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**ORIGINAL**

# A novel mechanism for the inhibition of type 2 iodothyronine deiodinase by tumor necrosis factor α: involvement of proteasomal degradation

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**Abstract.** Thyroxine ($T_4$) needs to be converted to 3,5,3'-triiodothyronine ($T_3$) by iodothyronine deiodinase to exert its biological activity. Recent studies revealed the presence of type 2 iodothyronine deiodinase (D2) in human thyroid tissue, human skeletal muscle and other tissues, suggesting that D2 is involved in maintaining plasma $T_3$ level in human. Tumor necrosis factor α (TNFα) is an inflammatory cytokine of which production is elevated in patients with nonthyroidal illness. Although several lines of evidence suggest the causal role of TNFα in nonthyroidal illness, detailed nature of the effect of TNFα on D2 remains unclear. In the present study, we identified D2 activity and D2 mRNA in TCO-1 cells, which were derived from human anaplastic thyroid carcinoma, and studied the mechanisms involved in the regulation of D2 expression by TNFα. The characteristics of the deiodinating activity in TCO-1 cells were compatible with those of D2 and Northern analysis demonstrated that D2 mRNA was expressed in TCO-1cells. D2 activity and D2 mRNA expression were rapidly increased by dibutyryl cAMP ($(Bu)_2$cAMP). TNFα showed an inhibitory effect on $(Bu)_2$cAMP-stimulated D2 activity in spite of little effect on $(Bu)_2$cAMP-stimulated D2 mRNA expression. MG132, a proteasome inhibitor abolished TNFα suppression of D2 activity whereas BAY11-7082 or 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline, inhibitors of nuclear factor-κB (NF-κB) failed to attenuate the effect of TNFα on D2 activity. These data suggest that a posttranslational mechanism through proteasomal degradation but not NF-κB activation is involved in the suppression of D2 by TNFα.

*Key words*: Nonthyroidal illness, Type 2 iodothyronine deiodinase, Tumor necrosis factor α, Proteasome

**THYROXINE ($T_4$)**, a major secretory product of thyroid gland, needs to be converted to 3,5,3'-triiodothyronine ($T_3$) by iodothyronine deiodinase to exert its biological activity [1, 2]. In rats, while D1 (type 1 iodothyronine deiodinase) activity is present in thyroid gland, liver, kidney and many other tissues, D2 (type 2 iodothyronine deiodinase) activity is present in limited number of tissues including brain, anterior pituitary, brown fat and pineal gland [1, 2]. Since it is known that D2 activity increases in hypothyroid state, while D1 activity decreases in hypothyroid state, D2 is considered to play a significant role in providing a local intracellular $T_3$ [1, 2]. In humans, D2 mRNA was also detected in thyroid gland, skeletal muscle and heart and vascular smooth muscle [3-8]. The presence of D2 activity in these tissues suggests that D2 may contribute to the circulating $T_3$ level in humans.

Tumor necrosis factor α (TNFα) is a multifunctional inflammatory cytokine of which production is elevated in patients with nonthyroidal illness [9]. Nonthyroidal illness is characterized by low serum $T_3$ level and is known to be associated with sepsis, malignancy, acute myocardial infarction and starvation [10]. It is reported that the degree of the depression of serum $T_3$ level is correlated with the severity of the illness [11]. It has been demonstrated that TNFα plays a pivotal role in the pathophysiology of nonthyroidal illness. Administration of TNFα results in a decrease in serum $T_3$ and TSH concentrations in humans [12], and a decrease in serum $T_3$ level and liver D1 activity in rats [13]. Furthermore, TNFα has been shown to decrease D2 mRNA expression and D2 activity in

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cultured human skeletal muscle cells [7]. These data suggest that the suppressive effect of TNFα on D2 may play a pathophysiological role in nonthyroidal illness. However, a detailed mechanism of the suppression of D2 activity by TNFα has not been clarified.

In the present study, we have investigated the mechanisms for the suppressive effect of TNFα on human D2 activity in TCO-1 cells, which have been established from human anaplastic thyroid carcinoma cells [14].

## Materials and Methods

### *Materials*

[γ-$^{32}$P] uridine triphosphate (UTP) and [$^{125}$I]$T_4$ were purchased from NEN Life Science Products Corp. (Boston, MA, USA). LH-20, ECL reagent and HYPERfilm-ECL were from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA). AG 50W-X2 resin, protein assay kit and PVDF membrane were from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Human recombinant TNFα was obtained from R&D systems, Inc. (Minneapolis, MN, USA). BAY11-7082 and 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline were purchased from Calbiochem (La Jolla, CA, USA). MG132 was from Peptide Institute (Osaka, Japan). Inhibitor of NF-κB-α (IκB-α) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and Phospho- IκB-α antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). All other chemicals at the highest quality were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise indicated.

