Novel aspects of T₃ actions on GH and TSH synthesis and secretion: physiological implications

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Abstract

Thyroid hormones (THs) classically regulate the gene expression by transcriptional mechanisms. In pituitary, the encoding genes for growth hormone (GH) and thyroid-stimulating hormone (TSH) are examples of genes regulated by triiodothyronine (T₃) in a positive and negative way, respectively. Recent studies have shown a rapid adjustment of GH and TSH synthesis/secretion induced by T₃ posttranscriptional actions. In somatotrophs, T₃ promotes an increase in *Gh* mRNA content, poly(A) tail length and binding to the ribosome, associated with a rearrangement of actin cytoskeleton. In thyrotrophs, T₃ reduces *Tshb* mRNA content, poly(A) tail length and its association with the ribosome. In parallel, it promotes a redistribution of TSH secretory granules to more distal regions of the cell periphery, indicating a rapid effect of T₃ inhibition of TSH secretion. T₃ was shown to affect the content of tubulin and the polymerization of actin and tubulin cytoskeletons in the whole anterior pituitary gland, and to increase intracellular alpha (CGA) content. This review summarizes genomic and non-genomic/posttranscriptional actions of TH on the regulation of several steps of GH and TSH synthesis and secretion. These distinct mechanisms induced by T₃ can occur simultaneously, even though non-genomic effects are promptly elicited and precede the genomic actions, coexisting in a functional network within the cells.

Key Words

- ▶ triiodothyronine
- ▶ GH
- ▶ TSH
- ▶ non-genomic actions
- ▶ secretion

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Nuclear thyroid receptors and the gene expression regulation

Most of the well-characterized actions of thyroid hormones (THs) are mediated by thyroid hormone receptors (THRs), the alpha (THRA) and beta (THRB) receptors, which belong to the superfamily of nuclear receptors. THRs act as transcriptional factors interacting with specific sequences of the DNA called thyroid hormone response elements (TRES) and promoting the activation or inhibition of target gene transcription. THRs can also indirectly bind to DNA through proteins such as AP1 (jun/fos) complexes,

influencing the gene expression (Flamant *et al.* 2017). The presence of THRA and THRB isoforms in humans, mice, rats, chickens and *Xenopus laevis* was described and indicates that this mechanism is conserved across a variety of species (Tata & Widnell 1966, Samuels & Tsai 1973, Sap *et al.* 1986, Weinberger *et al.* 1986, Yaoita *et al.* 1990).

The THRs are encoded by *Thra* and *Thrb* genes. The heterogeneous mRNAs are subjected to alternative splicing or alternative use of promoters generating nine

different transcripts: *Thra1*, *Thra2*, *Thra3*, *ThrΔa1*, *ThrΔa2*, *Thrb1*, *Thrb2*, *Thrb3* (found only in the kidney, liver and lung of rats) and *ThrΔb* (O'Shea & Williams 2002, Ortiga-Carvalho *et al.* 2014). The expression of these transcripts changes during the development and cellular differentiation processes, being also transcriptionally and posttranscriptionally regulated. The THR_s are differentially expressed in most tissues. *Thrb2* is highly expressed in the pituitary, paraventricular, ventromedial and arcuate nuclei of hypothalamus, inner ear and in some regions of the brain during development, while *Thrb1* mRNA is mainly detected in the brain, skeletal muscle, liver and kidney of rats (Lazar 1993, Ortiga-Carvalho *et al.* 2016). Mutations in THR isoforms are associated with different phenotypes according to tissue distribution of the mutated receptor (Mendoza & Hollenberg 2017).

THR_s bind to TREs under the form of homodimers or heterodimers, mainly with retinoid acid receptors and retinoid X receptors (RXRs) that also belong to the superfamily of nuclear receptors. The THR_s interact with TH, coactivators or corepressors modulating the gene transcription (Shupnik 2000, Yen 2001, Cheng *et al.* 2010).

In the anterior pituitary gland, TH_s downregulate the expression of genes that encode CGA (glycoprotein hormones alpha chain) and TSHB subunits of thyroid-stimulating hormone (TSH) (Franklyn *et al.* 1988) and upregulate *Gh* transcription (Crew & Spindler 1986, Koenig *et al.* 1987, Lavin *et al.* 1988). The THR_{B2} isoform participates in the positive and negative regulations of *Gh* and *Tshb* expressions, respectively (Abel *et al.* 1999, Barra *et al.* 2004). In humans, mutations in THR_B isoforms led to the classic form of resistance to thyroid hormone (RTHB) characterized by high TH levels and normal or slightly elevated serum TSH concentration, due to the resistance of pituitary and hypothalamus to respond to the negative feedback triggered by TH (Forrest *et al.* 1996, Refetoff *et al.* 2014). Mild-to-moderate growth retardation and delayed bone maturation have also been reported in RTHB (Refetoff *et al.* 1993, Kaneshige *et al.* 2000).

