Short-term effects of triiodothyronine on thyroid hormone receptor alpha by PI3K pathway in adipocytes, 3T3-L1

Efeitos rápidos da triiodotironina no receptor de hormônio tireoidiano alfa pela via PI3K em adipócitos, 3T3-L1

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ABSTRACT

Objective: The present study aimed to examine the effects of thyroid hormone (TH), more precisely triiodothyronine (T3), on the modulation of TH receptor alpha (TRα) mRNA expression and the involvement of the phosphatidyl inositol 3 kinase (PI3K) signaling pathway in adipocytes, 3T3-L1, cell culture. **Materials and methods:** It was examined the involvement of PI3K pathway in mediating T3 effects by treating 3T3-L1 adipocytes with physiological (P = 10nM) or supraphysiological (SI = 100 nM) T3 doses during one hour (short time), in the absence or the presence of PI3K inhibitor (LY294002). The absence of any treatment was considered the control group (C). RT-qPCR was used for mRNA expression analyzes. For data analyzes ANOVA complemented with Tukey's test was used at 5% significance level. **Results:** T3 increased TRα mRNA expression in P (1.91 ± 0.13, $p < 0.001$), SI (2.14 ± 0.44, $p < 0.001$) compared to C group (1 ± 0.08). This increase was completely abrogated by LY294002 in P (0.53 ± 0.03, $p < 0.001$) and SI (0.31 ± 0.03, $p < 0.001$). To examine whether TRα is directly induced by T3, we used the translation inhibitor cycloheximide (CHX). The presence of CHX completely abrogated levels TRα mRNA in P (1.15 ± 0.05, $p > 0.001$) and SI (0.99 ± 0.15, $p > 0.001$), induced by T3. **Conclusion:** These results demonstrate that the activation of the PI3K signaling pathway has a role in T3-mediated indirect TRα gene expression in 3T3-L1 adipocytes. Arq Bras Endocrinol Metab. 2014;58(8):833-7

Keywords
Triiodothyronine; adipocytes; TRα; PI3K

RESUMO

Objetivo: O objetivo do presente estudo foi analisar os efeitos do hormônio tireoidiano (HT), triiodotironina (T3), na modulação da expressão de mRNA do receptor alfa (TRα) de HT e o envolvimento da via de sinalização da via fosfatidilinositol 3-quinase (PI3K) em adipócitos, 3T3-L1. **Materiais e métodos:** Foi examinado o envolvimento da via PI3K nos efeitos do T3 nos tratamentos de adipócitos, 3T3-L1, nas doses fisiológica (P = 10nM) ou suprafisiológica (SI = 100 nM) durante uma hora (tempo curto), na ausência ou na presença do inibidor da PI3K (LY294002). A ausência de qualquer tratamento foi considerada o grupo controle (C). RT-qPCR foi utilizado para analisar a expressão do mRNA. Para as análises dos dados, utilizou-se ANOVA complementada com o teste de Tukey a 5% de significância. **Resultados:** O T3 aumentou a expressão de mRNA de TRα em P (1,91 ± 0,13, $p < 0,001$) e SI (2,14 ± 0,44, $p < 0,001$) em comparação com o grupo C (1 ± 0,08). Esse aumento foi completamente abolido por LY294002 em P (0,53 ± 0,03, $p < 0,001$) e SI (0,31 ± 0,03, $p < 0,001$). Para examinar se a expressão de TRα foi diretamente induzida pelo T3, utilizou-se o inibidor de tradução, ciclohexamida (CHX). A presença de CHX reduziu os níveis de mRNA de TRα em P (1,15 ± 0,05, $p > 0,001$) e SI (0,99 ± 0,15, $p > 0,001$), induzidos pelo T3. **Conclusão:** Esses resultados demonstram que a ativação da via de sinalização de PI3K tem um papel importante na expressão do gene TRα mediada indiretamente pelo T3, em adipócitos 3T3-L1. Arq Bras Endocrinol Metab. 2014;58(8):833-7

Descritores
Triiodotironina; adipócitos; TRα; PI3K

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INTRODUCTION

Thyroid hormone (TH) influences the metabolism and development of adipose tissue (AT) and modulates the proliferation and differentiation of adipocytes (1), mainly 3,5,3'-triiodothyronine (T3) (2). TH also stimulates lipolysis and lipogenesis (3) and is involved in regulation of lipid and carbohydrate metabolism in liver, skeletal muscle and heart tissues (4).

