < 0.001.

Immunofluorescent thyroglobulin detection

PC-Cl3 cells were seeded in 8-well Lab-TekTM II-Chamber SlideTM slides (Nunc, Langenselbold, Germany) in complete medium until reaching 60% confluence and then treated with melatonin (100 µM) in 0.5% FBS at the different times and conditions. To visualize thyroglobulin immunostaining, cells were fixed for 6 minutes in cold 100% methanol (Merck 64271 Damstadt, Germany), washed three times in PBS and blocked by incubation with donkey normal serum for 1 h at room temperature.

Cells were then incubated with anti-thyroglobulin antibody (dilution 1/500, A 0251 Dako, Denmark) overnight at 4°C. Afterwards, slides were washed in PBS and incubated for 30 min at room temperature with Cy-2 labelled secondary antibody (1/100, Jackson Immunoresearch Laboratories, Suffolk, UK). Controls for immunoreaction specificity were performed by omitting the primary antibody step.

Finally, after PBS washing, slides were mounted in 90% glycerol, 2% n-propylgallate (Sigma, St. Louis, MO, USA) and observed under a fluorescence microscope (BX50, Olympus, Japan). Immunofluorescent outputs were generated using a Hamamatsu ORCA-03G camera (Hamamatsu Photonics, Japan) and Image Pro Plus 7.0 software (Media Cybernetics, USA) under the same microscope and software parameter settings and images were compared for intensity.

Statistical analyses

For qRT-PCR gene expression and western blot analyses, relative expressions were expressed in arbitrary units as mean ± S.D. from at least three replicates from fully independent cell treatments or animals per group. Data were compared using Student's t-test or ANOVA followed by the corresponding post-hoc tests. P values of less than 0.05 were accepted as significant.

RESULTS

Melatonin increases thyroglobulin expression

To investigate the role of melatonin in the biosynthesis of thyroglobulin by follicular cells, rat thyroid PC-Cl3 cells were cultured for 72 hours in 0.5% FBS medium containing 5H or 6H with or without 100 µM melatonin. The effect of melatonin the expression of thyroglobulin was then analyzed at mRNA level by RT-qPCR, and at protein level by semiquantitative Western-blot and immunofluorescence. As *Fig. 1* shows, significant increases in thyroglobulin mRNA expression were detected when cells were treated with 100 µM melatonin (*Fig. 1A*) for 48 and 72 h and were maximum at 72 h (*Fig. 1B*). Furthermore, the effect of melatonin was present under both 5H and 6H culture conditions (in the absence or presence of TSH). Melatonin effect was maximum in the presence of TSH (6H conditions) at which a 6-fold increase in thyroglobulin mRNA levels was observed (*Fig. 1C*). The upregulation of thyroglobulin expression was also observable at protein level as revealed by Western blot (*Fig. 2A*), and finally, in good agreement with mRNA and Western-blot data, the intensity of immunofluorescence staining in PC-Cl3 cells for thyroglobulin was higher after melatonin treatment when compared to control cells (*Fig. 2B*). Data indicate that melatonin regulates the expression of thyroglobulin in cultured rat thyroid follicular cells.

Melatonin does not increase basal and TSH-induced PAX8 or TTF1 expression

To investigate the role of PAX8 and TTF1 in melatonin-induced thyroglobulin increased expression, rat thyroid PC-Cl3 cells were cultured for 72 h in 0.5% FBS 5H and 6H media with or without 100 µM melatonin. As shown in *Fig. 3A* and *3B*, respectively, there were no significant differences in TTF1 or