Hypothyroidism induced by thyroidectomy in male rats increases the expression of both TSH subunits (Bargi-Souza *et al.* 2013, 2015), while the expression of GH, which is involved in linear growth and intermediary metabolism modulation, is markedly reduced (Hervas *et al.* 1975, Volpato & Nunes 1994). On the other hand, studies performed in hyperthyroid rodents and in T₃-treated TtT97 cells, a thyrotrophic tumor-transplantable cell line, have shown a marked increase in *Gh* mRNA content and a significant reduction in *Cga* and *Tshb* contents

(Shupnik *et al.* 1985, Chin *et al.* 1993, Volpato & Nunes 1994, Shupnik 2000, Bargi-Souza *et al.* 2013, 2015).

Transcriptional regulation of *Gh* by T₃

During the 1980s, several studies carried out in rats demonstrated that the *Gh* expression is positively regulated by TH_s (Crew & Spindler 1986, Larsen *et al.* 1986, Koenig *et al.* 1987, Lavin *et al.* 1988). The increase in the *Gh* transcription rate triggered by T₃ requires the interaction of the THR/T₃ complex with sequences located close to the transcription start site (TSS) of *Gh* and the presence of specific transcriptional factors expressed in somatotrophs, since the insertion of *Gh* promoter in fibroblast or kidney cells did not evoke *Gh* transcription in response to T₃ (Larsen *et al.* 1986, Koenig *et al.* 1987).

Studies on the mutational analysis of the TRE of the rat *Gh* gene (r*Gh*) and other positively T₃-regulated genes suggest that (G/A)GGT(C/G)A is a putative consensus hexamer half-site sequence (Flamant *et al.* 2017). Changes in the orientation, spacing and number of half-sites of TRE were also observed (Yen 2001, Ortiga-Carvalho *et al.* 2016). In the r*Gh* promoter, the sequences could be oriented as palindromes, or direct repeats separated by 4 nucleotides (Yen 2001, Cheng *et al.* 2010).

In the case of TH_s positively regulated genes, such as *Gh*, the THR_{B2} heterodimerizes mainly with the RXRs and in the absence of the ligand, the THR/RXR complex is linked to the DNA, repressing the transcriptional process through the interaction with corepressors such as NCoR1 (nuclear receptor corepressor-1) and the SMRT (silencing mediator for retinoid and thyroid receptor), a process that involves the alteration of histone deacetylases, such as HDAC1 or HDAC3 (Harvey & Williams 2002). Numerous corepressors interact with THR_s suggesting a high level of complexity in the basal suppression process in the absence of TH_s.

The presence of ligand promotes a conformational change in THR_{B2} that results in the dissociation of corepressors (CoR) and recruitment of coactivators (CoA) such as the nuclear receptor coactivator 1 (NCoA-1 or SRC-1) establishing a new complex that leads to histone acetylation or methylation, which, in turn, changes the interaction between RNA polymerase and other transcriptional factors inducing the gene transcription (Ortiga-Carvalho *et al.* 2014, Mendoza & Hollenberg 2017). It is worth mentioning that T₃-mediated activation of rat *Gh* gene expression presents a synergism between transcriptional factor Pit-1 (POU domain, class 1, transcription factor 1) and T₃,

increasing the *Gh* mRNA content (Schaufele *et al.* 1992, Sinha & Yen 2000, Yen 2001).

The regulation of GH synthesis and secretion mediated by T_3 differs in non-mammalian species. T_3 increases GH synthesis and secretion in fish, but decreases *Gh* mRNA expression in birds and GH secretion in chickens (Melamed *et al.* 1998). These findings could be related to a differential expression of coactivators and repressors in different species, which affects the response to TH, explaining the diversity of T_3 effects on GH regulation. Currently, the literature and knowledge are relatively poor for reptile and amphibian species.

Negative transcriptional regulation of *Cga* and *Tshb* genes by T_3

The involvement of THRA and THRB isoforms in the control of hypothalamus–pituitary–thyroid (HPT) axis has been evaluated in patients with natural mutations. Clinical observations have shown that in patients with a mutation in the THRA, the HPT axis is minimally or not impaired, while the opposite is observed in patients with mutation of THRB isoform (Ortiga-Carvalho *et al.* 2014).

These clinical observations are supported by studies performed in different models of *Thrb1* and *Thrb2* knockout mice. The lack of all THRB isoforms (*Thrb*^{−/−}) led to resistance to thyroid hormone (RTH-b) characterized by the enlargement of the thyroid gland (goiter), increased size and number of follicles, elevated T_4 and T_3 concentrations, normal or elevated serum TSH concentration and increased *Tshb* and *Cga* mRNA expressions due to defects in thyrotrophic response to THs (Forrest *et al.* 1996, O'Shea & Williams 2002, Mendoza & Hollenberg 2017). The generation of THRB2 knockout (*Thrb2*^{−/−}) mice provided evidence that this isoform plays a major role in the negative feedback exerted by T_3 in thyrotrophs and in the stimulation of *Gh* mRNA synthesis in somatotrophs (Abel *et al.* 1999, 2003).

