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T3 rapidly modulates TSHβ mRNA stability and translational rate in the pituitary of hypothyroid rats

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ABSTRACT

Whereas it is well known that T3 inhibits TSHβ gene transcription, its effects on TSHβ mRNA stability and translation have been poorly investigated. This study examined these possibilities, by evaluating the TSHβ transcripts poly(A) tail length, translational rate and binding to cytoskeleton, in pituitaries of thyroidectomized and sham-operated rats treated with T3 or saline, and killed 30 min thereafter. The hypothyroidism induced an increase of TSHβ transcript poly(A) tail, as well as of its content in ribosomes and attachment to cytoskeleton. The hypothyroid rats acutely treated with T3 exhibited a reduction of TSHβ mRNA poly(A) tail length and recruitment to ribosomes, indicating that this treatment decreased the stability and translation rate of TSHβ mRNA. Nevertheless, acute T3 administration to sham-operated rats provoked an increase of TSHβ transcripts binding to ribosomes. These data add new insight to an important role of T3 in rapidly regulating TSH gene expression at posttranscriptional level.

1. Introduction

Thyroid hormones (THs) act at the transcriptional level to regulate gene expression. Most of THs response genes are positively (Ishiguro et al., 2010; Calvo and Obregon, 2009), while few are negatively regulated by T3. TSH gene is one of THs down-regulated genes, and its expression has been shown to be controlled by T3 at the transcriptional and posttranscriptional levels (Yusta et al., 1998; Staton and Leedman, 1998), being the latter poorly explored. Usually, the posttranscriptional actions do not depend on THs binding to the nuclear receptors (TRs), are achieved in a short period of time, and occur even in the presence of drugs that block gene transcription, which point out to a non-genomic action of THs (Siegrist-Kaiser et al., 1990).

In fact, some binding sites for THs have been identified at the cytosol, plasma membrane and some organelles, like endoplasmic reticulum and mitochondria (Cao et al., 2009; Verga Falzacappa et al., 2009; Davis et al., 2005, 2009; Cheng et al., 1987; Psarra et al., 2006), and were shown to be involved in the rapid actions of THs, including those on the mRNA processing. Indeed, some pituitary transcripts have their stability modulated by THs, as GH and TSH, whose half-lives increase and decrease after T3 treatment, respectively (Diamond and Goodman, 1985; Krane et al., 1991).

The posttranscriptional modulation of gene expression requires proteins which recognize specific sequences at the end of 3′-untranslated region (3′-UTR) of the transcript, making it more or less sensitive to degradation (Nechama et al., 2009). Other proteins, which are responsible for the transcript poly(A) tail lengthening and integrity (Misquitta et al., 2006) were also shown to be involved with this event.

It is recognized that transcripts with long poly(A) tails are more stable and efficiently translated, and, as a consequence, they can generate more protein. However, this process depends, among other factors, on the transcript poly(A) tail association to translational factors, and of its arrestment to the ribosomal subunits (Algire and Lorsch, 2006; Kimball et al., 1999; Stipanuk, 2007), process in which the cytoskeleton plays a key role.

It is known that the association of the transcripts to cytoskeleton depends on specific proteins, like the elongation factor of polypeptide chain 1A (EF1A) (Liu et al., 2002). This process confers stability to the transcripts, and also allows them to be transported to different regions of the cells, where they can be translated (Mohr and Richter, 2004; Shestakova et al., 1993a,b).

Indeed, GH transcripts have their stability and translational rate rapidly increased in parallel to cytoskeleton rearrangement, in rats acutely treated with T3 (da Silva et al., 2006, 2010). Moreover, GH gene expression is positively regulated by thyroid hormone at both the transcriptional and posttranscriptional level.

Taking into account that TSH is the most important regulator of thyroid function, and that the gene that encodes TSHβ is down-regulated by T3 at the transcriptional level, we attempted to investigate whether T3 could rapidly act on TSHβ gene expression at the posttranscriptional level, modifying the stability and translation rate of TSHβ transcripts.

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2. Materials and methods

2.1. Materials

T3, methylmercaptoimidazole and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyvinylidene difluoride filters (PVDF Hybond-P) was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rhodamine-phalloidin was purchased from Molecular Probes (Eugene, OR, USA). The commercial kit RIA-*gnost* T3 was obtained from CIS Bio International (Gif-sur-Yvette, FR). TSH concentrations in serum were measured by chemiluminescence immunoassay using Luminex xMAP technology (LINCOplex kit; Luminex Corp., Austin, TX, USA). BSA, β-mercaptoethanol, oligo dT, dNTP, 5× First-Strand buffer, reverse transcriptase (M-MLV) and Random Primers DNA Labeling System kit were purchased from Invitrogen Life Technology (Grand Island, NY, Carlsbad, CA and Gaithersburg, MD, USA). GoTaq® DNA Polymerase and 5× Green GoTaq® Flexi Buffer were purchased from Promega (Madison, WI, USA). SYBR® Green PCR master mix was purchased from Applied Biosystems (Foster City, CA, USA). Maxi Script in vitro transcription kit was purchased from Ambion (Austin, TX, USA). All other reagents were purchased from Labsynth (São Paulo, SP, Brazil).

