

New insight into the mechanisms associated with the rapid effect of T₃ on *AT1R* expression

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Abstract

The angiotensin II type 1 receptor (AT1R) is involved in the development of cardiac hypertrophy promoted by thyroid hormone. Recently, we demonstrated that triiodothyronine (T₃) rapidly increases AT1R mRNA and protein levels in cardiomyocyte cultures. However, the molecular mechanisms responsible for these rapid events are not yet known. In this study, we investigated the T₃ effect on *AT1R* mRNA polyadenylation in cultured cardiomyocytes as well as on the expression of microRNA-350 (miR-350), which targets *AT1R* mRNA. The transcriptional and translational actions mediated by T₃ on *AT1R* levels were also assessed. The total content of ubiquitinated proteins in cardiomyocytes treated with T₃ was investigated. Our data confirmed that T₃ rapidly raised AT1R mRNA and protein levels, as assessed by real-time PCR and western blotting respectively. The use of inhibitors of mRNA and protein synthesis prevented the rapid increase in AT1R protein levels mediated by T₃. In addition, T₃ rapidly increased the poly-A tail length of the *AT1R* mRNA, as determined by rapid amplification of cDNA ends poly-A test, and decreased the content of ubiquitinated proteins in cardiomyocytes. On the other hand, T₃ treatment increased miR-350 expression. In parallel with its transcriptional and translational effects on the *AT1R*, T₃ exerted a rapid posttranscriptional action on *AT1R* mRNA polyadenylation, which might be contributing to increase transcript stability, as well as on translational efficiency, resulting to the rapid increase in AT1R mRNA expression and protein levels. Finally, these results show, for the first time, that T₃ rapidly triggers distinct mechanisms, which might contribute to the regulation of AT1R levels in cardiomyocytes.

Journal of Molecular Endocrinology (2012) **49**, 11–20

Introduction

Thyroid hormones (THs), in addition to elevating the basal metabolism, stimulate the synthesis of cardiac proteins, which results in a significant increase in cardiac muscle mass (Kahaly & Dillmann 2005, Pantos *et al.* 2008, Dillmann 2009). A growing body of evidence indicates that the renin–angiotensin system (RAS) has a critical role in the development of cardiac hypertrophy found in hyperthyroidism and the local RAS, present in the cardiomyocytes, is directly implicated in this process (Diniz *et al.* 2009, Carneiro-Ramos *et al.* 2010). In this sense, the contribution of the RAS in this pathological condition has been demonstrated by several studies reported in the literature, which showed that AT1R receptor blockade and angiotensin-converting enzyme inhibition attenuate or prevent the development of cardiac hypertrophy induced by THs (Kobori *et al.* 1997, Asahi *et al.* 2001, Basset *et al.* 2001, Hu *et al.* 2003, Pantos *et al.* 2005).

For a long time, TH effects were considered to be mediated by its interaction with TH receptors present on specific regions of target genes, by means of which

it can modulate gene expression (Ribeiro *et al.* 1995, Pantos *et al.* 2008). Although the effects of THs that are mediated by the modulation of gene expression are known to occur with a time lag of hours or even days, research that has been carried out mainly in the past decade has identified other responses to TH that are more rapid and take place in seconds or minutes (Davis & Davis 2002, Hiroi *et al.* 2006, Davis *et al.* 2008). 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 to a nongenomic action of THs (Siegrist-Kaiser *et al.* 1990). In fact, some binding sites for THs have been identified in the cytosol, plasma membrane, and some organelles, like endoplasmic reticulum and mitochondria (Cheng *et al.* 1987, Davis *et al.* 2005, Psarra *et al.* 2006, Cao *et al.* 2009, Verga Falzacappa *et al.* 2009), and have been shown to be involved in the rapid actions of THs, including those on mRNA processing.

We have recently demonstrated that the angiotensin II type 1 receptor (AT1R) is a critical mediator of the ability of triiodothyronine (T₃) to induce

cardiomyocyte hypertrophy as well as the rapid effect that T_3 has on the activation of the Akt/GSK-3 β /mTOR signaling pathway (Diniz *et al.* 2009). However, the molecular mechanisms underlying these rapid T_3 -promoted events are not yet known. Considering that this effect was achieved in a short period of time (5 or 10 min) and that there is no consensus sequence for TH receptor binding in the *AT1R* gene promoter, we hypothesize that this action could occur as a consequence of posttranscriptional effects. In this sense, besides regulating the transcription of several genes, it has been found that THs may also have posttranscriptional effects on the stability of several mRNAs (Poddar *et al.* 1996, Staton & Leedman 1998, Kamegai *et al.* 2001, Liu & Waxman 2002, Danzi & Klein 2005, Minamisawa *et al.* 2006, Silva *et al.* 2010). One of the posttranscriptional mechanisms that influence the stability of mRNA is the increase in mRNA poly-A tail length, which protects the mRNA from degradation by RNases (Salles *et al.* 1999). Then, the increase in mRNA poly-A tail length followed by an improvement of the transcript stability and translation might contribute to the high *AT1R* synthesis observed in hyperthyroidism.