The absence of both *Thra1* and *Thra2* in mice led to the development of an impaired thyroid gland and to the reduction of thyroxine (T_4) synthesis (O'Shea & Williams 2002), while the overexpression of THRA1 results in low levels of T_3 and T_4 and normal levels of TSH (Mendoza & Hollenberg 2017). Mice with double knockout for *Thra* and *Thrb* (*Thra*^{−/−} *b*^{−/−}) present higher T_4 , T_3 and TSH serum concentrations than *Thrb*^{−/−} mice (Weiss *et al.* 1997, Abel *et al.* 1999, Chiamolera *et al.* 2012). In TαT1 cells, the THRB is essential for T_3 inhibition of *Tshb* mRNA expression, while the THRA plays an important role only in the absence of THRB (Chiamolera *et al.* 2012).

In humans, some RTH patients with mutations in THRA presented normal or low TSH levels and reduced T_4 / T_3 ratio. All these data together suggest that the THRA isoform plays an important role in the negative feedback mechanism and that THRA and THRB are required for the adequate regulation of the HPT axis (Mendoza & Hollenberg 2017).

Although the precise sequence of negative TREs (nTRE) has not been described yet (Ortiga-Carvalho *et al.* 2016), previous studies demonstrated that they appear to be near the TSS of *Cga/CGA* and *Tshb/TSHB* genes of rats, mice and humans (Carr *et al.* 1987, Shupnik 2000). However, recent studies have shown that the negative regulation of human *TSHB* gene exerted by T_3 is independent of nTRE (Matsushita *et al.* 2007, Matsunaga *et al.* 2015).

In the pituitary, the glycoprotein alpha subunit (*CGA*) is expressed in thyrotrophs and gonadotrophs and composes the mature molecule of TSH, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) together with the beta-subunit, which, in turn, guarantees hormonal specificity (Pierce & Parsons 1981).

Regarding the transcriptional effects of triiodothyronine on the *Cga* expression, it is known that in the rat and mouse genomes, there are several hexameric TRE half-site motif regions in the *Cga* gene, supporting the hypothesis that there is a suppression of *Cga* transcription by T_3 (Burnside *et al.* 1989). Studies in the human choriocarcinoma cell line JEG-3, transfected with THR, have shown that T_3 treatment markedly suppresses the *CGA* expression. The presence of a binding site for THR located between −22 and −7 base pairs of TSS of *CGA* gene immediately downstream from the TATA box suggests a possible interference of THs/TATA box with transcriptional factors reducing the synthesis of *CGA* mRNA (Chatterjee *et al.* 1989).

Considering that the *Cga* subunit is negatively regulated by THs (Shupnik & Ridgway 1987, Bargi-Souza *et al.* 2015), it might be expected that during hypothyroidism and hyperthyroidism, significant alterations in gonadotrophin synthesis and secretion would occur since *CGA* is essential for the composition of FSH and LH and that binding between alpha and beta subunits is important for hormonal maturation, biological activity and the recognition of these glycoproteins by specific receptors on gonads (Pierce & Parsons 1981, Bargi-Souza *et al.* 2015). Our recent studies indicate that alterations in the HPT axis strongly affect the hypothalamus–pituitary–gonad axis in rats, which might explain some reproductive disorders observed under hypothyroid and hyperthyroid conditions (Romano *et al.* 2013).

Despite the efforts, the precise mechanism implicated on the molecular basis of T_3 negative regulation of *Tshb* and *Cga* remains unknown. Four models were proposed for the negative regulation triggered by T_3 involving the nTREs as follows: (a) the position of nTRE, which prevents the formation of RNA polymerase II complex and consequently the initiation of transcription; (b) the interaction of the THR-TH complex with co-coactivators altering the conformation of the DNA molecule and thus preventing the transcription process; (c) the interaction of THR-TH with transcription inhibitors and (d) the presence of an nTRE inside an enhancer sequence, thus preventing transcription (Chin *et al.* 1993). Moreover, the mutation in the activation domain of THRB abolishing CoA recruitment but preserving normal T_3 binding and CoR interactions has shown that this portion is required for positive and, paradoxically, for negative regulation by TH, increasing the complexity of TH gene expression regulation (Ortiga-Carvalho *et al.* 2005). It is noteworthy to mention that transcriptional alterations occur concomitantly with alterations in GH and TSH secretion in rats, which implies that different mechanisms are triggered for the proper synthesis/secretion regulation of these hormones (Zoeller *et al.* 2007).