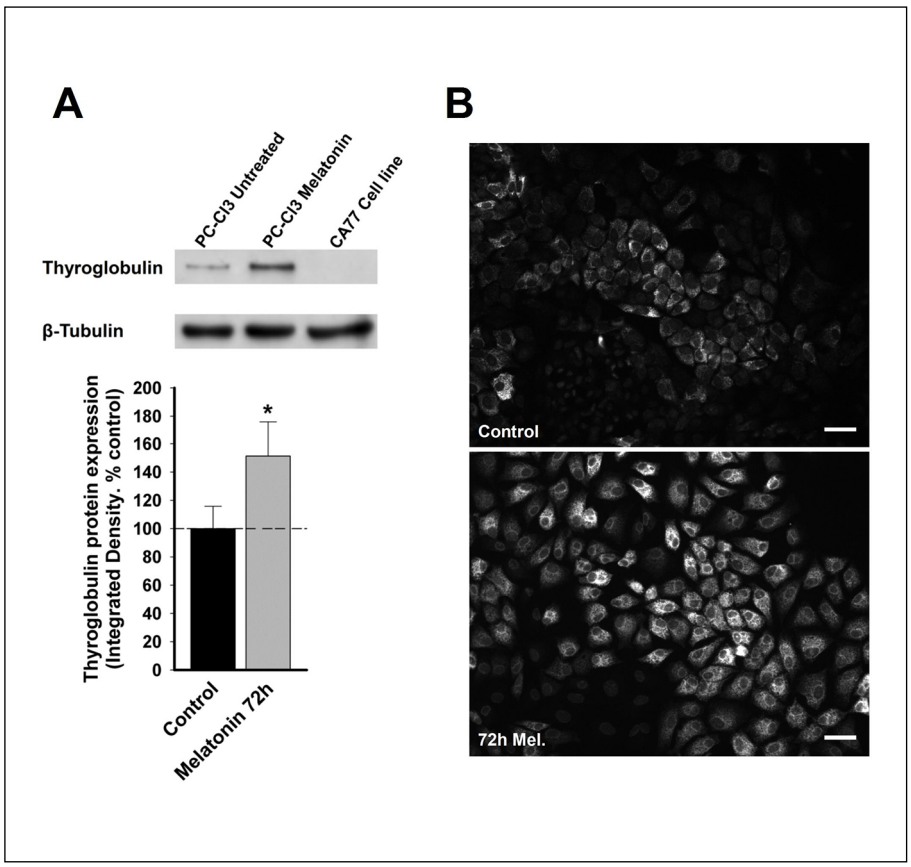

Fig. 2. Detection of thyroglobulin protein in PC-Cl3 after treatment with melatonin. Cells were cultured in 6H medium, treated with 100 µM melatonin for 72 h. (A): Western blot analysis of thyroglobulin protein expression. Graph shows an increase in thyroglobulin protein level after treatment with melatonin. The values are presented as percentage of the level of untreated cells (control), using β-tubulin as house-keeping protein. Rat thyroid C-cell line CA77 was used as negative control. Data shown in this figure represent the average of three independent experiments and are expressed as mean ± S.D. Student's T-test *P < 0.05 vs. control. (B): Immunofluorescence detection of thyroglobulin in PC-Cl3 cells after 72 h-melatonin treatment. Compatibly with western blot data, the cytoplasmic immunofluorescent signal intensity for thyroglobulin was qualitatively higher in the presence of melatonin when compared to non-treated cells. Photographs show one representative of three independent experiments.. Bar = 30 µm.