An additional mechanism that tightly controls the level of many key proteins involved in transcriptional regulation is the ubiquitin–proteasome system (UPS), the major pathway for intracellular protein degradation in eukaryotic cells. Activation of the UPS has been described in different models of cardiac hypertrophy, and its inhibition may prevent and reverse cardiac hypertrophy and remodeling *in vivo* (Schlossarek & Carrier 2011). Then, with a few known exceptions, polyubiquitination is required for a protein to be degraded by the proteasome (Su & Wang 2010), which consequently influences the regulation of protein content in cells. Considering the effects of UPS on different models of cardiac hypertrophy, the effect of T_3 on the content of ubiquitinated proteins in cardiomyocytes was also evaluated in this study.

Indeed, recent studies have uncovered a potentially important role for a family of small RNAs, known as microRNAs (miRs), in the regulation of gene expression at the posttranscriptional level by mRNA degradation, translation repression, or miR-mediated mRNA decay (Cordes & Srivastava 2009). The miRs have been implicated in the control of diverse biological and pathological processes, including cell differentiation, apoptosis, cell proliferation, and organ development (Plasterk 2006). Bioinformatic approaches have been used to predict that each miR may regulate hundreds of targets, indicating their important role in most biological processes (Elia *et al.* 2009). In this regard, a recent study demonstrated that miR-155 interacts with the 3'-UTR of the human *AT1R* mRNA and inhibits the expression of the *AT1R* in fibroblasts (Martin *et al.* 2006). Moreover, a search

for predicted miR targets indicated that the rat *AT1R* is a target of miR-350. Considering this finding, we hypothesized that T_3 might modulate this miR in cardiomyocytes, which could be indirectly contributing to the increase in *AT1R* levels after rapid T_3 treatment.

Taking this into account, in this report, we attempted to investigate whether TH could rapidly trigger distinct mechanisms to regulate the *AT1R* levels in cardiomyocytes. We observed for the first time that, in parallel with its transcriptional and translational effects on the *AT1R*, T_3 also exerts a rapid posttranscriptional action on *AT1R* mRNA polyadenylation, which might be contributing to increased transcript stability, as well as translational efficiency, resulting in the rapid increase in *AT1R* mRNA expression as well as in its protein levels. Additionally, our study demonstrates that T_3 rapidly reduces the ubiquitin protein content and, on the other hand, increases miR-350 expression in cardiomyocyte cultures. Finally, this study shows, for the first time, some mechanistic insights into the rapid effect induced by T_3 on *AT1R* levels.

Materials and methods

Primary cultures of cardiomyocytes

All protocols were performed in accordance with the Ethical Principles in Animal Research set forth by the Brazilian College of Animal Experimentation and were approved by the Biomedical Sciences Institute/USP Ethics Committee for Animal Research. Primary cultures of neonatal rat ventricular cardiac myocytes were prepared by enzymatic disaggregation, as described previously (Barreto-Chaves *et al.* 2000). Briefly, hearts from neonatal (1- to 3-day old) Wistar rats were excised and ventricles were minced and subjected to multiple enzymatic digestions at 37 °C using a mixture of collagenase type 2 (Worthington, Lakewood, NJ, USA) and pancreatin (Gibco). Dissociated cells were layered on a discontinuous Percoll (Amersham Biosciences) density gradient, consisting of two phases. Myocytes migrated to the interface of the discontinuous layers, while other cells migrated to the surface of the layer of Percoll. The myocytes were collected and washed to remove all traces of Percoll. Cell viability was estimated by the Trypan blue method, after which the cells were counted and plated. Myocytes were cultured in DMEM (Gibco) containing 5% newborn calf serum (NCS; Gibco) and 10% horse serum (Gibco), supplemented with antibiotics (penicillin and streptomycin; Gibco). The cells were maintained at 37 °C under humidified conditions of 95% air and 5% CO₂.

Treatment of the cells

Seventy-two hours after plating, the cells were transferred to DMEM containing NCS 0.5% and maintained overnight. Then, the medium was discarded and the cells were incubated with serum-free medium (control cells) or serum-free medium containing T₃ (10 nmol/l; Sigma) for 5, 10, 15, and 30 min. Moreover, the cardiomyocytes were incubated for 15 min with the translational inhibitor cycloheximide (Cyclo 5 µg/ml; Sigma) or with the transcription inhibitor actinomycin D (Actino 5 µg/ml; Sigma) (Frias *et al.* 2007) before stimulation with T₃ (10 nmol/l) for 5 min.

