Expression of thyroid hormone receptor α in 3T3-L1 adipocytes; triiodothyronine increases the expression of lipogenic enzyme and triglyceride accumulation

Wei Jiang, Takahide Miyamoto, Tomoko Kakizawa, Takahiro Sakuma, Shin-Ichi Nishio, Teiji Takeda, Satoru Suzuki and Kiyoshi Hashizume

Department of Aging Medicine and Geriatrics, Institute of Aging and Adaptation, Shinshu University Graduate School, 3-1-1 Asahi, Matsumoto 390-8621, Japan

(Requests for offprints should be addressed to Takahide Miyamoto; Email: miyamoto@hsp.md.shinshu-u.ac.jp)

Abstract

Thyroid hormone receptors (TR) are members of the nuclear receptor superfamily. There are at least two TR isoforms, TRα and TRβ, which act as mediators of thyroid hormone in tissues. However, the relative expression of each TR isoform in target tissues is still elusive. Herein, we have developed an RT-PCR and restriction enzyme digestion method to determine the expression of TRα1 and TRβ1. We analyzed the expression of TR isoforms in 3T3-L1 preadipocytes induced to differentiate by an adipogenic cocktail in the presence or absence of 100 nM triiodothyronine (T3). The TRα1 isoform was predominantly expressed in 3T3-L1 adipocytes, and its expression was increased at the stage of development concomitant with the emergence of lipid droplets. Little, if any, TRβ1 mRNA was detected in adipocytes. Administration of T3 to the differentiating 3T3-L1 cells enhanced the accumulation of triglyceride. The expression profile of TRα1 in T3-treated adipocytes was similar to that in non-treated cells. The transcripts of adipogenic factors, CCAAT/enhancer binding protein β (C/EBPβ) and peroxisome proliferator activated receptor γ (PPARγ), were not altered by T3. Lipid binding protein, aP2, that is downstream of these transcription factors was also unaffected by T3. In contrast, the lipogenic enzyme, glyceraldehyde-3-phosphate dehydrogenase mRNA was significantly increased in the presence of T3. Therefore, T3 appears to be a hormone capable of modulating the expression of lipogenic enzyme and augments the accumulation of lipid droplets. We conclude that the TRα isoform might play an important role in the generation and maintenance of the mature adipocyte phenotype, regulating the expression of lipogenic enzymes.


Introduction

Thyroid hormone, 3,5,3′-triiodothyronine (T3), exerts profound effects via nuclear thyroid hormone receptors (TRs) on energy metabolism, homeostasis, development and differentiation in all vertebrates (Evans 1988, Lazar 1993). There are at least two thyroid hormone receptor (TR) genes, designated as TRα and TRβ, each of which generates two or more mRNAs and polypeptide products through alternate splicing or alternative promoter (Sap et al. 1986, Weinberger et al. 1986, Benbrook & Pfahl 1987, Wrutniak et al. 1995). To date, three functional TRs have been described: TRα1, TRβ1 and TRβ2, which can bind T3 and regulate transcription by binding to specific thyroid hormone response elements within promoters of their target genes (Lavin et al. 1988, Harvey & Williams 2002). In addition, a variant molecule, TRα2, does not bind T3 and may act to inhibit T3 action due, in part, to missing a critical portion of the amino terminal ligand-binding region (Koenig et al. 1989, Lazar et al. 1989). Despite a high degree of structural homologies, minor divergence in the amino-terminal sequences of TR isoforms may modulate an isoform-dependent function.

The extensive and tissue-specific action of thyroid hormone has been well documented, but little is known about the relative contribution of each TR isoform to T3 action in target tissues. Initial attempts to determine the role of each of the TR proteins indicated the wide distribution of TRα1, TRα2, and TRβ1 mRNAs among target tissues, whereas TRβ2 mRNA is limited to the anterior pituitary or to a certain region of the central nervous system (Thompson et al. 1987, Hodin et al. 1989, Wood et al. 1991). Schwartz et al. (1992) compared the concentration of TRα and TRβ mRNA to nuclear T3 binding capacity in rat tissues by using specific antibodies against each TR type. Marked variation in the ratio of the
expression of TR isoforms among tissues raises the question of redundancy and specificity of TR isoforms in target tissues.