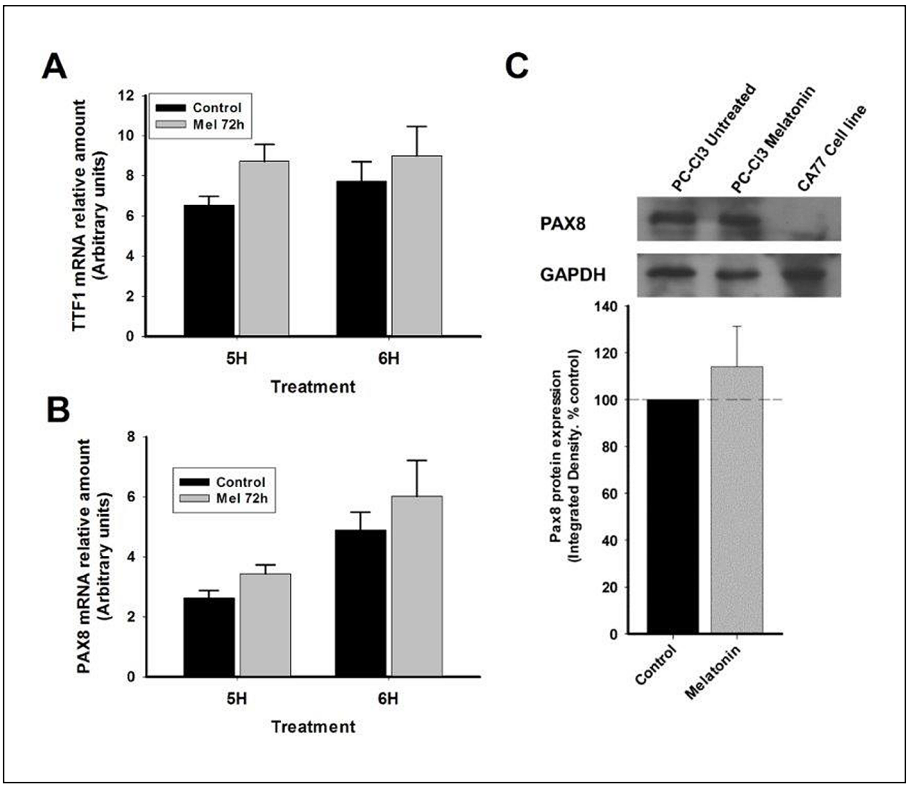

Fig. 3. Effect of melatonin on TTF1 and PAX8 expression in PC-Cl3 cells. For mRNA analyses, cells were cultured in the absence (5H) or presence (6H) of TSH and treated with 100 µM melatonin for 72 h. Total RNA was subjected to qRT-PCR analysis. Graphs show TTF1 (3A) and PAX8 (3B) mRNA relative amounts using appropriate primers and normalized to the β-actin housekeeping gene. Data represent mean ± S.D. (n = 4). For Western blot protein analysis of PAX8 (3C), cells were cultured in 6H medium and treated with 100 µM melatonin for 72 h. PAX8 protein was detected in melatonin treated cells without significant differences as compared to controls, using GAPDH as house-keeping protein. Rat thyroid C-cell line CA77 was used as negative control. Data shown in this figure represent the average of three independent experiments and are expressed as mean ± S.D.