Rapid amplification of cDNA ends poly-A test

The *AT1R* mRNA poly-A tail length was determined by a rapid amplification of cDNA ends poly-A test (RACE-PAT), according to a standard protocol (Salles *et al.* 1999) used previously (Serrano-Nascimento *et al.* 2010). Initially, 200 ng oligo(dT) anchor (5'-GCGAGCTCCGCGGCCGCG-T12) were added to 2 µg total RNA in a sterile RNase-free microfuge tube. The samples were denatured at 70 °C for 5 min, transferred to 42 °C, and reverse transcribed for 60 min, using a solution containing 10 mmol/l dNTPs mix, 5× first-strand buffer, 0.01 mol/l dithiothreitol (DTT), and 1 µl of 200 U/µl reverse transcriptase MMLV (Invitrogen Life Technologies). Three microliters of the reverse-transcribed reaction product were mixed with a reaction buffer containing 25 mmol/l MgCl₂, 10 mmol/l dNTPs mix, 25 pmol/l of each primer (*AT1R*: 5'-TTC CAT ACA GTC TGC CTT GCT-3' and anchor: 5'-GCGAGCTCCGCGGCCGCG-T12-3'), 1.25 U Taq DNA polymerase (Invitrogen), and 10× Taq DNA polymerase buffer. The nucleotide location of the *AT1R* primer within the *AT1R* mRNA reference sequence is from 2055 to 2075 nucleotides. The reaction was performed for 40 cycles and the PCR cycling conditions were as follows: 95 °C for 30 s (denaturation), 64 °C for 1 min (annealing), and 72 °C for 1 min (extension) followed by a final 7 min elongation step at 72 °C. The amplification products were separated by electrophoresis in a 2.5% agarose gel containing ethidium bromide. Amplicon sizes were estimated by densitometry and compared with a 100 bp DNA ladder (Invitrogen) using an image analysis system. The PCR-amplified mRNAs appeared as a smear indicating that there was a mixture of *AT1R* mRNA with different poly-A tail lengths. The top of the smear of each sample indicated the longest amplified fragment, which represents the poly-A tail size plus 106 bases upstream, according to the *AT1R* primer used. The poly-A tail length of the *AT1R* mRNA was expressed as a percentage of the control value.

Real-time RT-PCR

Total RNA was obtained using the Trizol reagent (Invitrogen) following the manufacturer's instructions. For reverse transcription, we employed 1 µg total RNA using SuperScript II Reverse Transcriptase (Invitrogen). Real-time RT-PCR was performed in a thermocycler (Corbett Research, Sydney, NSW, Australia) using the SYBR Green PCR master mix (Invitrogen) according to the manufacturer's recommendations. The primer sequences used for *AT1R* were 5'-CAC TTT CCT GGA TGT GCT GA-3' and 5'-CCC AGA AAG CCG TAG AAC AG-3', while those for β-actin were 5'-AGT TCG CCA TGG ATG ACG AT-3' and 5'-AAG CCG GCC TTG CAC AT-3'. The nucleotide location of the *AT1R* primers within the *AT1R* mRNA reference sequence is from 1041 to 1181 and the nucleotide location of the β-actin primers within the β-actin mRNA reference sequence is from 74 to 143.

Samples were run in duplicate, and the real-time RT-PCR data were normalized to β-actin, as we have previously certified that variations in TH levels do not alter β-actin mRNA levels (Diniz *et al.* 2009, Carneiro-Ramos *et al.* 2010).

miRNA stem loop RT-PCR

miR-350 target prediction was performed with the use of the following algorithm: TargetScan (<http://www.targetscan.org>). The expression of miRs was analyzed by quantitative miR stem loop RT-PCR (TaqMan MicroRNA Assays, Applied Biosystems) as described previously (Thum *et al.* 2007). The RNA enriched for small species was obtained from cells (mirVana miRNA isolation kit; Ambion, São Paulo, SP, Brazil) following the manufacturer's instructions. We used a highly target-specific stem loop structure and reverse transcription primer, and after reverse transcription, specific TaqMan hybridization probes for miR amplification were used. The cDNA was reverse transcribed from RNA samples using specific miR primers from the TaqMan MicroRNA Assays (4381117 for miR-350 and 4395470 for RNU6-1; Applied Biosystems) and reagents from the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's recommendations. PCR products were amplified from cDNA samples using the TaqMan MicroRNA Assay together with the TaqMan Universal PCR Master Mix (Applied Biosystems) following the manufacturer's instructions. Considering that *U6* snRNA has been generally used as an internal control for normalizing the levels of target miRs obtained by RT-PCR (Nie *et al.* 2011, Wang *et al.* 2011), we evaluated the effect exerted by TH on the *U6* snRNA and we have certified that variations in TH levels were not able to modulate *U6* snRNA. Samples were run in triplicate, and the real-time RT-PCR data were normalized to *U6* snRNA.

Western blot analysis

Cell lysates were obtained using digestion buffer (90 mmol/l KCl, 10 mmol/l HEPES, 3 mmol/l MgCl₂⁺, 5 mmol/l ethylenediaminetetraacetic acid, 1% glycerol, 1 mmol/l DTT, 0.04% SDS, 20 mmol/l aprotinin, 20 mmol/l pepstatin, 20 mmol/l leupeptatin, 40 μmol/l phenylmethylsulfonyl fluoride, and 100 mmol/l orthovanadate), and immunoblotting was performed as described previously (Diniz *et al.* 2009). Protein concentrations were analyzed by the Bradford method. Total protein (150 μg) was resolved by electrophoresis on 5% stacking/15% polyacrylamide–SDS gels, and the resolved proteins were transferred to nitrocellulose membrane (Bio-Rad). The membrane was stained with Ponceau solution to demonstrate that the protein concentration was similar in the different lanes. The membrane was then washed with TBST (50 mM Tris, 150 mM NaCl, pH 7.5, and 2% Tween 20) for 10 min at room temperature. After this, the membrane was incubated at 4 °C overnight with polyclonal antibody against the following primary antibodies: AT1R (AT1Ra and AT1Rb, which were used for quantification of *AT1R* expression) and α-actinin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in TBST. A polyclonal antibody against ubiquitin (Boston Biochem, Cambridge, MA, USA) was used to determine the content of ubiquitinated proteins. After washing, the membranes were added to peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham Biosciences) for 1 h at room temperature at a 1:10 000 dilution in TBST. The membrane was again washed with TBST and was incubated with ECL detection reagents (Amersham Biosciences), which produced a chemiluminescence signal that was detected by exposure to X-ray film. The protein bands were quantified using densitometry, and the band density was then calculated and expressed as a percentage of control. The samples were normalized to α-actinin. The multiple bands for the ubiquitinated proteins were quantified using densitometry and then were normalized by the actinin levels and the results were expressed as percentage in relation to control.