TH receptors (THR) are proteins that belong to the nuclear hormone receptor superfamily and originate from TH receptor alpha (TRα) and TH receptor beta (TRβ) genes (5,6) located on chromosomes 17 and 3 in humans, respectively. These nuclear receptors act on certain genes according to nucleotide sequences called TH responsive elements (TRE) located in the promoter sites of target genes (7). Although THR are primarily nuclear receptors, approximately 10% are located in the cytoplasm (8). All isoforms of THR, TRα1, TRα2, and TRβ1, are expressed in white and brown adipose tissue, being TRα1 the predominant THR isoform (9,10). T3 and other hormones regulate the different TR isoforms.

TH may function through other mechanisms than THR/TRE (11). Alternative mechanisms may be referred as a non-classical or non-genomic because initiation sites are located in the plasma membrane, such as activation of αvβ3 integrin, or the cytoplasm where TH may activate mitogen-activated protein (MAPK) or the phosphatidyl inositol 3-kinase (PI3K) pathway. Initiation sites are proteins that are characterized as iodothyronine receptors (12,13).

As lipid metabolism is closely associated with a number of health problems, the regulation of adipocytes represents an area of emerging interest. So far, TR has been implicated as a major factor in the regulation of the development and function of adipose tissue (14-16). In this study it was assessed the effects of different levels of T3 (physiological and supraphysiological) on TRα gene expression in adipocyte cell culture, 3T3-L1, by real-time PCR (RT-qPCR), during one-hour exposure. The influence of protein synthesis on regulation of TRα transcription by T3 and possible activation of a non-classical pathway (PI3K), by T3, to modulate this gene was also evaluated. Our observations suggest that mRNA TRα levels modulated by T3 depends on activation of the PI3K pathway.

MATERIALS AND METHODS

Chemicals

Isobutylmethylxanthine (IBMX), dexamethasone, insulin, cycloheximide (CHX), triiodothyronine (T3), LY294002 (LY), dimethyl sulfoxide (DMSO), sodium hydroxide (NaOH) and Charcoal Stripped fetal bovine serum (FBS) were purchased from Sigma (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and antibiotic-antimycotic 100X solution were purchased from Gibco BRL (Grand Island, NY, USA).

Cell culture and differentiation

The experimental protocol was approved by the Ethics Committee on Animal Experiments of the Botucatu School of Medicine-Unesp (protocol nº 752).

Mouse 3T3-L1 pre-adipocytes were obtained from the Cell Bank of Rio de Janeiro (Rio de Janeiro, RJ, Brazil) and grow in polystyrene 6-well plates at 37°C in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic 100× solution. Upon cell confluence (designated as day 0), differentiation was initiated with 1 µg/mL insulin, 1 µM DEX, and 0.5 mM IBMX in DMEM containing 10% FBS. After a 4-day incubation, culture media was replaced by DMEM supplemented with 10% FBS and 1 µg/mL insulin, and the cells were fed every two days with DMEM containing 10% FBS. 3T3-L1 cells were fully differentiated by day 8. After differentiation, cells were incubated for 24 hours in DMEM supplemented with 10% charcoal stripped FBS (to deplete T3) and 1 µg/ml insulin. After incubation, cells were treated with physiological T3 (10 nM; P group) or supraphysiological T3 (100 nM; SI) (17-19) for one hour. A non-treated group, that received 0.1% NaOH (diluent T3), served as the control (C). P and SI groups were also treated with CHX (10 µg/mL) (13) and LY (50 µM) (13) for one hour.