The regulation of HPT in non-mammals appears to be very similar to that in mammals. In teleost and amphibians, the activity of HPT is important for the normal development and metamorphosis of the animals and their larvae are sensitive to slight disruption of HPT function (Carr & Patino 2011). In the few fish and amphibians species studied, both T_4 and T_3 decrease TSH secretion by reducing *Tshb* gene transcription (Blanton & Specker 2007). In birds, THs present a shorter half-life than that in mammals but the negative feedback triggered by THs at the pituitary level is similar to that observed in mammals (McNabb 2007).

Non-genomic actions of THs

The non-genomic or posttranscriptional actions of THs are not dependent on the nuclear receptor interaction with TRE, even though the signaling resulting from these mechanisms could lead to the regulation of gene expression (Davis *et al.* 2013, 2016). These actions are rapidly triggered even in the presence of transcription inhibitors (Yusta *et al.* 1998, Lorenzo *et al.* 2002) by TH interactions with THRs coupled to specific enzymes in the cytosol or to other binding sites located on the plasma membrane and organelles such as mitochondria and

endoplasmic reticulum (Moeller *et al.* 2006, Davis *et al.* 2008, 2009, Goulart-Silva *et al.* 2011).

There are a substantial number of studies connecting non-genomic actions of THs with complex physiological processes like the traffic of intracellular protein, the basal activity of transporter proteins, the regulation of the half-life of specific mRNAs (Davis *et al.* 2005, Hammes & Davis 2015), the cellular metabolism (Moeller *et al.* 2006), the brain development (Anton *et al.* 1999, Leonard 2008, Cao *et al.* 2009), the cardiac growth (Kenessey & Ojamaa 2005), angiogenesis (Bergh *et al.* 2005) and the osteoblast proliferation (Kalyanaraman *et al.* 2014).

In 2005, a binding site for THs was identified in the extracellular domain of $\alpha V\beta 3$ integrin present on the plasma membrane, which has a crucial role in pro-angiogenic actions triggered by THs (Bergh *et al.* 2005, Cody *et al.* 2007, Lin *et al.* 2011). This integrin has two binding sites, S1 and S2, for THs with different affinities for T_4 and T_3 . The S1 site only recognizes T_3 and, in the presence of the ligand, activates the Src and PI3K pathways, whereas the S2 site recognizes both T_4 and T_3 with higher affinity for T_4 and activates the ERK1/2 pathway (Davis *et al.* 2011). Parts of TH actions elicited via $\alpha V\beta 3$ integrin are blocked by RGD (arginine-glycine-aspartate) peptide which strongly suggests that this TH binding site is located at or near the RGD recognition site on the $\alpha V\beta 3$ integrin (Davis *et al.* 2005, Cody *et al.* 2007). It was shown that the presence of RGD peptide prevented the trafficking of THRs from the cytosol to the nucleus induced by T_3 treatment (Cheng *et al.* 2010), pointing out a crucial participation of the $\alpha V\beta 3$ integrin in gene transcription regulation.

More recently, a THRA of 30kDa (p30 THRA) was evinced in osteoblasts associated with plasma membrane domains rich in caveolin. T_3 binding to this receptor leads to an increase in intracellular Ca^{++} concentration and subsequent activation of NO-cGMP-PKGII followed by MEK-ERK and PI3K-Akt signaling cascades resulting in increased proliferation and survival of osteoblasts (Kalyanaraman *et al.* 2014). Another non-genomic mechanism of TH actions involves their interaction with nuclear receptors coupled to the regulatory p85 subunit of phosphoinositide 3-kinase (PI3K) in the cytoplasm regulating important proteins associated with glucose uptake, protein synthesis and proteolysis inhibition (Saji & Ringel 2010, Kimura *et al.* 2014). Truncated isoforms of THRs present in the cytoplasm also mediate non-genomic actions of T_4 and reverse T_3 (rT_3) (Cheng *et al.* 2010). The interaction of THRAA1 with T_4 or rT_3 is associated

with the rearrangement of actin cytoskeleton and with the release of laminin, a matrix protein, by astrocytes (Farwell *et al.* 1995). The laminin and the rearrangement of the actin cytoskeleton are related to the induction of neuronal migration and growth of neurites, respectively (Farwell *et al.* 1995).

Thus, the identification of binding sites of THs at the plasma membrane and cytosol indicates a complex mechanism triggered by THs to establish their biological effects and the elucidation of TH non-genomic actions raises the possibility of therapeutic intervention through pharmacological agent actions in these binding sites.

Non-genomic regulation of GH and TSH synthesis and secretion

Posttranscriptional effects of T₃ on Gh synthesis

In the beginning of 1990s, few studies addressed the non-genomic actions of THs and they were restricted to T₄ and rT₃. These studies pointed out the rapid modulation of type 2 deiodinase (DIO2) activity triggered by T₄ and rT₃ in astrocytes, but not by T₃, suggesting that genomic actions were triggered by T₃, while T₄ and other metabolites were responsible for the non-genomic actions (Leonard 2008).