Statistical analysis

The data obtained are presented as mean ± s.d. of at least three independent experiments and are expressed as percentages in relation to control. The number of experiments (*n*) refers to the number of different cell extractions and each experiment was performed in triplicate. Data were analyzed using one-way ANOVA, followed by Tukey's *post-hoc* test, and values of *P* < 0.05 were considered statistically significant.

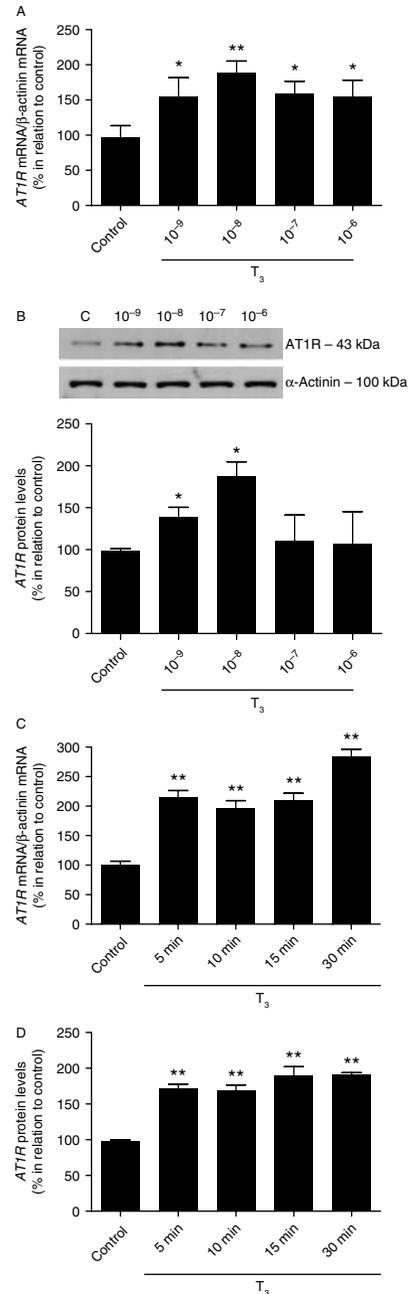


Figure 1 Modulation of AT1R by T₃ in cultured cardiomyocytes. Initially, a dose–response curve of T₃ was performed to evaluate mRNA (A) and protein levels (B) of AT1R in cells treated with serum-free medium in control group or serum-free medium containing different concentrations of T₃ (10⁻⁹ to 10⁻⁶ M/l) for 5 min. Then, the cells were treated with serum-free medium containing 10 nM/l T₃ for 5, 10, 15, or 30 min or serum-free medium alone in control cells. β-Actin mRNA expression was used for normalization of *AT1R* mRNA levels and α-actinin protein expression was used for normalization of AT1R protein levels. Values represent the *AT1R* mRNA/β-actin mRNA level ratio (C), AT1R/α-actinin protein ratio (D). **P* < 0.05; ***P* < 0.01 vs control. Number (*n*) represents the different cardiomyocyte preparations (*n* = 4).

Results

T₃ rapidly raises the AT1R levels in cardiomyocytes

We have recently demonstrated that T₃ induces a rapid increase in AT1R mRNA and protein levels in isolated cardiomyocytes (Diniz *et al.* 2009). In order to investigate the possible mechanisms implicated in these rapid events, the cardiomyocytes were treated with T₃ (10 nmol/l) for 5, 10, 15, and 30 min. Then, the AT1R mRNA expression was quantified by real-time RT-PCR and the protein levels were determined by western blotting (Fig. 1). The dose (10 nmol/l) was chosen based on a dose–response curve of T₃ treatment on AT1R mRNA and protein levels in cardiomyocytes in culture as shown in Fig. 1A and B respectively. As expected, the AT1R mRNA levels were significantly increased in T₃-treated cells ($P < 0.01$ vs control) in comparison with the control values (Fig. 1C). Similarly, the protein levels of AT1R were higher after T₃ treatment for 5 min ($P < 0.01$ vs control) and were maintained through 30 min ($P < 0.01$ vs control) of T₃ treatment (Fig. 1D) when compared with control cells. In order to get further mechanistic insight, we analyzed whether the effect of T₃ upon AT1R levels requires translational and transcriptional actions. To access this information, specific translational and transcriptional inhibitors were used (cycloheximide and actinomycin D respectively). Treatment with 10 nmol/l T₃ along with cycloheximide was able to block the increase in AT1R levels observed in cells treated with T₃. The same result was observed when the cells were treated with T₃ and the specific transcription inhibitor actinomycin D (Fig. 2). These results demonstrate that the rapid increase in AT1R levels in cardiomyocyte cultures induced by T₃ involves transcriptional and translational mechanisms.