LY was used to determine if the non-classical pathway (PI3K) was involved in T3 action on TRα mRNA levels. CHX was used to determine if T3 directly or indirectly modulates TRα during the one-hour exposure.

Oil red O staining

3T3-L1 cells were grown on 6-well plates and induced to differentiate as previously described. After an 8-day incubation (day 8), plates were washed twice with phosphate-buffered saline (PBS), fixed with 37% formalde-

hyde for 30 minutes at room temperature, and washed twice again with PBS. After fixation, cells were stained for two hours at room temperature with a filtered oil red O solution (0.5 g oil red O (Sigma) in 100 mL isopropanol), washed twice with distilled water, and visualized to confirm differentiation.

Gene expression

Whole RNA was extracted from 3T3-L1 cells using Trizol (Invitrogen) according to the manufacturer's instructions. A high capacity cDNA reverse transcription kit for RT-PCR® (Invitrogen, Sao Paulo, Brazil) was used to synthesize 20 µL of complementary DNA (cDNA) from 1,000 ng of whole RNA.

TRα (assay Mm00617505_m1 – Applied Biosystems) mRNA levels were determined by real-time polymerase chain reaction (RT-qPCR). Quantitative measurements were determined with the Applied Biosystems StepOne Plus detection system using a TaqMan qPCR commercial kit (Invitrogen) according to the manufacturer's instructions. Cycling conditions were as follows: enzyme activation at 50°C for 2 min, denaturation at 95°C for 10 min, cDNA product amplification for 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. Gene expression was quantified relative to C group values after normalization by an internal cyclophilin (assay Mm00434759_m1- Applied Biosystems) control using the $2^{-\Delta\Delta Ct}$ method as previously described (20).

Statistical analysis

Gene expression was analyzed using analysis of variance (ANOVA) followed by Tukey's test. Data are expressed as mean ± standard deviation. The significance level was set at 5%.

RESULTS

Different T3 concentrations up-regulation TRα mRNA by PI3K pathway

Figure 1 shows the up-regulation of TRα levels in 3T3-L1 adipocytes, and to verify PI3K pathway involvement in mediating the action of T3 on TRα mRNA expression the P and SI groups were treated with the PI3K pathway inhibitor LY. We showed that LY associated with T_3 led to decreased TRα mRNA levels (Figure 1A and B).