However, our group showed that a single T₃ injection in thyroidectomized (Tx) rats promoted an increase in Gh mRNA content in 15 min compared to hypothyroid animals (Volpato & Nunes 1994), and in the molecular weight of Gh mRNA characterized by its slow migration on the agarose gel 30 min thereafter. After removing the poly(A) tail, Gh mRNA from Tx rats treated with T₃ migrated on the gel to the same height of Gh mRNA from untreated Tx rats. In fact, sequencing analyses of Gh mRNA from T₃-treated Tx rats revealed an increased number of adenine residues in its poly(A) tail, evincing a posttranscriptional action of T₃ in this step of GH processing (*unpublished data*).

It is worth to highlight that Gh mRNA from Tx rats presents longer poly(A) tail making this transcript more stable than that of euthyroid animals (Jones *et al.* 1990, Murphy *et al.* 1992). It appears that in the absence of TH, mechanisms are triggered to ensure minimal GH synthesis since the transcriptional rate of Gh gene is reduced under the hypothyroidism condition. Furthermore, it has also been shown that suppression of GH synthesis and secretion induced by IGF-I were abolished in hypothyroidism (Melmed & Yamashita 1986), which reinforces that other mechanisms operate to regulate the GH expression in Tx animals.

In our study, T₃ further induced a rapid increase in Gh mRNA poly(A) tail length, ensuring more stability for Gh transcripts (*unpublished data*), and GH labeling at the perinuclear region of somatotrophs of Tx rats suggesting that GH synthesis was stimulated by T₃ (Silva *et al.* 2006). Our group has proceeded with the investigations on the molecular mechanisms involved in the rapid actions of T₃ on Gh mRNA stability and we have also studied the involvement of the cytoskeleton in T₃ non-genomic actions, as discussed later.

Rapid and negative posttranscriptional regulation of Cga and Tshb

Considering the positive effects of T₃ on the posttranscriptional steps of Gh synthesis, our group has investigated whether T₃ could trigger rapid effects on Tshb mRNA content and poly(A) tail length (Goulart-Silva *et al.* 2011, Bargi-Souza *et al.* 2013).

Using Tx rats, we observed that in hypothyroidism, the poly(A) tail length of Tshb mRNA is increased, followed by a significant rise in the translational rate of Tshb, besides the expected increase in the transcriptional process (Bargi-Souza *et al.* 2013). Thirty minutes after treatment with T₃, the poly(A) tail length of Tshb mRNA of Tx rats was reduced to values similar to those observed in the poly(A) tail length of euthyroid rats and was positively correlated with a marked reduction in the content of Tshb in polysome fractions indicating that T₃ rapidly reduced mRNA translation and TSH protein synthesis (Goulart-Silva *et al.* 2011, Bargi-Souza *et al.* 2013).

Previous studies regarding T₃ effects on the Tshb mRNA poly(A) tail length have already shown a decrease in its polyadenylation degree in hypothyroid pituitary cell cultured with T₃ for 8 h and this reduction was more pronounced after 24 h (Krane *et al.* 1991, Staton & Leedman 1998). Euthyroid animals treated for 4 h with higher doses of T₃ also presented a reduction of Tshb mRNA poly(A) tail length (Krane *et al.* 1991).

In hypothyroidism, we have also observed an increase in the Cga mRNA content and in its association with ribosomes, indicating a rise in the translational rate of CGA. Thirty minutes after T₃ administration, the content of Cga mRNA seen in the polysome fraction was reduced suggesting that the translation of both alpha and beta subunits is posttranscriptionally modulated by T₃ in hypothyroid animals (Bargi-Souza *et al.* 2015).

In contrast to the results observed for Tshb, the poly(A) tail length of Cga was not altered under different thyroidal conditions, which led us to hypothesize that

T₃ acts through different pathways modulating the elongation of *Tshb* poly(A) tail and the translation of both transcripts. Thus, the regulation of poly(A) tail length by T₃ is specific for *Tshb* and it could be speculated that such specificity would reduce the impact on *Cga* synthesis since this subunit is also important for LH and FSH hormones.

Cytoskeleton as a target of non-genomic actions of T₃: consequences to GH and TSH secretion

The cytoskeleton is directly involved with the mechanical support system of a cell, its shape and the release of molecules stored in secretory granules by exocytosis (Apodaca 2002), being a target of hormones such as T₃ (Banovac & Koren 2000).

The first experiments showing cytoskeletal rearrangement induced by THs were performed in cultured astrocytes. The treatment with T₄ or rT₃ promoted a cytoskeleton reorganization characterized by an increase in polymerized actin content and led to reduction of the DIO2 enzymatic activity in astrocytes (Siegrist-Kaiser *et al.* 1990).

Considering that DIO2 is responsible for the intracellular generation of T₃ from T₄ and that T₃ is an important *Gh* and *Tshb* gene expression modulator (Ortiga-Carvalho *et al.* 2016), our group attempted to investigate the repercussions of hypothyroidism and T₃ effects in the cytoskeleton of pituitary cells and the consequences to GH and TSH secretion (Silva *et al.* 2006, Bargi-Souza *et al.* 2013).