T₃ rapidly increases the poly-A tail length of the AT1R mRNA

Considering that T₃ promoted a rapid increase in AT1R mRNA and protein levels and that the length of the poly-A tail of mRNAs is directly related to its stability and translation rate, we have investigated whether this hormone influenced the poly-A tail length of the AT1R mRNA in cardiomyocyte cultures. To address this question, the cells were previously treated with T₃ (10 nmol/l) for 5, 10, 15, and 30 min and subsequently RACE-PAT experiments were performed. The poly-A tail length was significantly increased after T₃ treatment for 5 min (~60%, $P < 0.01$ vs control), and this was sustained through 30 min of T₃ treatment ($P < 0.01$ vs control), as revealed by the analysis of the smearing pattern of PCR products obtained by RACE-PAT (Fig. 3). This analysis showed a higher number of base pairs, which is evidenced by the smear, in the AT1R transcript poly-A tail in T₃-treated cardiomyocytes than

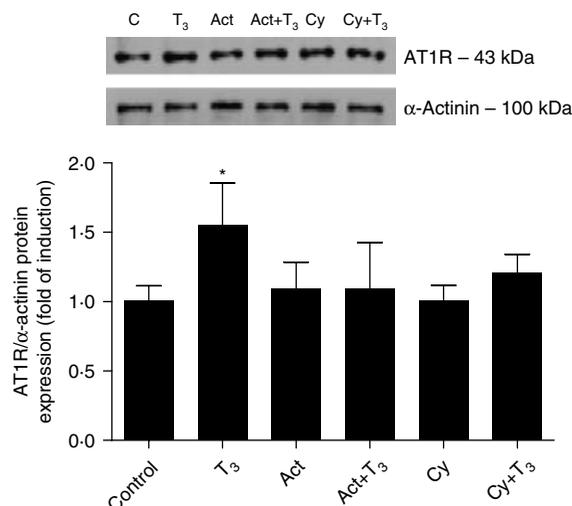


Figure 2 AT1R expression is abolished by cycloheximide and actinomycin D treatments. Cardiomyocytes were incubated for 15 min with the translational inhibitor cycloheximide (Cy, 5 μg/ml; Sigma) or the transcription inhibitor actinomycin D (Act, 5 μg/ml) before stimulation with T₃ (10 nmol/l) for 5 min. AT1R protein levels were analyzed by western blotting and α-actinin protein expression was used as controls for equal loading in cellular extracts. Values represent the AT1R/α-actinin protein ratio. * $P < 0.05$ vs control. Number (*n*) represents the different cardiomyocyte preparations ($n = 4$).

in their respective controls. These results demonstrate for the first time that T₃ rapidly increases the poly-A tail length of the AT1R mRNA in cultured cardiomyocytes.

T₃ rapidly decreases the content of ubiquitinated proteins

It is well known that one of the mechanisms involved in the regulation of protein content is polyubiquitination, which is required for a protein to be degraded by the proteasome (Su & Wang 2010). In order to investigate the possible rapid effect of TH on ubiquitinated protein content, the cardiomyocyte cultures were treated with T₃ (10 nmol/l) for 5, 10, 15, and 30 min. Then, we analyzed the content of ubiquitinated proteins by western blotting using a specific antibody. The cardiomyocytes briefly treated with T₃ showed lower levels of ubiquitinated proteins ($P < 0.01$) when compared with control cells (Fig. 4). These results demonstrate for the first time that T₃ was able to reduce the total level of protein ubiquitination in isolated cardiomyocytes.

T₃ rapidly increases miR-350 levels in isolated cardiomyocytes

Considering that T₃ modulates different miRs in cardiomyocytes and that AT1R is a target of miR-350, we hypothesized that T₃ might modulate this miR in