2.2. Animals and treatments

Male Wistar rats weighing 200–250 g were obtained from our own breeding colony and maintained on rat chow and tap water *ad libitum*. They were housed in a room kept at constant temperature (23 ± 1 °C) and on a 12 h-light, 12-h dark (lights on at 0700 h) schedule. The animals were made hypothyroid by surgical thyroidectomy, after being deeply anesthetized with ketamine and xylazine, and received 0.03% methylmercaptoimidazole (MMI), plus 4.5 mM calcium chloride in drinking water during 20 days. The animals were assorted in two experimental groups, named euthyroid sham-operated (SO) and hypothyroid, which were subjected to acute (30 min) T3 administration (100 μg T3 per 100 g BW) (da Silva et al., 2010; Pachucki et al., 1999) or saline. At least three animals per group were used. Rats were weighed and killed by decapitation 30 min after T3 or saline intravenous treatment. The pituitaries were excised and used for the analysis of TSHβ mRNA poly(A) tail length and translation rate, by rapid amplification of cDNA ends – poly(A) test (RACE-PAT) and polysome profile, respectively. The TSHβ transcripts binding to cytoskeleton was also evaluated. The experimental protocol conformed with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation and was approved by the Institute of Biomedical Sciences/University of São Paulo–Ethical Committee for Animal Research.

2.3. Evaluation of TSHβ mRNA poly(A) tail length

TSHβ mRNA poly(A) tail length was measured by RACE-PAT, according standard protocol (Sallés et al., 1999; Serrano-Nascimento et al., 2010). In brief, 200 ng of an oligo(dT) anchor (5′-GCGAGCTCCGCGGCCGCG-T_{12}) were added to 2 μg of total RNA in a sterile RNase-free microfuge tube. The samples were denatured at 65 °C for 5 min, transferred to 42 °C and reverse-transcribed for 60 min, using a mix containing: 10 mM of each dNTP, 5× First-Strand buffer, 0.01 M of DTT, 1 μl of reverse transcriptase M-MLV Reverse Transcriptase (Invitrogen Life Technology). The inactivation of the reaction was performed at 70 °C for 15 min. Three microliters of the RT reaction product were mixed to a reaction buffer containing 25 mM of $MgCl_2$, 10 mM of each dNTP, 25 pmol of each primer [TSHβ 5′-CTGCAAGTGTGGCAAGTGTAA-3′ and oligo(dT) anchor], 1.25 U of GoTaq® DNA Polymerase and 5× Green GoTaq® Flexi Buffer (Promega). The reaction was performed for 40 cycles; each cycle consisted of 95 °C for 30 s (denaturation), 68 °C for 1 min (annealing), and 72 °C for 1 min (extension), followed by a final 7 min elongation step at 72 °C. The PCR-amplified products were submitted to denaturing electrophoretic separation on 2.5% (w/v) agarose gels stained by ethidium bromide. Amplicon sizes were estimated by densitometry and compared with a 100-bp DNA ladder (Invitrogen Life Technologies) using the Image-Quant TL software (Version 2005 Amersham Biosciences). The top of the smear indicated the longest amplified fragment, which represents the poly(A) tail size plus 213 bases upstream, according to the TSHβ primer used. The changes in poly(A) tail length were detected by analyzing the length of the cDNA smears obtained in all experimental groups studied.

2.4. Actin fractionation for the evaluation of TSHβ mRNA bound to F-actin

The protocol was adapted from Posern et al. (2002), Haller et al. (2004), and Liu et al. (2002). In order to evaluate the amount of TSHβ mRNA bound to F-actin the pituitaries were lysed in 0.3 ml ice lysis buffer containing 50 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 20 mM HEPES (pH 7.9), and phalloidin (132 nM), a fungal toxin that specifically binds to and preserves F-actin from depolymerization (da Silva et al., 2010). After 10 min, the homogenate was centrifuged at 100,000 × *g* for 30 min at 4 °C, and the supernatant (non-polymerized actin) and pellet fractions (polymerized actin) were obtained. The pellet, which represents the F-actin fraction, was resuspended in 0.1 ml of actin lysis buffer and sonicated. To investigate the TSHβ mRNA binding to actin cytoskeleton the transcript was taken out from F-actin by guanidine–phenol–chloroform method (Chomczynski and Sacchi, 1987), and submitted to real-time PCR, as described afterwards.

Table 1
RT-PCR primers.