### *Cell culture*

TCO-1 (JCRB0239) cells established from human anaplastic thyroid carcinoma cells were obtained from Human Science Research Resources (Osaka, Japan). TCO-1 cells were seeded to 12-well plastic culture plates for the measurement of deiodinase activity and real-time PCR or 60 mm plastic culture dishes for Western and Northern analyses, and then the cells were cultured in DMEM supplemented with 10% fetal calf serum. When the cells reached to confluence, the medium was replaced with DMEM supplemented with 10 % thyroid hormone depleted fetal calf serum [15] for 24 h, then the cells were incubated in the thyroid hormone depleted medium containing compounds to be tested for indicated hours.

### *Measurement of iodothyronine deiodinase activity*

Iodothyronine deiodinase activity was measured as previously described [16] with minor modifications [17]. Briefly, TCO-1 cells per each well were washed twice with the washing buffer (100 mM potassium phosphate, pH 7.0), scraped off and transferred into 1mL of ice-cold buffer (100 mM potassium phosphate, pH 7.0, containing 20 mM dithiothreitol). After centrifugation at 3,000 rpm for 15 min at 4 ˚C, the supernatants were discarded. Pellets were sonicated in 100 μL of the assay buffer (100 mM potassium phosphate, pH 7.0, containing 1 mM EDTA and 20 mM dithiothreitol) per tube and were incubated with indicated amount of [$^{125}$I]$T_4$, which was purified using LH-20 column chromatography on the day of experiment, in the presence or absence of 1 mM 6-propyl-2-thiouracil (PTU) or 1 mM iopanoic acid (IOP) for indicated hours at indicated temperature in duplicate. The reaction was terminated by adding 100 μL of 2% BSA and 800 μL of 10% trichloroacetic acid. After centrifugation at 3,000 rpm for 10 min at 4 ˚C, the supernatant was applied onto a small column packed with AG 50W-X2 resin (bed volume = 1 mL) and eluted with 2 mL of 10% glacial acetic acid. Separated $^{125}$I was counted with a γ counter. Non-enzymatic deiodination was corrected by subtracting $I^-$ released in sample-free tubes. The protein concentration was determined by Bradford's method using BSA as a standard [18]. The deiodinating activity was calculated either as percent $I^-$ released or as femtomoles of $I^-$ released/mg protein/h after multiplication by a factor of 2 to correct random labeling at the equivalent 3' and 5' positions. In some experiments, reaction products were analyzed by HPLC (Hitachi, Tokyo, Japan) [19]. Briefly, the incubation mixtures were extracted with 2 vol absolute ethanol, evaporated, dissolved in acetonitrile/water (32:68), applied to C18 column (Shimazu Co., Kyoto, Japan), and eluted with acetonitrile/water/phosphoric acid (32:68:0.1). The flow rate was 1 mL/min, and each 0.5-min fraction was collected and counted for radioactivity.

### *Western blot analysis*

TCO-1 cells per each dish were washed twice with PBS, scraped off and centrifuged at 3,000 rpm for 15 min at 4 ˚C, then the supernatant was discarded. Pellets were sonicated in 100 μL of buffer A [20 mM Tris-HCl (pH 7.4), 5 mM EGTA, 2 mM EDTA, 10 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride and 10 mg/mL aprotinin]. The sonicate was added to

Laemmli buffer [120 mM Tris-HCl (pH 6.8), 4% SDS, and 20% glycerol] (1 vol sonicate: 1 vol Laemmli buffer) and heated for 5 min at 100 ˚C. Aliquots were size-separated by SDS-polyacrylamide electrophoresis using 12% gels and electrophoretically transferred to PVDF membranes. Membranes were blocked with 5% milk powder and incubated with the primary antibodies for 16 h at 4 ˚C. After incubation with primary antibodies, the membranes were washed, incubated with the horseradish peroxidase-conjugated secondary antibody for 45 min, and washed again. The bound secondary antibody was detected with ECL reagent. Chemiluminescence was photographed with HYPERfilm-ECL.

***RNA preparation and Northern analysis***

Total RNA was isolated from each dish by the modified acid guanidinium thiocyanate phenol-chloroform method and Northern analysis was performed as previously described [4, 7, 8]. Briefly, 40 µg of total RNA per lane were electrophoresed on a 1.4% agarose gel containing 0.66 M formaldehyde and transferred overnight in 20 X SSC (1 X SSC = 150 mM sodium chloride and 15 mM trisodium citrate) to a nylon membrane (Biodyne, Pall BioSupport Corp., East Hills, NY, USA). RNA was cross-linked to the nylon membrane with a UV Stratalinker (Stratagene, San Diego, CA, USA). The membrane was prehybridized with the hybridization buffer (50% formamide, 0.2% SDS, 5% dextran sulfate, 50 mM HEPES, 5 X SSC, 5 X Denhart's solution, and 100 mg/mL denatured salmon sperm DNA) at 68 ˚C for 2 h. Subsequently, the membrane was hybridized at 68 ˚C overnight with the hybridization buffer containing a human D2 cRNA probe. The membrane was washed twice in 2 X SSC-0.1% SDS at 25 ˚C for 15 min and twice in 0.1 X SSC-0.1% SDS at 68 ˚C for 1 h. Autoradiography was established by exposing the filters for 7–14 d to x-ray film (XAR-2, Eastman Kodak Co., Rochester, NY, USA) at -70 ˚C. After the detection of D2 mRNA, the probe was stripped off, and blots were rehybridized with human G3PDH cRNA probe as a control. Hybridization and washing were performed as described above, and the membrane was exposed for 1 h. RNA samples for comparison were analyzed on the same blot.