Adipose tissue that consists of white adipose tissue (WAT) and brown adipose tissue (BAT) plays a vital role in lipid metabolism and energy balance. Thyroid hormone modulates the development and metabolism of adipose tissue in both physiological and pathological conditions. Previous studies showed that the numbers of adipocytes were increased or decreased in hyper- or hypothyroid animals (Picon & Levacher 1979). Recently, it has been reported that thyroid hormone is an adipogenic factor in the Ob17 adipocyte cell line where T3 regulates adipocyte proliferation, as well as induced preadipocyte differentiation (Darimont et al. 1993, Gharbi-Chihi et al. 1993). On the other hand, some studies supported the view that the other hand, some studies supported the view that thyroid hormone increased basal oxygen consumption and lipolysis (Oppenheimer et al. 1991, Viguere et al. 2002). The contradictory data might be obtained by using different cell types or distinct experimental conditions, which indicate the intricate effects of thyroid hormone on adipose tissue. It has been shown that TRs are expressed in WAT and BAT (Reyne et al. 1996), but the precise isoform-dependent function in adipose tissue has not been clarified.

In the present study, we have developed an RT-PCR and restriction enzyme digestion method to define the relative roles of the TRα1 and TRβ1 isoforms in 3T3-L1 adipocytes. Our observations suggest that TRα1 plays a role in adipocytes through regulating the expression of lipogenic enzymes.

Materials and Methods

Cell culture and differentiation

The mouse 3T3-L1 preadipocytes were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin in 24-well plates or 100 mm² plates. Two days after cell confluence (designated as day 0), differentiation was initiated with 1 µg/ml insulin, 1 µM dexamethasone (DEX), and 0·5 mM isobutylmethylxanthine (IBMX) in DMEM containing 10% fetal bovine serum (FBS) in the presence or absence of 100 nM T3. After incubation for 2 days (day 2), the culture media were replaced with DMEM supplemented with 10% FBS and 1 µg/ml insulin, and the cells were then fed every two days with DMEM containing 10% FBS. 3T3-L1 cells were fully differentiated by day 8. For T3-treated cells, T3 was added to the media over the full course of the differentiation.

In the above mentioned experiments, FBS was depleted from T3 by anion exchange resin (Teboul et al. 1991).

3T3-L1 cells were grown on 24-well plates and induced to differentiate as described above. After incubation for 8 days (day 8), plates were washed three times with phosphate-buffered saline (PBS), fixed with 7% formaldehyde for 5 min at room temperature, then replaced with fresh fixing solution and incubated for at least one hour. After fixation, cells were stained with a filtered oil red O solution (0·5 g oil red O (Sigma) in 100 ml isopropanol) for 15 min at room temperature. Cells were then washed twice with distilled water and visualized under the microscope.

After the microscopic examination, we quantified the amount of triglyceride in each well. Isopropanol (300 µl/well) was added to the staining plates and the plates were incubated for 10 min at room temperature, then the optical density (OD) of the solution at 500 nm was measured.

To distinguish between TRα and TRβ isoforms using RT-PCR and the restriction enzyme digestion method

We designed one common primer set for the amplification of the ligand-binding domain (LBD) of the TRα and TRβ isoforms. The primer sequences are: TRα P1 5'-GAAGACCAAGATCATCCTCCT-3' and P2 5'-AGG AAGCGCTGGC-3'; PCR amplification of both TRα and TRβ isoforms produces a 426 bp product, but after digestion with the TRα-specific restriction enzyme, Pst I, the TRα transcript is converted into two fragments of 222 bp and 204 bp; meanwhile, the TRβ transcript is left intact as a 426 bp fragment.