We have observed a marked disarrangement of the cytoskeleton characterized by a deep reduction of actin and tubulin filament staining in anterior pituitary cells of hypothyroid rats and an evident increase in blood vessel diameter (Silva *et al.* 2006, Bargi-Souza *et al.* 2013). Actin and tubulin polymerization leads to the assembly of microfilaments and microtubules, respectively. The latter is involved in the transport of vesicles and granules within the cell and their release to the extracellular milieu (Fokin *et al.* 2014). Thus, changes in the arrangement of the cytoskeleton in pituitary could be associated with alterations in pituitary hormone secretion.

Studies carried out by different research groups have shown a link between the animal thyroid condition and the microtubule polymerization in the central nervous system (Chaudhury *et al.* 1985, Ravindra & Grosvenor 1990, Lorenzo *et al.* 2002, Gutierrez 2012), as well as a reduction in the amount of actin filaments in astrocyte

cytoskeleton (Siegrist-Kaiser *et al.* 1990) in the absence of T₄. The results obtained in the anterior pituitary of hypothyroid rats showed a disarrangement of actin and tubulin filaments, which reinforces the importance of THs in the cytoskeleton integrity of cells.

The repercussions on pituitary hormone secretion were then evaluated by immunohistochemistry. The results showed a decrease in actin filament content (F-actin) in GH immunoreactive cells of hypothyroid rats in which GH granules were predominantly observed at the cell periphery. Thus, cytoskeleton disarrangement in somatotrophs could contribute to the well-established reduction of GH secretion observed in hypothyroidism (Silva *et al.* 2006). However, 30 min after T₃ administration of hypothyroid rats, an increase was observed in the F-actin content mainly near the plasma membrane, characterizing a rapid cytoskeleton rearrangement, even though the total actin protein remained unchanged, which led us to suppose that T₃ only affected actin rearrangement (Silva *et al.* 2006). In parallel, there was a remarkable increase in GH labeling at the perinuclear region and a decrease in GH staining at the cell periphery, strongly indicating that GH was released, which was confirmed by a rapid increase in liver *Igf1* mRNA expression in these animals (Silva *et al.* 2010).

Furthermore, a possible relationship between T₃ effects on the cytoskeleton and the repercussions in GH synthesis cannot be discarded. In fact, the cytoskeleton enables the polysome to anchor to it. Polysomes correspond to the functional units of the translational machinery and they arrest the mRNAs to initiate the translation mechanism. Thus, anchoring polysomes to the cytoskeleton also means attaching and distributing mRNAs to specific cell regions, where they will be translated (Campbell & Hesketh 1996, Veyrune *et al.* 1996, Czaplinski & Singer 2006, Coulon *et al.* 2013). In this sense, the cytoskeleton may be involved in the increased GH synthesis observed after hypothyroid rats had been acutely treated with T₃ and in the marked GH staining at the somatotroph perinuclear region. In the hypothyroid state, immunostaining for GH was almost absent near the nucleus due to the reduced transcriptional rate of the *Gh* gene (Crew & Spindler 1986, Larsen *et al.* 1986, Lavin *et al.* 1988, Samuels *et al.* 1988).

In addition to the changes observed for the actin cytoskeleton, T₃ also promoted a microtubule rearrangement in pituitary as well as an increase in tubulin content and a rapid reduction of the pituitary blood vessel diameter, which, in turn, could alter the dynamics of pituitary blood flow (Bargi-Souza *et al.* 2013). Considering

the role of microtubules in hormone secretion, our findings strengthen the important role of THs in the secretion of pituitary hormones consequently affecting metabolism, growth and organic system functions, considering the central position of pituitary in the control of other endocrine glands' activity.

Different from the somatotrophs, no colocalization of TSH and F-actin or microtubule labeling was observed. In fact, despite F-actin and microtubule disarrangement in the pituitary in hypothyroidism, TSH secretion is highly increased. Indeed, the immunohistochemistry and electron microscopy analysis of hypothyroid rat pituitaries showed strong TSH staining near and inside the pituitary blood vessels which is in accordance with the high serum TSH concentration detected in the hypothyroid animals (Bargi-Souza *et al.* 2013).

In contrast, T₃ promptly reduced TSH labeling inside blood vessels and promoted a rapid redistribution of TSH granules in thyrotrophs, increasing their abundance in the whole cytoplasm and reducing their presence in regions close to the plasma membrane. These findings indicate a rapid T₃ effect inhibiting TSH secretion (Bargi-Souza *et al.* 2013).