***Reverse transcription and real-time PCR***

Reverse transcription was performed as previously described [20] with minor modifications. Briefly, single strand cDNA synthesis was performed on 1µg total RNA using random hexamers and murine leukemia virus reverse transcriptase (GeneAmp RNA PCR kit, Roche, Branchburg, NJ, USA) in 20 µL. Subsequently, 5 µL of the cDNA was amplified in a TaqMan 7000 real-time PCR apparatus using human D2 and human G3PDH TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA). mRNA levels were expressed as arbitrary units after the correction for human G3PDH.

***Statistics***

All values are expressed as means ± SD. Statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by Fisher's PLSD test.

## Results

***Deiodinating activity in TCO-1 cells***

The deiodinating activity in TCO-1 cells was measured by the release of $I^-$ from 2 nM [$^{125}$I]$T_4$ in the presence of 20 mM DTT and 1 mM PTU. $T_4$ deiodination was dependent on an incubation period up to 2 h (Fig. 1a), and protein concentration of TCO-1 cells (Fig. 1b). Incubation at 4°C or preheating the cell sonicate at 56°C for 30 min completely abolished the deiodination. These results indicate the presence of $T_4$ deiodinating activity in TCO-1 cells. $T_4$ deiodinating activities were not influenced by 1 mM PTU, but abolished by 1 mM IOP. From the double reciprocal plot, kinetic constants for $T_4$ were calculated to be Km = 5.0 nM and Vmax = 333.3 fmoles $I^-$ released/mg protein/h in TCO-1 cells as shown in Fig. 1c. The reaction products were analyzed by HPLC, only three peaks corresponding to $I^-$, $T_4$, and $T_3$ were defined, and radioactivity in the $I^-$ peak was comparable to that in the $T_3$ peak (data not shown). These results indicate that the characteristics of $T_4$ deiodinating activity in TCO-1 cells are compatible with those of D2.

***Effect of TNFα on D2 activity in TCO-1 cells***

Treatment with 1 mM of dibutyryl cAMP ($(Bu)_2$cAMP) for 6 h increased D2 activity in TCO-1 cells. TNFα (0.1-100 ng/mL), while having no significant effect on basal D2 activity, suppressed $(Bu)_2$cAMP-stimulated D2 activity in a dose dependent manner as shown in Fig. 2a. The maximum suppression was achieved by 100 ng/mL of TNFα to approximately 50 % of $(Bu)_2$cAMP- stimulated D2 activity. Fig. 2b demonstrates time-course of the effect of TNFα on D2