RNA isolation and RT-PCR

Total RNA was extracted from 3T3-L1 cells harvested at the indicated time points during differentiation using an RNasy kit (Qiagen) and quantified by spectrophotometry. First-strand cDNA was synthesized by MuLV reverse transcriptase with oligo (dT) primer (Roche). The cDNA was used as a template for PCR to amplify the specific product. The sequences of each set of primers are listed below: peroxisome proliferator activated receptor γ (PPARγ) P1, 5'-ATGTCTCTCATAATGGCCATC-3' and P2, 5'-CTAGTACAAGTGCTTTGTA-3'; CCAAT enhancer binding protein β (C/EBPβ) P1, 5'-AGGAC TTCTCTCCGAC-3' and P2, 5'-GCAGCTGCTT GAACAAG-3'; aP2 P1, 5'-GAACCTGGAAGCTT GTC-3' and P2, 5'-ACTCTTGTGAAAGTCCAG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) P1, 5'-CCCCAACTCATCCTCCT-3' and P2, 5'-TTC CTCTTGGAAAGCC-3'; elongation factor-α P1, 5'-CCATGGAAGCTTGTAGTGAAGCTCT-3' and P2, 5'-TAGCCCTTCTGAGCTTTTGGGCAG-3'. Each set of primers was designed to include at least one intron in
order to allow the discrimination of contaminating genomic DNA from cDNA. In control experiments, RT-PCR was performed under the same experimental conditions but lacking reverse transcriptase. RT-PCR products were separated by 1% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate pH 8.0, 2 mM EDTA) and stained with ethidium bromide. Identities of the PCR products were confirmed by using an ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA).

Northern blot analysis

Total RNA was isolated from 3T3-L1 cells at the indicated time points during the differentiation process using the RNeasy kit (Qiagen). Fifteen micrograms total RNA were size fractionated in 1% denaturing agarose-formaldehyde gel, then transferred to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) and cross-linked with UV light (Stratalinker, Stratagene, La Jolla, CA, USA).

Hybridizations were performed in ExpressHybTM hybridization solution (Clontech, Palo Alto, CA, USA) with cDNAs labeled with [32P]dCTP using a random hexamer labeling kit (Amersham Pharmacia Biotech). The results were visualized using a Phosphor Imager (Fuji BAS 1500). Blots were stripped and reprobed with cDNA for elongation factor-α that was considered as an internal control.

Statistical Analysis

Comparison of the accumulation of triglyceride from 3T3-L1 cells grown in the absence or presence of T3 was performed using the unpaired Student’s t test. A P<0.05 was considered as statistical significance. The data represent as mean ± s.d.

Results

*T₃ increases the accumulation of lipid droplets in 3T3-L1 adipocytes*

To assess the role of thyroid hormone receptors in adipocytes, we first tried to examine the effect of thyroid hormone on the differentiating 3T3-L1 cells. Two days after cell confluence (day 0), differentiation was induced by adipogenic stimuli in the absence or presence of 100 nM T₃. Cytoplasmic triglyceride droplets were visible by day 3. The oil red O staining of these cells on day 8 is shown in Fig. 1A. In the presence of an adipogenic cocktail, 3T3-L1 cells revealed a morphological appearance of mature adipocytes, converting from a fibroblast-like to a spherical shape and containing large lipid-laden droplets in the cytoplasm (Fig. 1A). Compared with non-treated cells, T₃-treated adipocytes exhibited more extensive deposits of lipid droplets (Fig. 1A), but the time course of differentiation was unchanged. We then quantified the amount of triglyceride and found that...
Figure 2  T₃ exerts its effect at the late stage of differentiation. Quantitative analysis of triglyceride accumulation. 3T3-L1 preadipocytes were treated with differentiation-permissive medium (DEX, IBMX and insulin) and cultured for 8 days. The data are derived from two independent experiments and represent the means ± S.D Control, without adipogenic mixture and T₃; T₃(−), cells were cultured in the differentiating media in the absence of T₃; T₃ (+), 100 nM T₃ were added to the differentiating media on day 0; T₃ (+), 100 nM T₃ were added to the differentiating media on day 3.

Although the PCR products from both transcripts are amplified by a single PCR reaction as a 426 bp product (Fig. 3, lane 1 and lane 2), using a common primer set. After digestion of the PCR product with Pst I enzyme, the TRα1 transcript remained intact (Fig. 3, lane 4). Thus, in these experiments, a reliable simplified method for the comparison of the expression of TRα1 with TRβ1 isoform has been established.