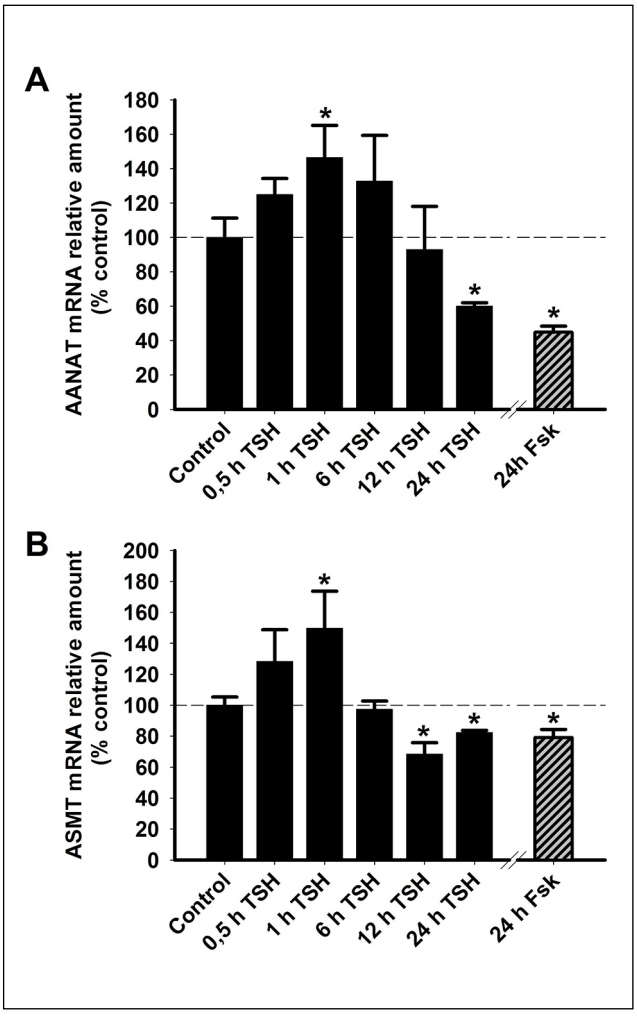

Fig. 4. TSH regulation of both AANAT and ASMT mRNA steady-states in the CA77 rat thyroid C-cell line. Graphs show SYBR® Green RT-qPCR based analyses of the 1 mIU/mL time course TSH and 10 µM forskolin treatments AANAT (4A) and ASMT (4B) mRNAs. Graphs show the specific mRNA relative amount using appropriate primers and normalized to the β-actin housekeeping mRNA. Data are presented as percentage of untreated cell levels (control), and expressed as mean ± S.D. (n = 3). ANOVA on ranks one-way multiple comparison vs. control (Dunnett's method) *P < 0.05.

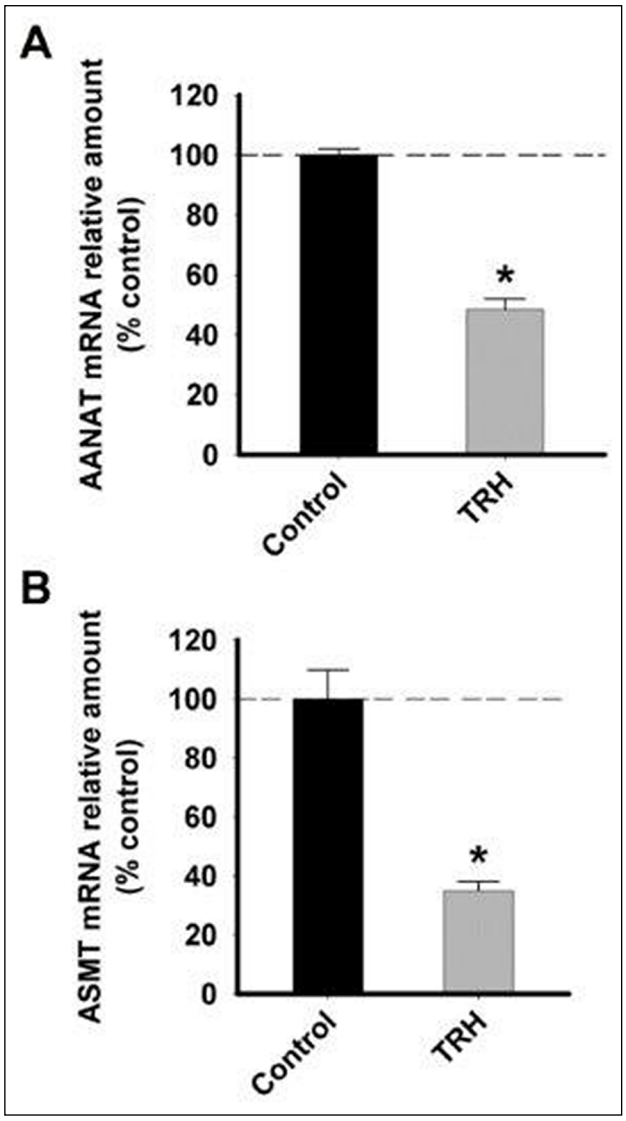

Fig. 5. AANAT and ASMT mRNA steady states in thyroid glands from rats subjected to TRH-induced hyperthyroidism. SYBR® Green RT-qPCR based analysis of the effect of TRH treatment. Graphs show the specific AANAT (5A) and ASMT (5B) mRNA relative amounts using appropriate primers and normalized to the β-actin housekeeping mRNA. Data are presented as percentage of untreated group levels (control), and expressed as mean ± S.D. (n = 5). Paired Student's T-test P values: *P < 0.05 vs. control.

PAX8 mRNA levels when cells were treated with melatonin under any conditions. Based on data in literature regarding the effect of TSH on PAX8 expression, as expected TSH-treated cells with or without melatonin showed significant 2-fold increases in PAX8 mRNA levels as compared to controls (*Fig. 3B*). Finally, in agreement with mRNA data, PAX8 protein analysis by Western blot revealed no differences after melatonin treatment (*Fig. 3C*).