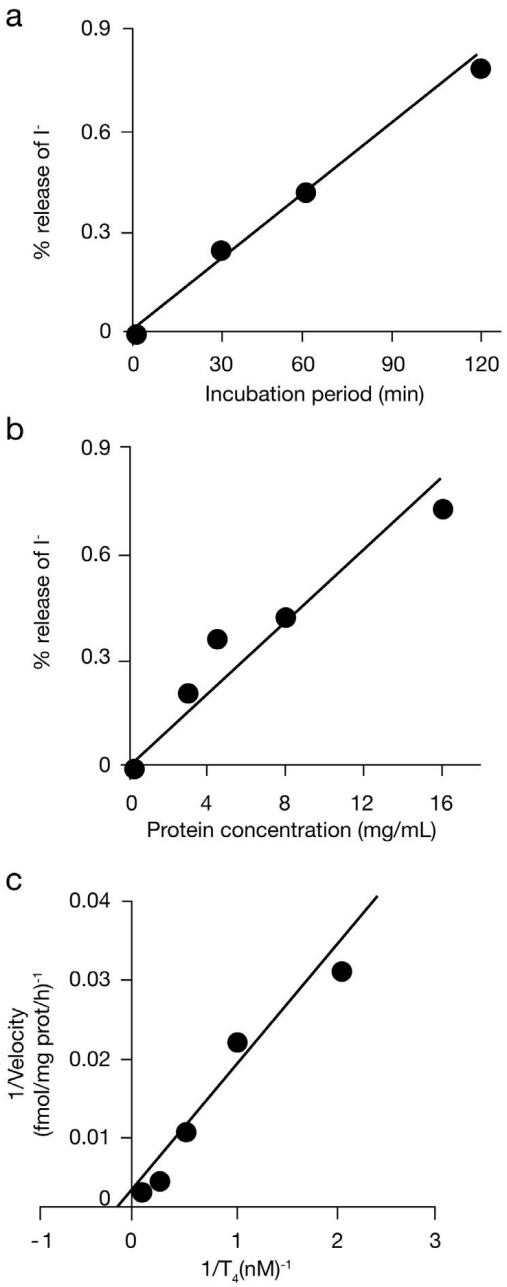


**Fig. 1** Characterization of deiodinating activity in TCO-1 cells
TCO-1 cells were incubated with DMEM containing $(Bu)_2$cAMP($10^{-3}$ M) for 6 h. Deiodinating activity was measured in the cell sonicates, as described in Materials and Methods. The deiodinating activity shown represents the mean of two wells. **a**) Deiodinating activity in TCO-1 cells measured by various incubation periods up to 2 h. **b**) Deiodinating activity measured in various protein concentrations of TCO-1 cells. Incubation was performed for 1 h. **c**) Double reciprocal plot of $T_4$ deiodination by $(Bu)_2$cAMP-stimulated TCO-1 cells. Incubation was performed for 1 h with various concentrations of [$^{125}$I]$T_4$. Kinetic constants were calculated to be: Km 5.0 nmol/L, Vmax 333.3 fmol I$^-$ released/mg protein/h.

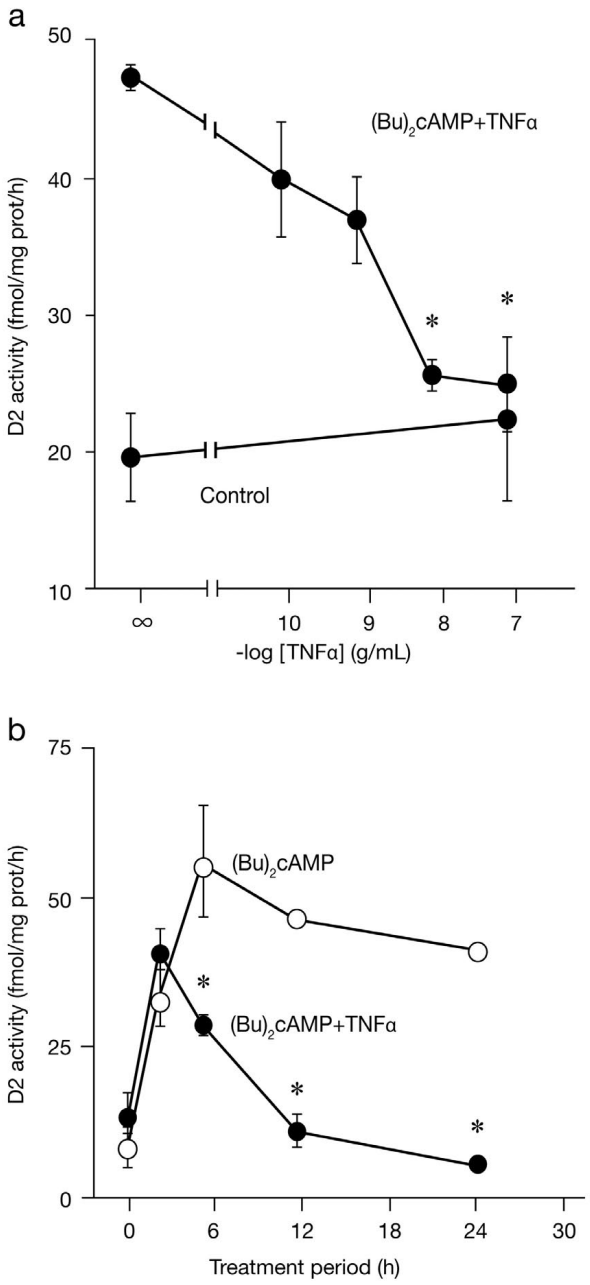


**Fig. 2** Effect of TNFα on D2 activity in TCO-1 cells
**a**) D2 activity in TCO-1 cells stimulated by $(Bu)_2$cAMP ($10^{-3}$M) in the presence of various concentrations of TNFα for 6 h. The D2 activity shown represents the mean ± SD of 3 wells. * $p<0.05$ (compared with control TCO-1 cells stimulated by $(Bu)_2$cAMP alone). **b**) Time course of TNFα effect on $(Bu)_2$cAMP-stimulated D2 activity in TCO-1 cells. D2 activity in TCO-1 cells incubated with $(Bu)_2$cAMP ($10^{-3}$ M) for various hours in the presence or absence of TNFα ($10^{-8}$ g/mL). D2 activity shown represents mean ± SD of 3 wells in one experiment.
* $p<0.05$ (compared with $(Bu)_2$cAMP alone).

activity in TCO-1 cells. Treatment with $(Bu)_2$cAMP resulted in 4-fold increase in D2 activity in TCO-1 cells, which reached the peak level at 6 h. TNFα suppressed $(Bu)_2$cAMP-stimulated D2 activity at 6 h and its inhibitory effect was sustained until 24 h. At 12 h and 24 h, TNFα suppressed $(Bu)_2$cAMP-stimulated D2 activity to the basal level.