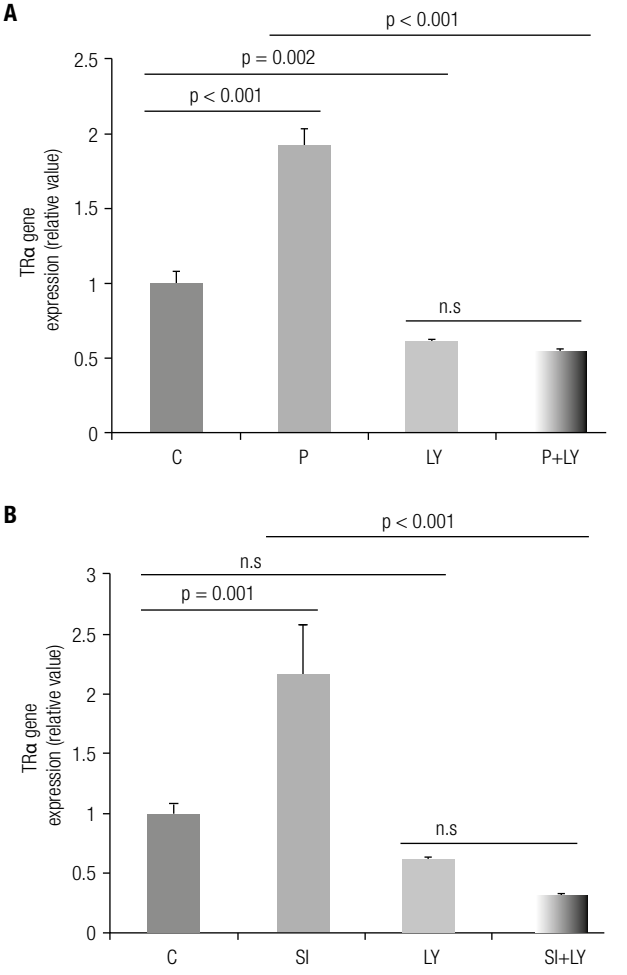

Figure 1. Effects of T3 and LY294002 on modulation of TRα mRNA levels in one hour. P = 10 nM T3, SI = 100 nM T3, C = no T3, and LY = 50 µM LY294002. (**A**) T3 influence (10 nM) on TRα gene expression in the presence/absence of LY. (**B**) T3 influence (100 nM) on TRα gene expression in the presence/absence of LY. ANOVA with Tukey's test. n.s. = non-significant; n = 3 per treatment.

Effect of inhibition of protein synthesis on T3-induced modulation of TRα mRNA levels

3T3-L1 adipocytes were cultured with T3 at doses that influenced TRα mRNA levels to determine the need for protein synthesis during one hour exposure. CHX was added to the P and SI groups. Complete abrogation of the T3-induced mRNA increase by CHX indicates that the TRα gene is indirectly induced by TH and requires prior protein synthesis (Figure 2A and B). However, CHX alone had significant influence on TRα mRNA levels.

DISCUSSION

AT is a target of TH and expresses THR that are important factors in regulating tissue development and func-

tion (16,21). In this paper we evaluated AT responses to different T_3 levels based on TRα mRNA expression without interference from systemic factors that occur *in vivo*. As an experimental model, we used 3T3-L1 cells (embryonic *Mus musculus* cells) that were differentiated *in vitro* into adipocytes. TRα mRNA levels were quantified using RT-qPCR.

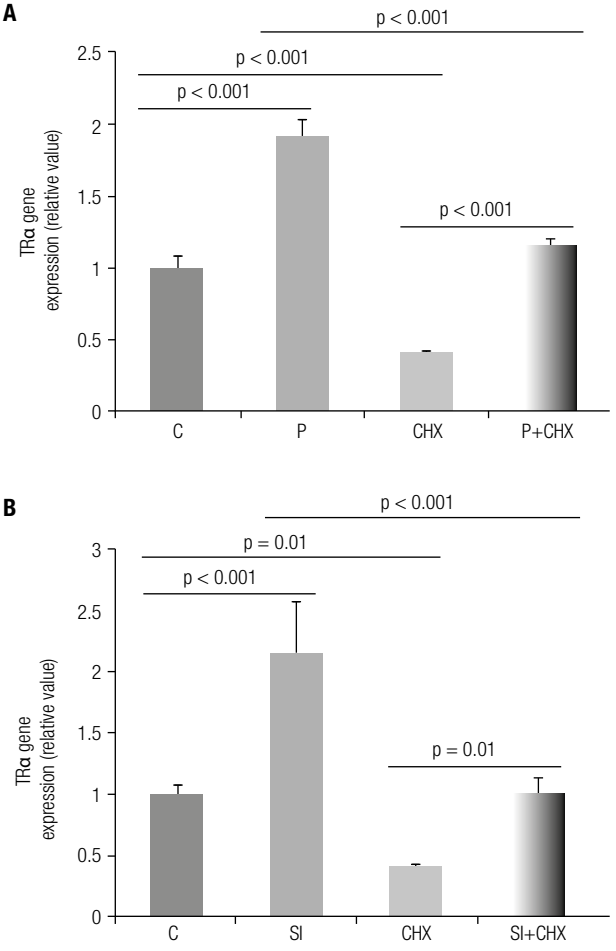

Figure 2. Effects of T3 and CHX on modulation of TRα mRNA levels in one hour. P = 10 nM T3, SI = 100 nM T3, C = no T3, and CHX = 10 µg/mL. (**A**) T3 influence (10 nM) on TRα gene expression in the presence/absence of CHX. (**B**) T3 influence (100 nM) on TRα gene expression in the presence/absence of CHX. ANOVA with Tukey's test. n.s. = non-significant; n = 3 per treatment.