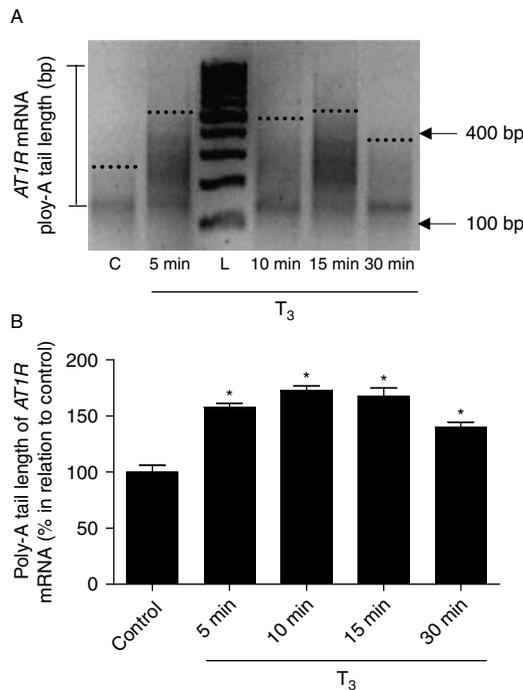


Figure 3 Analysis of the poly-A tail length of the *AT1R* mRNA by RACE-PAT. Image (A) shows a single gel representative of all experiments. Smearing pattern of PCR products of samples in ethidium bromide-stained 2.5% agarose gel. The top of the smear (line broken) indicates the longest amplified fragment, which represents the poly-A tail size plus 106 bases upstream, according to the *AT1R* primer used. Image (B) shows the poly-A tail length of the *AT1R* mRNA expressed as a percentage of control. The cardiomyocytes were treated with serum-free medium in control cells, or with serum-free medium containing 10 nM/1 T_3 for 5, 10, 15, and 30 min. The letter 'L' indicates the DNA ladder of 100 bp. * $P < 0.01$ vs control. Number (n) represents the different cardiomyocyte preparations ($n = 5$).

cardiomyocytes and indirectly contribute to the increase in *AT1R* levels after rapid T_3 treatment. Then, we investigated whether there was an effect exerted by T_3 on miR-350 expression in cardiomyocyte short-term cultures. The cardiomyocytes were treated briefly with T_3 (10 nmol/l) and the miR-350 expression was analyzed by real-time RT-PCR. The miR-350 expression was increased after T_3 treatment for 5 min ($P < 0.05$ vs control), and this increase was maintained through 30 min ($P < 0.05$ vs control) of T_3 treatment (Fig. 5). This result shows that T_3 rapidly increases the miR-350 in isolated cardiomyocytes.

Discussion

We have recently reported that T_3 rapidly increases *AT1R* levels in cultured cardiomyocytes (Diniz *et al.* 2009), and this increase may be associated with hypertrophic effect of TH in cardiac tissue. Taking this

into account, this study was designed to gain further insight into the possible molecular mechanisms involved in this effect. In this study, we confirmed that T_3 rapidly increases both the mRNA and protein levels of *AT1R* and demonstrated that different mechanisms might be involved in this rapid effect promoted by TH.

Initially, we analyzed whether the effect of T_3 upon *AT1R* levels involves translational and transcriptional events. The use of inhibitors of mRNA synthesis and protein synthesis showed that the transcriptional and translational mechanisms are involved in the rapid modulation of *AT1R* induced by T_3 in cardiomyocyte cultures. In fact, there are some studies in the literature demonstrating that rat *AT1R* expression is regulated by translational mechanisms via cytosolic proteins that interact with the 5'-UTR of the receptor mRNA (Krishnamurthi *et al.* 1999, Ji *et al.* 2004, Lee *et al.* 2006). Although there is no consensus sequence for TH in the *AT1R* gene promoter, this gene promoter has

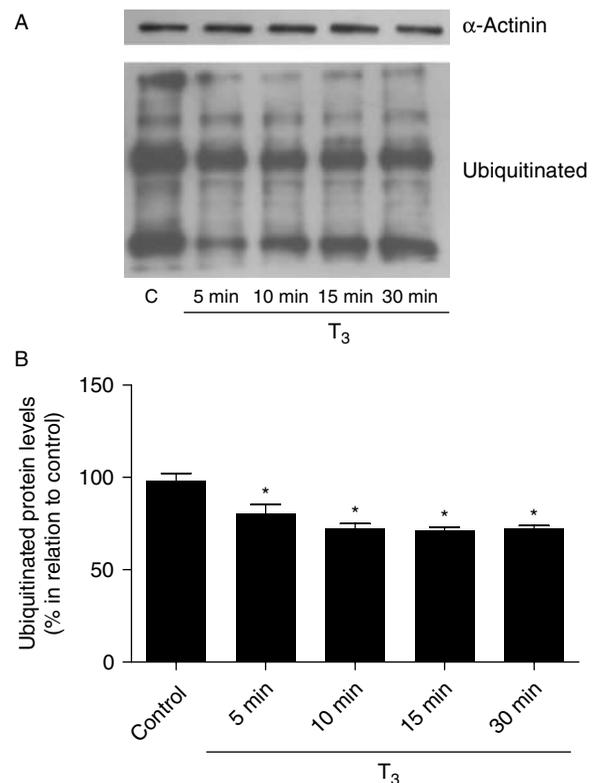


Figure 4 Ubiquitinated protein content in cardiomyocyte cultures determined by western blotting. Image (A) shows a single gel representative of all experiments and image (B) shows the data quantification as a percentage of control. The cells were treated with serum-free medium containing 10 nM/1 T_3 for 5, 10, 15, and 30 min or serum-free medium alone in control cells. The multiple bands for the ubiquitinated proteins were quantified using densitometry and then were normalized by the actinin levels and were expressed as percentage in relation to control. * $P < 0.01$ vs control. Number (n) represents the different cardiomyocyte preparations ($n = 4$).