### *Effect of TNFα on $(Bu)_2$cAMP-stimulated D2 mRNA expression in TCO-1 cells*

Since our previous observation indicated that TNFα inhibited D2 expression through a pretranslational mechanism in cultured human skeletal muscle cells [7], the effect of TNFα on D2 mRNA expression was also investigated in TCO-1 cells. Northern analysis of total RNA extracted from TCO-1 cells using human D2 cRNA probe demonstrated the hybridization signal with an approximately 7.5 kb in size, which is compatible with D2 mRNA [7, 8]. D2 mRNA was clearly increased by $(Bu)_2$cAMP (1 mM) treatment for 3h, indicating that cAMP regulates D2 expression through a pretranslational mechanism (Fig. 3a). Similar results of $(Bu)_2$cAMP-stimulation of D2 mRNA expression were obtained by using real-time PCR method suggesting that real-time PCR method is adequate for exploring D2 mRNA expression in TCO-1 cells (Fig. 3b). TNFα (10 ng/mL) showed no significant effect on basal D2 mRNA expression and $(Bu)_2$cAMP-stimulated D2 mRNA expression up to 12h suggesting that TNFα might not be involved in the regulation of D2 mRNA expression in TCO-1 cells (Fig. 3b, 3c).

### *Involvement of proteasome in the TNFα suppression of $(Bu)_2$cAMP-stimulated D2 activity in TCO-1 cells*

Since it has been shown that proteasomal degradation is involved in the regulation of D2 activity [21, 22], the effect of MG132, an inhibitor of proteasome on TNFα inhibition of D2 activity in TCO-1 cells was examined. MG132 (10 μM) increased $(Bu)_2$cAMP-stimulated D2 activity and abolished the inhibitory effect of TNFα on D2 activity (Fig. 4a). Further, MG132 remained effective in abolishing the inhibitory effect of TNFα on $(Bu)_2$cAMP-stimulated D2 activity in TCO-1 cells up to 24h (Fig. 4b). These data suggest that proteasomal degradation may be involved in the inhibitory effect of TNFα on D2 activity in TCO-1 cells. Therefore, it is suggested that TNFα inhibits D2 activity through proteasomal degradation at the posttranslational level.

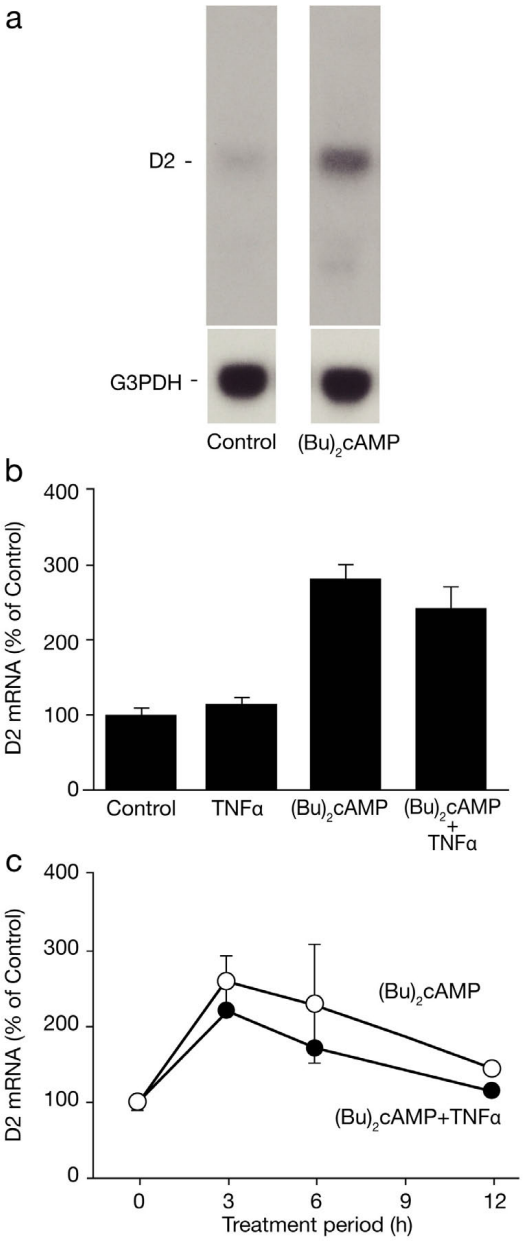


**Fig. 3** Effect of TNFα on D2 mRNA in TCO-1 cells
**a**) Northern analysis of D2 mRNA in TCO-1 cells incubated with $(Bu)_2$cAMP ($10^{-3}$ M) for 3 h. Each lane represents 40 μg total RNA obtained from cells in an individual dish. **b**) Real-time PCR of D2 mRNA in TCO-1 cells. Cells were stimulated by $(Bu)_2$cAMP in the presence or absence of TNFα for 3 h. D2 mRNA expression shown represents the mean ± SD of 3 wells. $*p < 0.05$ (compared with control TCO-1 cells). **c**) Time course of TNFα effect on $(Bu)_2$cAMP-stimulated D2 mRNA expression in TCO-1 cells. D2 mRNA expression in TCO-1 cells incubated with $(Bu)_2$cAMP ($10^{-3}$ M) for various hours in the presence or absence of TNFα ($10^{-8}$ g/mL). D2 mRNA expression shown represents mean ± SD of 3 wells in one experiment. $*p<0.05$ (compared with $(Bu)_2$cAMP alone).