3T3-L1 pre-adipocytes represent a well-established model for adipogenesis (22). TRα expression is higher than TRβ in 3T3-L1 adipocytes (18), which is in accordance with the findings of Jiang and cols. (2004) (23). Studies from our group showed that a T3 physiological and supraphysiological dose, at different times, increase TRα in adipocytes, 3T3-L1 (18).

It is known that TH may act by mechanisms other than the classical TR/Thyroid Hormone Responsive Elements (TRE) (11). These mechanisms can be called non-classical or non-genomic because their initiation sites may be in the plasma membrane, like the activation of integrin αvβ3 pathway, or in the cytoplasm, where the TH activates the mitogen-activated protein kinase (MAPK) or PI3K pathway. The initiation sites are proteins that are characterized as iodothyronine receptors (13,21).

PI3K participates in a wide variety of cellular process, including intracellular trafficking, organization of the cytoskeleton, cell growth and transformation, and prevention of apoptosis (24,25). PI3K has a role in differentiation of several cell lines (26,27), including adipocytes. PI3K pathway activation by TH originates in the cytoplasm and involves TRα or TRβ, resulting in specific gene transcription, including hypoxia inducing factor (HIF-1α), glucose transporter 1 (GLUT1), calcineurin inhibitor (ZAKI4α) and leptin (11,13,21,22). In this study, we used the LY294002 inhibitor to evaluate the need for PI3K pathway activation in modulating TRα mRNA by T3 during a one hour time period. Within the one hour period, the increased on TRα mRNA levels in the P and SI groups were suppressed by LY (Figure 1A and B), proving that pathway activation is necessary for T3 to increase TRα levels. However, the inhibition of PI3K decreased basal TRα mRNA expression demonstrating a need this pathway to TRα mRNA expression in normal cell condition.

In addition, inhibition of protein synthesis with CHX completely blocked T3-induced increase in TRα mRNA levels (Figure 2A and B), showing that it is a gene indirectly up-regulated by T3, depending on the synthesis of a yet unknown protein. Moreover, CHX group (without T3) compared with control group decreases the basal levels of TRα mRNA, indicating the existence of certain short-lived proteins essential for the expression of this gene in normal conditions. Monden and cols. (28) demonstrated that CHX blocks the reduction in TRα levels caused by T3 in HTB-185 cells, suggesting that down-regulation of TRα by T3 requires synthesis of a new protein.

In summary, during one hour of treatment, increased TRα levels in the P and SI groups were indirectly modulated by T3 and depended on activation of the PI3K pathway. This is the first study to demonstrate TRα modulation by T3 in a very short time period (one hour) and assess PI3K pathway activation by TH on this gene in adipocytes, 3T3-L1.