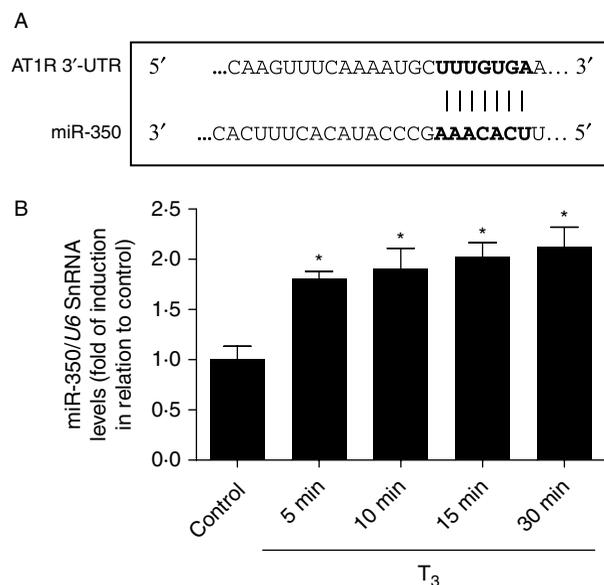


Figure 5 miR-350 targets AT1R. Sequence alignment between miR-350 (bottom) and target region (top) at position 539–545 of the AT1R 3'-UTR (A). miR-350 expression in cultured cardiomyocytes determined by real-time RT-PCR (B). *U6* snRNA was used for normalization of miR-350 expression and the results are expressed as fold of induction in relation to control. The cells were treated with serum-free medium containing 10 nM T₃ for 5, 10, 15, and 30 min or serum-free medium alone in control cells. **P* < 0.05 vs control. Number (*n*) represents the different cardiomyocyte preparations (*n* = 4).

several binding sites for SP1, a transcription factor that interacts with TH receptor binding protein-associated factors and is proposed to stabilize the transcription machinery and to activate transcription (Kim *et al.* 1999).

Considering that T₃ promoted a rapid increase in AT1R levels, we hypothesize that, in parallel with eliciting transcriptional events, T₃ could act posttranscriptionally, influencing the *AT1R* transcript stability, which would contribute to the high AT1R levels. Taking into account that transcript stability is directly correlated with its poly-A tail length, we evaluated whether acute T₃ treatment could increase the length of *AT1R* mRNA. Herein, we demonstrated for the first time that T₃ rapidly increases the polyadenylation of the *AT1R* transcript in isolated cardiomyocytes, suggesting that this posttranscriptional mechanism may be contributing to the rapid T₃-mediated regulation of *AT1R* expression. There is an increasing body of evidence that posttranscriptional rather than transcriptional mechanisms govern modulation of *AT1R* gene expression. In this context, angiotensin II and growth factors, which downregulate *AT1R* gene regulation, cause destabilization of *AT1R* mRNA, whereas insulin and LDL, which lead to upregulation of *AT1R* expression, induce stabilization of the *AT1R* mRNA (Nickenig *et al.* 2001). Poly-A tail elongation is one of the most important posttranscriptional mechanisms

known to increase transcript stability, as it makes the mRNA less sensitive to degradation by RNases (Munroe & Jacobson 1990, Salles *et al.* 1999). The addition of adenine residues to the transcript's 3'-UTR confers stability to the latter, as the poly-A binding protein binds to the poly-A tail, protecting it from exoribonuclease attack (Bernstein *et al.* 1989, Sachs 1990, Ross 1995) and also enabling better attachment of the transcript to the cap region, generating higher translation efficiency (Kapp & Lorsch 2004). The exact means by which T₃ rapidly increases the *AT1R* mRNA polyadenylation is unknown. However, some studies have demonstrated that mRNA stability dependent on the poly-A tail length is regulated by T₃ (Krane *et al.* 1991, Brunetto *et al.* 2012). In parallel to its transcriptional and translational effects on the *AT1R*, T₃ also exerts a rapid posttranscriptional action on *AT1R* mRNA polyadenylation, which might be contributing to increase transcript stability, as well as translational efficiency, resulting in the rapid increase in *AT1R* mRNA expression as well as in its protein levels. In fact, we have detected an increased AT1R protein content in cardiomyocytes treated for 5–30 min with T₃.

It is already well established that the cytoskeleton exerts an important role as a regulator of mRNA stability (Singer 1992, Shannon *et al.* 1998) and that TH acutely induces actin cytoskeleton rearrangement (Silva *et al.* 2006). Moreover, an alternative microtubule-dependent endocytic pathway plays an important role in AT1R-mediated uptake of extracellular angiotensin II (Li *et al.* 2009). Taking this into account, it is possible that the cytoskeleton rearrangement mediated by T₃ might also influence the rapid increase in AT1R levels in cardiomyocytes. This is an important point, and future studies are needed to address this question.

A second aim of this study was to identify whether ubiquitination might be altered after treatment with TH. The UPS represents the main mechanism of degradation of intracellular cytosolic and nuclear proteins, with two major biological functions, i.e. ubiquitylation and proteasome-mediated proteolysis (Etlinger & Goldberg 1977). Briefly, before degradation, a target protein undergoes a three-step process that covalently links a polyubiquitin chain to the substrate. Three enzyme components are involved in this process, Ub-activating enzyme (E1), Ub-conjugating enzymes (E2), and ubiquitin protein ligase (E3), which present substrate recognition sites (Hedhli & Depre 2010). Considering that polyubiquitination is an important mechanism required for protein breakdown by the proteasome (Stütt *et al.* 2004, Su & Wang 2010), our results showed that cardiomyocytes treated with T₃ presented a reduction in the polyubiquitinated protein content. Although these results demonstrate relevant insights related to the rapid effect promoted by T₃ on cardiomyocytes cultures, it is not possible to conclude

whether the reduction in the ubiquitination protein content may be contributing to the rapid increase in AT1R levels. However, we cannot discard this possibility.