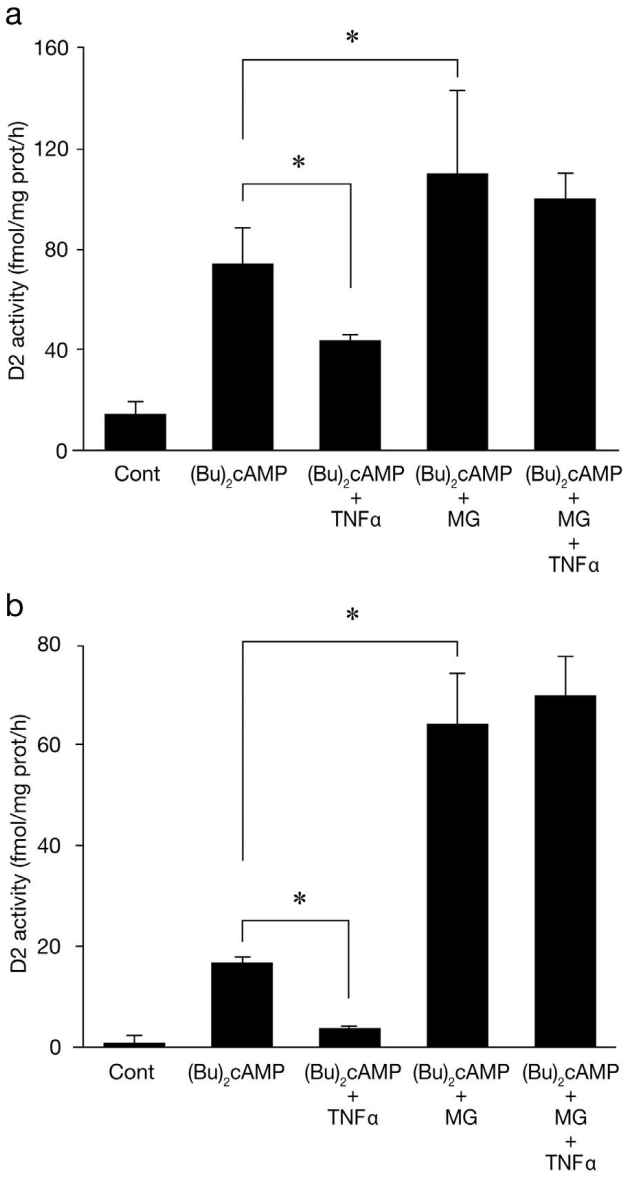


**Fig. 4** Effect of MG132 on TNFα suppression of D2 expression in TCO-1 cells
**a**) TNFα ($10^{-8}$ g/mL) effect on $(Bu)_2$cAMP ($10^{-3}$ M)-stimulated D2 activity in the presence or absence of MG132 ($10^{-5}$ M) in TCO-1 cells. Cells were pretreated with MG132 for 1 h, then stimulated by $(Bu)_2$cAMP in the presence or absence of TNFα for 6 h. D2 activity shown represents the mean ± SD of 3 wells. * $p$ <0.05 (compared with control TCO-1 cells stimulated by $(Bu)_2$cAMP alone).
**b**) TNFα ($10^{-8}$ g/mL) effect on $(Bu)_2$cAMP ($10^{-3}$ M)-stimulated D2 activity in the presence or absence of MG132 ($10^{-5}$ M) in TCO-1 cells. Cells were pretreated with MG132 for 1 h, then stimulated by $(Bu)_2$cAMP in the presence or absence of TNFα for 24 h. D2 activity shown represents the mean ± SD of 3 wells. * $p$ <0.05 (compared with control TCO-1 cells stimulated by $(Bu)_2$cAMP alone).

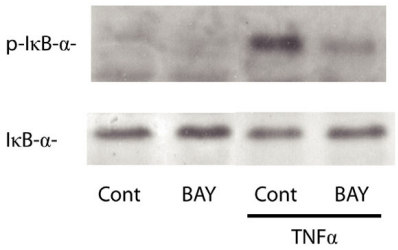


**Fig. 5** TNFα-induced phosphorylation of IκB-α in TCO-1 cells
Western analysis of phosphorylated form of IκB-α in TCO-1 cells. Cells were stimulated by TNFα ($10^{-8}$ g/mL) for 10 min.