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REFERENCES

1. Darimont C, Gaillard D, Ailhaud G, Negrel R. Terminal differentiation of mouse preadipocyte cells: adipogenic and antimitogenic role of triiodothyronine. Mol Cell Endocrinol. 1993;98:67-73.
2. Ailhaud G, Grimaldi P, Negrel R. Cellular and molecular aspects of adipose tissue development. Annu Rev Nutr. 1992;12:207-33.
3. Oppenheimer JH, Schwartz HL, Lane JT, Thompson MP. Functional relationship of thyroid hormone-induced lipogenesis, lipolysis, and thermogenesis in the rat. J Clin Invest. 1991;87:125-32.
4. Santini F, Marsili A, Mammoli C, Valeriano R, Scartabelli G, Pelosini C, et al. Serum concentrations of adiponectin and leptin in patients with thyroid dysfunctions. J Endocrinol Invest. 2004;27:RC5-7.
5. Williams GR. Cloning and characterization of two novel thyroid hormone receptor beta isoforms. Mol Cell Biol. 2000;20:8329-42.
6. Macchia PE, Takeuchi Y, Kawai T, Cua K, Gauthier K, Chassande O, et al. Increased sensitivity to thyroid hormone in mice with complete deficiency of thyroid hormone receptor alpha. Proc Natl Acad Sci U S A. 2001;98:349-54.
7. Glass CK. Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. Endocr Rev. 1994;15:391-407.
8. Baumann CT, Maruvada P, Hager GL, Yen PM. Nuclear cytoplasmic shuttling by thyroid hormone receptors. Multiple protein interactions are required for nuclear retention. J Biol Chem. 2001;276:11237-45.
9. Hernandez A, Obregon MJ. Presence and mRNA expression of T3 receptors in differentiating rat brown adipocytes. Mol Cell Endocrinol. 1996;121:37-46.
10. Bianco AC, Silva JE. Cold exposure rapidly induces virtual saturation of brown adipose tissue nuclear T3 receptors. Am J Physiol. 1988;255:E496-503.
11. Moeller LC, Broecker-Preuss M. Transcriptional regulation by nonclassical action of thyroid hormone. Thyroid Res. 2011;4 Suppl 1:S6.
12. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. Endocr Rev. 2010;31:139-70.
13. Moeller LC, Dumitrescu AM, Refetoff S. Cytosolic action of thyroid hormone leads to induction of hypoxia-inducible factor-1alpha and glycolytic genes. Mol Endocrinol. 2005;19:2955-63.
14. Flores-Delgado G, Marsch-Moreno M, Kuri-Harcuch W. Thyroid hormone stimulates adipocyte differentiation of 3T3 cells. Mol Cell Biochem. 1987;76:35-43.
15. Chawla A, Lazar MA. Induction of Rev-ErbA alpha, an orphan receptor encoded on the opposite strand of the alpha-thyroid hormone receptor gene, during adipocyte differentiation. J Biol Chem. 1993;268:16265-9.
16. Yen PM. Physiological and molecular basis of thyroid hormone action. Physiol Rev. 2001;81:1097-142.
17. Yoshida T, Monkawa T, Hayashi M, Saruta T. Regulation of expression of leptin mRNA and secretion of leptin by thyroid hormone in 3T3-L1 adipocytes. Biochem Biophys Res Commun. 1997;232:822-6.
18. de Oliveira M, Luvizotto R de A, Olimpio RM, de Sibio MT, Silva CB, Conde SJ, et al. Modulation of thyroid hormone receptors, TRα and TRβ, by using different doses of triiodothyronine (T3) at different times. Arq Bras Endocrinol Metabol. 2013;57:368-74.
19. de Oliveira M, Luvizotto Rde A, Olimpio RM, De Sibio MT, Conde SJ, Biz Rodrigues Silva C, et al. Triiodothyronine increases mRNA and protein leptin levels in short time in 3T3-L1 adipocytes by PI3K pathway activation. PLoS One. 2013;18;8(9):e74856.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25:402-8.
21. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. Endocr Rev. 2010;31:139-70.
22. Green H, Meuth M. An established pre-adipose cell line and its differentiation in culture. Cell. 1974;3:127-33.
23. Jiang W, Miyamoto T, Kakizawa T, Sakuma T, Nishio S, Takeda T, et al. Expression of thyroid hormone receptor alpha in 3T3-L1 adipocytes; triiodothyronine increases the expression of lipogenic enzyme and triglyceride accumulation. J Endocrinol. 2004;182:295-302.
24. Toker A, Cantley LC. Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature. 1997;387:673-6.
25. Vanhaesebroeck B, Leevers SJ, Panayotou G, Waterfield MD. Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci. 1997;22:267-72.
26. Aubin D, Gagnon A, Sorisky A. Phosphoinositide 3-kinase is required for human adipocyte differentiation in culture. Int J Obes. 2005;29:1006-9.
27. Fang J, Ding M, Yang L, Liu LZ, Jiang BH. PI3K/PTEN/AKT signaling regulates prostate tumor angiogenesis. Cell Signal. 2007;19:2487-97.
28. Monden T, Nakajima Y, Hashida T, Ishii S, Tomaru T, Shibusawa N, et al. Expression of thyroid hormone receptor isoforms down-regulated by thyroid hormone in human medulloblastoma cells. Endocr J. 2006;53:181-7.