It is known that in the event of cardiovascular disease, the UPS has been implicated in both protecting and exacerbating cardiac outcomes (Willis *et al.* 2010). Therefore, further experiments should be performed to better understand the involvement of UPS in the rapid action of T₃ in cardiomyocytes and the real participation of each enzyme (E1, E2, and E3) in this process. A recent study evidenced that hyperthyroidism stimulates multiple proteolytic pathways in skeletal muscle (O'Neal *et al.* 2009). In this sense, it is possible that TH may have different effects on the ubiquitination protein levels depending on the tissue and the model used. Considering that modulation on the ubiquitinated protein levels is observed in some models of cardiac hypertrophy (Hedhli & Depre 2010), it is possible to hypothesize that TH-mediated cardiac growth may also be influenced by alteration on the ubiquitinated protein content independent of the AT1R effect *per se*.

Several studies have demonstrated the importance of miRs not only in cardiovascular development but also in cardiovascular diseases (Thum *et al.* 2007, 2008, Wang *et al.* 2009). The miRs have been implicated in the control of diverse biological and pathological processes, including cell differentiation, apoptosis, cell proliferation, and organ development (Plasterk 2006). They are short, noncoding RNA molecules that act as negative regulators of gene expression by inhibiting mRNA translation or promoting mRNA degradation (Bartel 2004).

A search for predicted miR targets indicated that the rat AT1R is a target of miR-350. Considering this finding, we hypothesized that T₃ might modulate this miRNA in cardiomyocytes, which could be eventually contributing to the increase in AT1R levels after rapid T₃ treatment. In this study, we demonstrated for the first time that miR-350 expression is rapidly increased after T₃ treatment in isolated cardiomyocytes. The possible mechanisms responsible for the increase in miR-350 expression by T₃ are unknown. Additionally, whether the altered miR-350 expression observed in cardiomyocytes treated with T₃ is involved in mediating physiological or pathological effects of TH in these cells is also not clear yet. In addition, it is important to emphasize that future experiments should be performed to analyze whether miR-350 is able to directly interact with AT1R mRNA and influences its expression. Indeed, a recent study demonstrated that miR-155 interacts with the 3'-UTR of the human AT1R mRNA and inhibits the expression of the AT1R in fibroblasts (Martin *et al.* 2006). Taking this into account, it is possible that other miRs might contribute to the regulation of AT1R levels, as this transcript may also be a target for other miRs, according to target prediction using the TargetScan algorithm.

Both adult and neonatal cardiomyocytes have been commonly used as *in vitro* models for some underlying cellular and molecular mechanisms of the cardiac tissue. However, adult cardiomyocytes are terminally differentiated primary cells and are therefore difficult to work with owing to their short duration of viability. Although neonatal myocytes are not truly representative of the adult phenotype, these cells are usually cultured at confluence allowing for formation of cell-cell contacts, which provide crucial survival signals (Vlahos *et al.* 2003). Considering that our study was performed in neonatal cardiomyocyte cultures, it is possible that, depending on the model used, TH may have different effects on the mechanisms investigated in this study. In addition, considering that we analyzed only the rapid effect promoted by TH, we may hypothesize that longer treatment with TH may have distinct effects on these different mechanisms.

It is well known that neonatal cardiomyocytes represent partially differentiated cells. Recent findings suggest that TH is a prime driver of prenatal cardiomyocyte maturation (Chattergoon *et al.* 2011). Indeed, angiotensin II is also implicated in the cardiomyocyte differentiation of rat bone marrow mesenchymal stem cells (Xing *et al.* 2012). Whether the increased AT1R levels might be partially contributing to the TH effect on cardiomyocyte maturation is unknown. In this sense, future studies are needed to address this question.

In summary, this study showed for the first time that, in parallel to its transcriptional and translational effects on the AT1R, T₃ also exerts a rapid posttranscriptional action on AT1R mRNA polyadenylation, which might be contributing to increase transcript stability as well as translational efficiency, resulting in the rapid increase in AT1R mRNA expression as well as in its protein levels. Additionally, our study demonstrates that T₃ rapidly reduces the ubiquitin protein content and, on the other hand, increases miR-350 expression in cardiomyocyte cultures. Finally, this study shows, for the first time, that T₃ rapidly modulates distinct mechanisms, which might contribute to the regulation of AT1R levels in cardiomyocyte cultures.

Declaration of interest

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

Funding

This work was supported by the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP, Foundation for the Support of Research in the State of São Paulo; grants 06/61523-7 and 08/01489-5) and the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq, National Council for Scientific and Technological Development).

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Received in final form 3 April 2012

Accepted 23 April 2012

Made available online as an Accepted Preprint 23 April 2012