### *TNFα-induced phosphorylation of IκB-α (inhibitor of NF-κB-α)*

It is well known that nuclear factor-κB (NF-κB) activation is involved in the biological effects of TNFα. It has been revealed that NF-κB activation is involved in TNFα suppression of D1 activity and mRNA expression in HepG2 cells [23]. Furthermore, recent studies have shown that the expression of p65 subunit of NF-κB induces human D2 promoter activity in HC11 cells and HEK cells [24], and NF-κB binding site has been identified in D2 gene in HC11 cells [25]. These data suggest that NF-κB may play an important role in the regulation of D2 mRNA expression by TNFα. In the present study, we investigated the effect of TNFα on NF-κB activation in TCO-1 cells. In the inactive state, NF-κB is located in the cytoplasm of the cells in association with the inhibitory subunit, IκB-α. In response to TNFα stimulation, IκB-α is phosphorylated, then ubiquitinated and degradated by a proteasome dependent mechanism to allow NF-κB to translocate to the nucleus where it can activate gene expression [26]. As shown in Fig. 5, treatment with TNFα (10 ng/mL) for 10 min induced phosphorylation of IκB-α in TCO-1 cells indicating that TNFα can activate NF-κB mediated pathways in TCO-1 cells. On the other hand, BAY11-7082 (20 μM), an inhibitor of IκB-α phosphorylation [27] inhibited TNFα-induced phosphoylation of IκB-α indicating that BAY11-7082 is capable to inhibit NF-κB activation by TNFα in TCO-1 cells.

### *Effect of NF-κB inhibition on TNFα suppression of D2 activity in TCO-1 cells*

To investigate the implication of NF-κB activation in the inhibitory effect of TNFα on D2 activity, we examined the effect of BAY11-7082 on the TNFα suppression of D2 activity in TCO-1 cells. In the pres-

ence of BAY11-7082, although $(Bu)_2$cAMP-stimulated D2 activity was suppressed, TNFα remained effective in inhibiting $(Bu)_2$cAMP-stimulated D2 activity (Fig. 6a). Since BAY11-7082 suppressed $(Bu)_2$cAMP-stimulated D2 activity, the effect of another NF-κB activation inhibitor, 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline [28], which had a distinct mechanism of inhibition of NF-κB mediated transcription from BAY11-7082 was also investigated. 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline (1 μM) having no effect on $(Bu)_2$cAMP-stimulated D2 activity, did not abolish the inhibition of $(Bu)_2$cAMP-stimulated D2 activity by TNFα in TCO-1 cells (Fig. 6b). Taken together, it was suggested that a mechanism apart from NF-κB activation might be involved in the inhibitory effect of TNFα on D2 activity.

## Discussion

In the present study, iodothyronine deiodinating activity was clearly demonstrated in TCO-1 cells, which were derived from anaplastic thyroid carcinoma and expressed telomerase reverse transcriptase and thyroid transcriptionfactor 1 mRNAs but not thyroglobulin mRNA [14]. The deiodinating activity had low Km for $T_4$ and was not inhibited by 1 mM PTU but was abolished by 1 mM IOP. MG132, an inhibitor of proteasome that has been reported to be involved in the degradation of D2 increased the deiodinating activity in TCO-1 cells. These data indicate that the characteristics of the $T_4$ deiodinating activity in TCO-1 cells are compatible with those of D2 [1, 2, 29]. Northern analysis using human D2 cRNA probe demonstrated hybridization signals approximately 7.5 kb in size in TCO-1 cells, which is compatible with D2 mRNA [4, 5]. Although the expression of D1 in human thyrocytes has been demonstrated [30] and in the present study, the expression of D1 mRNA has been detected in TCO-1 cells, the deiodinating activity from 0.5 μM [$^{125}$I]r$T_3$ was undetectable in the presence of 1 mM of EDTA and 0.5 mM DTT, which is an optimal condition for the measurement of D1 activity [23, 31] (data not shown). These data suggest that D2 may be mainly involved in the $T_4$ deiodinating activity in TCO-1 cells.

$(Bu)_2$cAMP stimulated D2 activity and D2 mRNA expression in TCO-1 cells, indicating the involvement of cAMP mediated pathway in the regulation of D2. Our previous observations have shown the existence of D2 expression, which is regulated through cAMP