Our results show that the melatonin-mediated increase in thyroglobulin expression is not accompanied by a concomitant increase in TTF1 or, particularly, PAX8 levels. Therefore, either additional factors and/or PAX8 protein 'activation' might be required for the melatonin-mediated TSH dependant thyroglobulin up-regulation to occur.

Melatonin-synthesizing enzymes are regulated by thyroid-stimulating hormone in C cells

Once the role played by melatonin on the thyrocyte activity was determined, as a second part of the study we analyzed the regulation of melatonin synthesis by thyroid C-cells. To investigate the role of TSH in the rate of melatonin production by C cells, relative-expression analyses for the melatonin-synthesizing enzymes were carried out. Specifically, RT-qPCR-based mRNA quantifications of AANAT and ASMT in 24 h time-course of 1 mIU/mL TSH-treated CA77 cells were performed. As *Fig. 4A* shows, TSH first significantly increased and then decreased both AANAT and ASMT gene-expressions in a time-dependent manner. The expression levels rose until 1 h and decreased between 12 and 24 h of TSH-treatment. In addition, the effects were more noticeable for AANAT mRNA, the rate-limiting melatonin-synthesizing enzyme, which increased up to 140% at 1 h and diminished to 60% approximately at 24 h. TSH-R is an adenylcyclase-coupled 7-transmembrane domain family receptor, therefore 10 μM forskolin was used as cAMP signalling positive-control. Our results show that TSH regulates mRNA levels of AANAT and ASMT in rat CA77 C-cells.

Melatonin-synthesizing enzymes are downregulated in the thyroid gland of TRH-induced hyperthyroid rats

To verify the effectiveness of the hyperthyroidism experimental model, we measured the serum concentrations of thyroxin in all experimental animals. When TRH was administered, a hyperthyroid state was induced in the rats, characterized by a dramatic increase in T_4 serum levels (*Table 1*). *Fig. 5* shows the relative expression of both AANAT and ASMT enzymes in the thyroid glands of TRH-treated rats as compared to controls. The induction of hyperthyroidism caused significant decreases of both AANAT (*Fig. 5A*) and ASMT (*Fig. 5B*) mRNA-expression within the thyroid gland. These results, observed in an experimental rat-model of TRH induced hyperthyroidism, in agreement with those obtained in the *in vitro* experiments and described above, show that melatonin enzymatic machinery is controlled by TSH, probably *via* cAMP, in rat thyroid C cells.

DISCUSSION

In the present work we have demonstrated that melatonin has a direct effect on the thyroid-hormone biosynthesis activity in rat-thyroid differentiated follicular-cells. Thus, we have shown that exogenous melatonin increases thyroglobulin expression at mRNA and protein levels. This stimulatory effect is not directly mediated by an augmented expression of the main thyroid tissue-specific transcription factors TTF1 and, in particular, PAX8. As a second part of the study, we have demonstrated that intrathyroidal biosynthesis of melatonin at C-cell level is regulated by TSH. This agrees with our previous studies proposing that C cells would be involved in the hypothalamus-pituitary-thyroid axis (11, 19, 20, 22, 45) and, that the melatonin direct-effect on follicular cells could be exerted by that synthesized by C cells, which, by means of the paracrine secretion of this neurohormone, may have a regulatory role on the surrounding follicular cells.

Melatonin was first described to play a direct role in the mouse thyroid gland in 1986 by Lewinski *et al.* (38, 46). These authors reported melatonin to have an inhibitory effect on thyrocyte basal and TSH-induced mitotic activities. Melatonin inhibitory role on the thyroid gland was then extended in amphibians where this neurohormone was reported to control