Recently, studies from our group have shown that the CGA subunit content and secretion are also regulated by T₃ (Bargi-Souza *et al.* 2015). In hypothyroidism, the CGA amount in the pituitary is decreased and, considering only thyrotrophs, this reduction was even more evident. Moreover, T₃ acute administration in physiological and supraphysiological doses promoted a rapid increase in pituitary CGA intracellular content which is highly suggestive of a blockage in CGA secretion, since the *Cga* mRNA content in polysomal fractions was rapidly reduced, suggesting a reduction in CGA protein synthesis (Bargi-Souza *et al.* 2015).

Indeed, studies carried out by our group in anterior pituitary primary culture showed a reduction of CGA content in the cell culture medium after acute T₃ treatment, reinforcing the inhibitory T₃ action on CGA secretion (Bargi-Souza *et al.* 2015). These data together with the observation that acute T₃ administration reduces the *Cga* mRNA translation rate strengthen the evidence that T₃ acts non-genomically reducing the synthesis and secretion of CGA, which might also impact LH and FSH synthesis and secretion, as suggested (Krassas & Pontikides 2004, Romano *et al.* 2013). Hence, the increase in pituitary TSHB and CGA contents acutely triggered by T₃ strongly indicates a rapid inhibition of TSH secretion. In this sense, studies carried out in GH4C1 pituitary cells shows that

T₃ increases the activity of the Potassium Voltage-Gated Channel (KCNH2) by non-genomic mechanisms that involve the PI3 kinase pathway activation, antagonizing the thyrotropin-releasing hormone effects on PRL secretion (Storey *et al.* 2006).

Interaction between cytoskeleton and mRNAs

Transcripts anchored to the cytoskeleton may be positioned in specific regions of the cell to be translated where the new protein may exert its function. In fact, the actin transcript has been shown to be attached to cytoskeleton and addressed to cell periphery to be translated to monomeric actin (G-actin), which could be polymerized to F-actin (Bassell *et al.* 1998, Eom *et al.* 2003).

The mRNA interaction with the cytoskeleton may alter transcript stability, which, in turn, affects protein synthesis efficiency (Kosmidou *et al.* 2007, Challa & Stefanovic 2011). T₃ increases the *Gh* transcript stability by adding adenine residues to the 3' end of its poly(A) tail and the F-actin content; both alterations could contribute to increased GH protein synthesis. On the other hand, there are reports showing that some mRNAs become more stable after cytoskeleton disarrangement as it occurs with proinflammatory cytokine mRNAs, expressed on airway epithelial cells, whose translation rate is increased under this condition (van den Berg *et al.* 2006). Based on these findings, we can infer that actin and microtubule disorganizations, both observed in the hypothyroid rat pituitary, could differentially regulate GH and TSH synthesis and secretion.

As mentioned earlier, mRNAs are anchored to the cytoskeleton through interaction with specific proteins. EF1 alpha is one of these proteins, and it is worth mentioning that it has an important role in the protein synthesis elongation phase by transporting the aminoacyl-tRNA to the ribosome via a GTP-dependent mechanism (Greganova *et al.* 2011).

As pointed out, in hypothyroid states, the actin cytoskeleton of pituitary cells is disarranged. Besides that, we observed a reduction of both EF1 alpha and *Gh* mRNA in the F-actin fraction of hypothyroid rat pituitaries (Silva *et al.* 2010), which indicates a reduction of GH synthesis. In fact, the polysome profile analysis of hypothyroid rat pituitary showed a decrease in *Gh* mRNA amount in polysomes. Even though there is a low rate of *Gh* gene transcription in hypothyroid states, we cannot exclude the contribution of posttranscriptional mechanisms

to this event since EF1 alpha and *Gh* mRNA binding to F-actin cytoskeleton was shown to be reduced (Silva *et al.* 2010). In fact, T_3 acutely administered to hypothyroid rats rapidly increased the EF1 alpha and *Gh* mRNA amount in the F-actin cytoskeleton, the *Gh* mRNA content in the polysome fraction and promoted cytoskeleton rearrangement (Silva *et al.* 2006, 2010). These events may favor the increase in GH synthesis, and could explain the increase in GH labeling in the perinuclear region of somatotrophs, both observed in pituitary of hypothyroid rats acutely treated with T_3 .

Final considerations

In summary, in parallel to the transcriptional regulation of *Gh*, *Tshb* and *Cga* genes triggered by triiodothyronine which was for a long period considered the unique mechanism for regulation of GH and TSH synthesis

and consequently secretion, T_3 acts non-genomically on thyrotrophs and somatotrophs regulating the polyadenylation of *Gh* and *Tshb* mRNAs, and GH and TSH translation/synthesis and secretion. These findings significantly increase the comprehension on novel aspects of the negative feedback mechanism exerted by T_3 on thyrotrophs, as well as add new elements to the understanding of T_3 induction of *Gh* gene expression and secretion which are processes that are considered to be mainly regulated by growth hormone-releasing hormone. These non-genomic actions of T_3 on GH and TSH synthesis/secretion may have an important physiological relevance for the optimal control of the rate of GH and TSH basal secretion, minute by minute. Figure 1 summarizes the alterations observed in GH and TSH expressions under hypothyroid conditions and the non-genomic actions triggered by T_3 modulating GH and TSH synthesis/secretion.