To determine the expression of TRα1 and TRβ1 in 3T3-L1 adipoctyes, PCR amplifications were performed on cells submitted to synchronization experiments. Figure 4A illustrates the RT-PCR results obtained at different days of differentiation (day 0 to 4). It appears that TR was already present in preadipocytes, and the expression level of TR mRNA was increased on day 3 and day 4 after inducing differentiation. TRα1 mRNA was hardly detectable at any stage, even when PCR cycles were extended up to 35 cycles to increase the sensitivity (Fig. 4A). These results indicate the importance of TRα1 isoform as a master regulator of thyroid hormone signaling in adipocytes. As shown in Fig. 4, TRα1 was expressed at all stages examined and its expression level was increased at the stage concomitant with emergence of lipid droplets on day 3 and day 4, which is in close agreement with the results shown in Fig. 2. The expression pattern of the TRα1 isoform was not altered by T₃ throughout the entire period of differentiation (Fig. 4B). In control experiments, elongation factor-α mRNA levels were constant during the differentiation period.
and C/EBPs families (Brun et al. 1996, Schwarz et al. 1997, Rosen et al. 2000). These results led us to investi-
gate whether T₃ regulates the expression of these transcrip-
tion factors.

To gain insight into the molecular mechanism of thy-
roid hormone action in 3T3-L1 cells, RT-PCR was per-
formed with RNA samples isolated at various time points of the di-
fferentiation process. Figure 5 illustrates the expression pro-
files of adipogenic factors during differenti-
tation in the absence or presence of 100 nM T₃. The expression of C/EBPα was increased very early and
transiently under standard conditions as reported by
Ntambi & Young-Cheul (2000). The expression level of C/EBPα was not a-
ffected by the administration of T₃. The transcript for PPARγ, whose induction may be regulated
by C/EBPα, began to be increased as early as day 1 and
rose progressively during the differenti-
tation process. As shown in Fig. 5, no significant difference in the expression pattern of the PPARγ transcript was detected between the absence and presence of T₃. The adipocyte-selective fatty
acid binding protein, aP2, that is known to be downstream of these adipogenic transcription factors, was increased over the basal level from day 2 to day 8. The expression of aP2 was also unaffected by T₃. However, T₃ increased the expression of the lipogenic enzyme GAPDH involved in lipid metabolism at the terminal phase of differenti-
tation. In control experiments, elongation factor-
α mRNA levels were expressed constantly.

As shown in Fig. 6, similar results were obtained in Northern blot analysis. We could not detect any consider-
able alteration by T₃ in the expression of the adipogenic factors C/EBPα and PPARγ, nor in their down-
stream factor, aP2. However, the peak levels and also the basal expression levels of the GAPDH transcript were augmented by the addition of T₃. Quantitative determi-
nation using Phospho Imager revealed that T₃ increased the expression of GAPDH by 2-fold and 1·7-fold on days 3 and 4 respectively (Fig. 7). Elongation factor-α mRNA
expression was not altered by T₃ (data not shown).

**Discussion**

As lipid metabolism is closely associated with a number of health problems, 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 (Flores-Delgado et al. 1987, Chawla & Lazar 1993, Dace et al. 1999, Yen 2001). In this paper, we demonstrate that triiodothyronine increases the accumulation of lipid droplets and the expression of lipogenic enzyme in 3T3-L1 adipocytes. Moreover, we show that the TRα isoform is predominantly expressed in adipocytes, suggesting that TRα seems to be involved in a complex process of lipid accumulation in adipocytes.

The mouse 3T3-L1 preadipocyte is a well-established model for studying adipogenesis in vitro (Green & Meuth
1974). When 100 nM T3 were added to the 3T3-L1 cells in the presence of adipogenic mixture, the numbers of triglyceride droplets were significantly augmented at the terminal phase. We tried to explain which TR isoform mediates the action of thyroid hormone in 3T3-L1 adipocytes. There are several reports regarding the determination of the expression of TR isoforms using specific antibodies or specific probes (Strait et al. 1990, Schwartz et al. 1992). In the present study, we have employed a simple and reliable method to determine the relative amount of TRα1 and TRβ1 transcripts in the cells. It has been shown that both TRα and TRβ are expressed in Ob17 preadipocytes, although there is a predominance of TRα (Teboul et al. 1991, Dace et al. 1999). In these experiments, we have come to the same conclusion that the TRα subtype is the predominant TR transcript in 3T3-L1 adipocytes. Because of its extremely low expression, the role of the TRβ isoform is less clear; however, TRβ might maintain a basal responsiveness to thyroid hormone in adipocytes (Dace et al. 1999). Furthermore, we have shown that TRα expression is increased at the time of conversion from the intermediate to the late stage