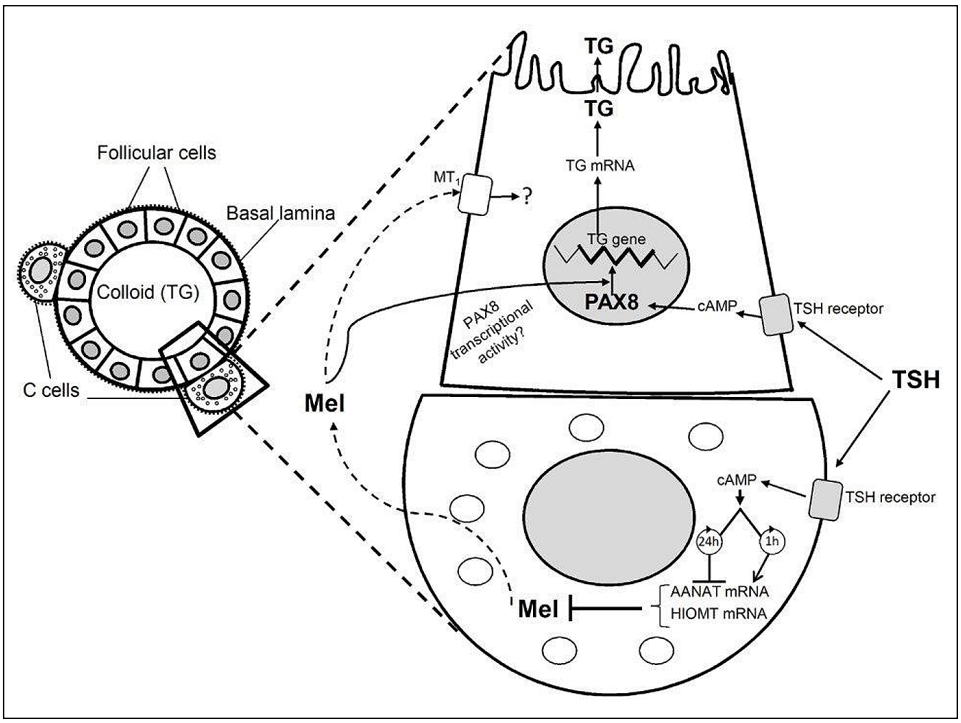

Fig. 6. Biological model of the thyroid gland as a melatoninergic system. Endogenous C-cell synthesized melatonin is regulated by TSH. Thus, TSH, binding to its membrane receptor in C cells, first upregulates and then downregulates the two key enzymes for melatonin synthesis, in a time-dependent fashion possibly through a cAMP-dependent mechanism. On the other hand, melatonin upregulates thyroglobulin (TG) synthesis in the presence of TSH, which may be caused by a melatonin redox mediated increase in PAX8 transcriptional activity.

metamorphosis and development by inhibiting thyroid response to T_4 and TSH (37, 47).

During the last decade, a large body of evidence, mainly reported by Lewinski and co-workers research group, has contributed to confirm the antioxidant and protective role played by melatonin in the normal as well as in the prooxidant-agent insulted thyroid-gland (40, 48-51). However, these studies reported no data regarding the capacity of the thyroid gland to self-synthesize melatonin to exert the above mentioned effects.

The first report describing immunoreactivity for melatonin within the thyroid gland was that by Kvetnoy (10). These results were recently confirmed by our research group who reported molecular evidence for, not only the presence of the enzymatic machinery for melatonin synthesis in the rat C cells, but also the expression of melatonin membrane-receptors in thyrocytes (11), which supported the studies describing the direct effect of melatonin in the thyroid gland mentioned above.

Pineal melatonin-synthesis is mainly regulated by norepinephrine (NE), which is released by nerve-endings at the pinealocyte vicinity during the dark period. AANAT expression has been described to be cAMP-dependent in a two phase fashion. In brief, a temporal switch activates sequentially both cAMP-dependent proteins: pCREB (cAMP-response element binding) and ICER (inducible cAMP early repressor). The chronologically programmed activation of these proteins is responsible for the pCREB-mediated increase in AANAT -the rate-limiting enzyme for melatonin (52) - expression at the early hours and for the ICER-mediated inhibition in AANAT expression which decreases at the late hours of the night and during the daylight (53).

Here we show that the expression of both AANAT and ASMT mRNAs decrease in 24 h TSH-treated C cells in culture and, moreover, in rats subjected to sustained TRH induced hyperthyroidism. These results, after long term exposure to TSH-cAMP, would be in agreement with the decrease of AANAT mRNA levels observed in the pineal gland at the late hours of the night after norepinephrine-induced sustained increase in cAMP levels. Nevertheless, further studies are needed to clarify the role of C-cell synthesized melatonin in thyroid activity and at this regard, our results might be of interest in those disorders, as for example in central hyperthyroidism, in which inappropriate elevated sustained TSH-values are exhibited (54).