Primers	Sequence
Antisense: TSHβ	5′-GTTGGTTTTGACAGCCTCGT-3′
Sense: TSHβ	5′-GGCAAACTGTTTCTTCCCAA-3′
Antisense: RPL19	5′-TCAGGCCATCTTTGATCAGCT-3′
Sense: RPL19	5′-CAATGAAACCAACGAAATCG-3′

2.5. Polysome profile by sucrose gradient

The protocol was adapted from Fourcroy et al. (1981). A pool of 10 pituitaries was homogenized in 0.6 ml lysis buffer containing 15 mM Tris–HCl (pH 7.4), 25 mM $MgCl_2$, 300 mM NaCl, 1% Triton X-100, 0.1 mg/ml cycloheximide, 1 mM dithiothreitol and 10 mg/ml heparin. After removal of cell debris by centrifugation at 10,000 × *g* for 10 min at 4 °C, the nucleic acid concentration of supernatant was quantified, and 14 AU (absorbance unit) were loaded onto a 10 ml sucrose gradient (7–47% w/v), and centrifuged at 39,000 rpm for 150 min (SW41 Ti rotor – Beckmann). The UV absorbance of the resulting gradients was monitored at 254 nm (Monitor UVis-920; GE HealthcareBio-Sciences AB, Uppsala, Sweden). The gradients were collected into 11 fractions of approximately 1 ml each for RNA isolation, immediately frozen on dry ice, and then stored at −70 °C. For statistical analysis, fractions 6 through 11 were classified as the polysome region. The first discernible ribosomal subunit peak generally fell in fraction 2 or 3. TSHβ mRNA bound to ribosome was determined by Northern blot analysis, as follows (da Silva et al., 2010).

2.6. Isolation of polysomal RNA

For RNA isolation, 1 ml from each polysomal fraction was mixed with 2 ml guanidine thiocyanate 4 M and 3 ml absolute ethyl alcohol, kept overnight at −70 °C and centrifuged at 10,000 × *g* for 20 min at 4 °C for RNA precipitation. RNA pellets were washed with 70% ethyl alcohol and dissolved in diethyl pyrocarbonate (DEPC)-treated water, at 65 °C, for 15 min. Then, 2 mM sodium acetate, ethyl alcohol (1:0.5 v/v) and 45 U of heparin were added to the samples, which were centrifuged at 13,000 rpm for 20 min at 4 °C (5415R – Eppendorf). RNA pellets were washed with 70% ethyl alcohol, dried, denatured with formaldehyde and formamide, and loaded onto a 1.0% agarose/formaldehyde gel for Northern blot analysis (da Silva et al., 2010). This protocol was adapted from Chomczynski and Sacchi (1987). The RNAs of first fractions of polysomal profile were extracted by guanidine–phenol–chloroform method (Chomczynski and Sacchi, 1987).

2.7. Northern blotting

The RNA samples were blotted from the gel to a Hybond N1 membrane (Amersham) by neutral capillary transfer, and baked at 80 °C for 1 h in a vacuum oven. Blots were then probed with ^{32}P-labeled rat TSHβ cDNA by random priming (Random Primers DNA Labeling System kit; Invitrogen Life Technology) for 16 h at 42 °C. The membrane was washed under highly stringent conditions, subjected to autoradiography, and quantified using the Image Master-1D-Pharmacia Biotech SW software. All blots were stripped and re-hybridized with a ^{32}P-labeled RNA probe specific for 18S ribosomal subunit (18S rRNA), synthesized by in vitro transcription (Maxi Script in vitro transcription kit; Ambion), to correct for the variability in RNA loading. The results were expressed as mean ± SEM of TSHβ mRNA/18S rRNA ratio.

2.8. Real-time PCR

This procedure was used to determine the amount of pituitary TSHβ mRNA bound to cytoskeleton. Total RNA extracted from pituitary (Chomczynski and Sacchi, 1987) were treated with RNase-free DNase I (Invitrogen Life Technology), and 2 μg of RNA were reverse-transcribed in a reaction containing oligo dT (100 μg/ml); 10 mM dNTP; 5× First-Strand buffer; 2 μl of 200 U M-MLV reverse transcriptase (Invitrogen Life Technology). For real-time PCR amplification, 2 μl of each reverse transcription product was diluted in a reaction buffer containing: 5 μl SYBR® Green PCR master mix (Applied Biosystems) and 900 nM primers in a final volume of 10 μl per sample. All primers used are listed in Table 1. The reaction conditions consisted in two steps at 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of three steps: 20 s denaturation at 95 °C, 60 s annealing at 58 °C, and 20 s at 72 °C. The threshold cycle (CT) of the real-time RT-PCR was measured in duplicate for each sample, and the threshold was set up at the beginning of the amplification, in the logarithm scale. The CT value represents the PCR cycle number at which the increase fluorescence intensity of amplified RT-PCR products of the target gene is exponential, and correlates inversely to the amount of the target mRNA in the sample. A ΔCT value was calculated for each sample by subtracting the CT value of the gene, treated as the reference gene, from the CT value of the gene of interest. To reduce interassay variation, all samples were normalized to the ΔCT value of a control sample derived from the same control animal, for all assays (ΔΔCT). The relative expressions of mRNAs were calculated, using the expression 2-ΔΔCT, and reported as AU. The endogenous control used was the RPL19 gene.