![Figure 5](image1.png)

**Figure 5** RT-PCR analysis of the mRNA of adipogenic factors in the absence (−) or presence (+) of T3 (T3). Total RNA was prepared from 3T3-L1 cells at different time points of the differentiation process in culture under conditions lacking T3 (left side) and with thyroid hormone (right side). The transcripts were estimated by RT-PCR analysis. The transcripts of elongation factor-α are shown as an internal control.

![Figure 6](image2.png)

**Figure 6** Northern blot analysis of mRNA of adipogenic factors in the absence (−) or presence (+) of T3 (T3). The mRNA samples from Fig. 5 were analyzed by Northern blot. Elongation factor-α levels are shown to demonstrate that equivalent amounts of RNA were loaded in each lane.
of differentiation, which is coincident with the accumulation of lipid droplets, suggesting a prominent role of TRα isofrom in the generation and maintenance of the adipocyte phenotype. Compared with previous reports showing that T₃ is an adipogenic factor necessary at an early stage of differentiation in Ob17 cells, we found that T₃ alone could not promote the conversion from preadipocyte to mature adipocyte (data not shown). These results suggest that T₃, by itself, is not an adipogenic hormone in 3T3-L1 cells, and that the role of T₃ in adipocytes might differ between cell types.

To understand the role of TRs in 3T3-L1 adipocytes, mRNA expression of the adipogenic factors was monitored. It is known that up-regulation of lipogenic enzymes is required for lipid storage (Dugail et al. 1992, Briquet-Laugier et al. 1994, Ratledge 2001). GAPDH is one of the lipogenic enzymes involved in glycolysis, which is a major metabolic pathway for providing lipogenic substrates (Barroso et al. 1999). Our results showed that administration of T₃ to the differentiating 3T3-L1 cells augmented the accumulation of lipid droplets and significantly increased transcripts of GAPDH and glycerol-3-phosphate dehydrogenase (data not shown). Similar regulation of malic enzyme in adipocytes was also documented in previous studies (Flores-Delgado et al. 1987, Lorenzo et al. 1988). Collectively, we speculate that T₃ enhances lipid accumulation in 3T3-L1 adipocytes by inducing the expression of these lipogenic enzymes. In the present experiments, we could not elucidate the molecular mechanism whereby T₃ could regulate the transcript of GAPDH. There is an hypothesis based on the fact that the promoter region of malic enzyme have response elements for TR and is regulated by thyroid hormone (Petty et al. 1990, Desvergne et al. 1991), and a similar mechanism might be involved in the regulation of GAPDH by T₃ (Barroso et al. 1999).

By contrast, T₃ does not affect the expression of an early transcription factor, C/EBPβ, which belongs to a family of transcription factors containing a highly conserved basic/leucine zipper (bZIP) domain and has been implicated in transcriptional control of cell growth and differentiation (Porse et al. 2001). This observation might be in conflict with earlier investigations showing that C/EBPβ and C/EBPα are regulated by thyroid hormone in the rat liver (Menendez-Hurtado et al. 2000). It is possible that hormonal control of C/EBP genes is regulated in a tissue- or cell-specific manner. Furthermore, thyroid hormone does not alter the expression of PPARγ, which is considered to be a potent adipogenic inducer, or of aP2, which is a downstream factor under the control of PPARγ (Gurnell et al. 2000, Tamori et al. 2002). These results are consistent with the finding that T₃ itself is not an adipogenic inducer.

The findings reported here, as well as recent studies suggest that TRα plays a pivotal role in mediating the action of thyroid hormone in adipocytes. It is possible that T₃ increases lipid accumulation through regulating the expression of lipogenic enzymes. However, the function of TRs in adipose tissue is not entirely clear, and the preadipocyte line does not precisely reflect the in vivo process. Further investigations will be required to fully elucidate the role of TRα in adipocytes.

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