Thyroid hormone synthesis operates through a complex network of coordinated interactions involving numerous enzymes, membrane transporters and transcription factors. Here we have focused our studies on the role played by melatonin in the regulation of thyroglobulin synthesis, in particular through the transcription factors PAX8 and TTF1. Thyroglobulin synthesis is mainly activated by TSH, whose binding to its specific receptor induces the coupling of different G proteins which activate the adenylate cyclase/cAMP cascade (14), and also the PLC cascade (55). Thyroglobulin promoter is well characterized to contain binding sites, among others, for both TTF1 and PAX8 transcription factors, both of them synergistically necessary for thyroglobulin expression (15).

Although TTF1 appears to be more constitutively expressed, TSH/cAMP has been shown to increase PAX8 at mRNA and protein levels, which are followed by an increase in thyroglobulin expression (17, 56). To date, data present in the literature strongly support a fundamental role of PAX8 in the TSH-mediated increased expression of thyroglobulin. However, other than PAX8, one or more additional factors have been suggested to be required for thyroglobulin TSH-mediated activation (17), such as synergistic interactions with other transcription factors as TTF1 or redox activation (57, 58).

Our results show that melatonin increases thyroglobulin expression at both mRNA and protein levels and that this effect is more marked in medium containing TSH and insulin. This effect is not accompanied by an increase in the transcription factors TTF1 and, noteworthy, PAX8, whose minor changes are also variable in relation to the hormonal -insulin and TSH- background. Therefore, in the present work we clearly show that the capacity of melatonin to up-regulate TSH-stimulated thyroglobulin is not mediated by an increased expression of PAX8.

The molecular mechanisms responsible for the effects of melatonin in target organs involve several actions: binding to high-affinity G-protein-coupled receptors at the membrane level, interaction with intracellular targets to modulate signal transduction pathways, redox-modulated processes, or the

scavenging of free radicals (8). The combination of them in one single cell or in the same system is sometimes responsible of the complex and pleiotropic effects described for this neurohormone (6). In previous studies, we demonstrated that the thyroid follicular cell line PC-Cl3 used for this study expresses the MT1 membrane receptor (11). In brief, both MT1 and MT2 have been described to signal through the coupling of different G proteins which inhibit the adenylate cyclase/cAMP and subsequent phosphorylation of the cAMP-responsive element binding protein (CREB), and therefore decreasing its downstream transcriptional activity (59). According to the latter, the observed effects in our study would seem not be due to MT1 or MT2 cAMP/CREB signalling. Interestingly, TTF-1 and specially PAX8 proteins have been reported to be activated through oxidoreductive modifications (58). In this context, Cao *et al.* (57) demonstrated that reduction of Cys-45 and Cys-57 residues were involved in the restoration of PAX8 transcriptional activity, and described Ref-1 as the enzymatic factor involved in the reductive activation of PAX8 in living cells. Noteworthy, thioredoxin is one of the hydrogen donors for Ref-1 (60) and this small redox protein has been reported to be increased by melatonin in cerebral ischemic injury treatment (61). Consequently, our results, together with data in the literature might suggest that the increase in thyroglobulin expression would be, at least in part, compatible with either direct or indirect oxidoreductive changes induced by melatonin. Furthermore, the fact that results in our study are more marked in the presence of TSH/cAMP, and plausibly PAX8, would be in agreement with a putative involvement of PAX8 redox-state. Nevertheless, further investigations are required to clarify the exact underlying mechanisms by which melatonin exerts that effect on the thyroid follicular cell and the roles played by melatonin in the overall scheme of thyroid function.

In conclusion, as depicted in *Fig. 6*, the present study shows that TSH regulates the expression of the enzyme machinery for melatonin synthesis in C cells. In addition, we found that melatonin directly regulates thyroid hormone biosynthetic activity in rat cultured thyrocytes. Although further studies are necessary, the observed effects could be, at least in part, carried out by C-cell synthesized melatonin and likely through a redox control mechanism.

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