Figure 1

Illustration of GH (A) and TSH (B) synthesis and secretion in hypothyroid states and summary of the non-genomic actions of triiodothyronine (T_3) on somatotrophs (C) and thyrotrophs (D). The actin and tubulin cytoskeleton of the whole pituitary is disarranged in thyroidectomized (TX) rats (A and B) and are rapidly rearranged by T_3 , which increases F-actin and alpha tubulin expression and microtubule polymerization (C and D). In hypothyroidism, *Gh* mRNA content and translation rates are reduced in somatotrophs. GH content is decreased and found in scarce vesicles close to the plasma membrane (A). In thyrotrophs (B), *Tshb* and *Cga* mRNA contents are increased, as well as *Tshb* poly(A) tail length. *Tshb* and *Cga* mRNA translation rates are increased under hypothyroid conditions, while intracellular TSHB and CGA protein contents are reduced due to the elevated TSH secretion rate evidenced by the increased serum TSH concentration and the increased TSHB labeling at the periphery of thyrotrophs and inside the blood vessels. T_3 acutely (30 min) increases *Gh* mRNA content and polyadenylation raising the efficiency of *Gh* translation. The enhanced labeling of GH in the cytoplasm mainly in the perinuclear region and the increased hepatic *Igf1* mRNA content are strong indicators of increased GH synthesis (C) and secretion. In thyrotrophs (D), the contents of both *Tshb* and *Cga* mRNAs are rapidly reduced by T_3 , as well as the *Tshb* mRNA poly(A) tail length. The translation rates of both *Tshb* and *Cga* mRNA are promptly reduced and TSHB and CGA protein contents are increased in response to a rapid inhibition of TSH secretion triggered by T_3 through non-genomic mechanisms. Thus, both genomic and non-genomic actions of T_3 positively and negatively modulate the synthesis and secretion of GH and TSH, respectively.

Figure 2

Physiological response to thyroid hormone (TH) actions. The non-genomic actions of TH are triggered by triiodothyronine (T_3), thyroxine (T_4), rT_3 and diiodothyronine (T_2). These actions have shown to affect the expression and activity of many target genes and proteins inducing rapid (few minutes) adjustments in cellular basal activity by activating different intracellular signaling pathways. In parallel to non-genomic actions, TH genomic actions, which are mainly triggered by T_3 , promote long-term control of the cellular activity increasing or reducing the transcription rate of T_3 target genes. Cellular responses to genomic actions are late compared to non-genomic actions since they depend on the changes in the transcriptional machinery activity as well as on specific transcriptional factors. The final cellular physiological response to TH is a result of a complex mechanism involving both genomic and non-genomic actions. This figure also highlights the overlap of genomic and non-genomic actions of T_3 .

It is worth mentioning that the non-genomic actions elicited by T_3 precede and are in line with its transcriptional actions on both genes since they lead to a positive and negative regulation of GH and TSH, respectively. Other genes/proteins that are targeted by T_3 also present an overlap of non-genomic and genomic mechanisms. The T_4 - $\alpha V\beta 3$ interaction promotes $\alpha V\beta 3$ internalization (Lin *et al.* 2013) and the αV monomer forms a complex with MAPK-p300 inside the cell that binds to the *Thrb1* promoter increasing its transcriptional rate (Hammes & Davis 2015). The increased amount of THRB1, in turn, increases the response to genomic actions of T_3 mediated by this isoform (Hammes & Davis 2015).

Thus, non-genomic actions are responsible for rapid adjustments of cellular basal activity, whereas in parallel, genomic actions promote long-term control of cellular activity, being responsible for the specific properties of a tissue/system (Hammes & Davis 2015). In this sense, the physiological response to TH is the result of both genomic and non-genomic actions, as illustrated in Figure 2.

The understanding of non-genomic molecular pathways activated by T_3 would provide new insights into the physiological role of TH at a cellular level enabling the development of new analogs that at certain degree could compensate the lack of proper TH regulation observed in THR mutation disorders or specific antagonists to avoid tumor cell proliferation and angiogenesis, triggered by non-genomic actions of T_4 through interaction with $\alpha V\beta 3$ integrin at the plasma membrane (Hammes & Davis 2015).

It is noteworthy to mention that the literature on the non-genomic actions of TH is recent and there is

still much to be investigated. Moreover, some actions are observed in a period of time of only a few seconds to minutes, which represents a limitation for clinical studies. The development of tools to explore the non-genomic actions in endocrine and metabolic disorders is also required. The use of microarray, RNA-Seq, proteomics and functional assays would also be useful to characterize/describe new non-genomic actions triggered by THs. Due to all these reasons, clinical applications of non-genomic actions are still unclear.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as hindering the impartiality of this review.

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