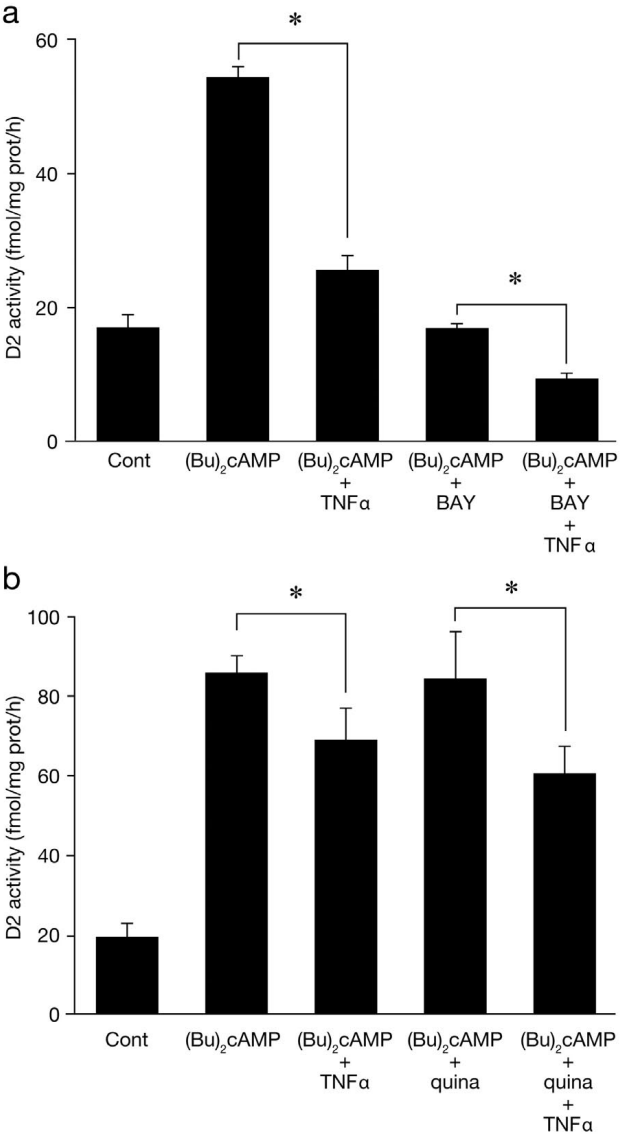


**Fig. 6** Effect of NF-κB inhibitors on TNFα suppression of D2 expression in TCO-1 cells

**a**) TNFα ($10^{-8}$ g/mL) effect on $(Bu)_2$cAMP ($10^{-3}$ M)-stimulated D2 activity in the presence or absence of BAY11-7082 ($2x10^{-5}$ M) in TCO-1 cells. Cells were pretreated with BAY11-7082 for 1 h, then stimulated by $(Bu)_2$cAMP in the presence or absence of TNFα for 6 h. D2 activity shown represents the mean ± SD of 3 wells. *$p$ <0.05 (compared with control TCO-1 cells stimulated by $(Bu)_2$cAMP alone).

**b**) TNFα ($10^{-8}$ g/mL) effect on $(Bu)_2$cAMP ($10^{-3}$ M)-stimulated D2 activity in the presence or absence of 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline ($10^{-6}$ M) in TCO-1 cells. Cells were pretreated with 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline for 1 h, then stimulated by $(Bu)_2$cAMP in the presence or absence of TNFα for 6 h. D2 activity shown represents the mean ± SD of 3 wells. *$p$ <0.05 (compared with control TCO-1 cells stimulated by $(Bu)_2$cAMP alone).

mediated pathways at the pretranslational level in human thyroid tissue, cultured human skeletal and vascular smooth muscle cells [4, 7, 8]. Since the presence of cAMP response element has been demonstrated in the promoter lesion of human D2 [4, 29], it is likely that pretranslational mechanisms are also involved in the regulation of D2 expression through cAMP mediated pathways in TCO-1 cells.

TNFα, which is thought to play a pivotal role in the pathophysiology of nonthyroidal illness, inhibited $(Bu)_2$cAMP-stimulated D2 activity in TCO-1 cells. IC50 of TNFα to inhibit $(Bu)_2$cAMP-stimulated D2 activity in TCO-1 cells was approximately 1.0 ng/mL. The effective dose of TNFα is comparable to that reported in the previous study [32] suggesting that the physiological concentration of TNFα is able to regulate $T_3$ production. Although $(Bu)_2$cAMP stimulation of D2 activity sustained up to 24h, the prolonged treatment with TNFα suppressed $(Bu)_2$cAMP-stimulated D2 activity to the basal level. These data suggest a possible involvement of a degradating mechanism in the regulation of D2 activity by TNFα.

The effects of cytokines on iodothyronine deiodinases have been controversial in the previous studies. In rat FRTL-5 thyroid cells, basal and TSH-stimulated D1 activities were inhibited by IL-1, IL-6, interferon-γ (IFN-γ) and TNFα [33-35]. Mouse liver D1 was stimulated by IL-1 [36]. TNFα increased D2 activity in rat anterior pituitary cells and GH3 cells [37]. In contrast, our previous study demonstrated the TNFα inhibition of D2 activity in cultured human skeletal muscle cells [7], and other investigators showed that IL-6, IFN-γ and TNFα inhibited D2 in human thyroid tissue [38]. Although these data suggest that the response of deiodinating activity against cytokines may vary by tissues or cells, it has been postulated that the result of these changes cause the decline of serum thyroid hormone level to decrease the basal metabolic rate and protein and fat catabolism in critical illness [39].

In the presence of MG132, the inhibitory effect of TNFα on $(Bu)_2$cAMP-stimulated D2 activity was abolished. These data indicate that a pathway which is sensitive to proteasomal degradation is involved in the posttranslational regulation of D2 activity by TNFα in TCO-1 cells. It has been shown that proteasomal degradation is involved in the regulation of D2 activity [21, 22], it is likely that TNFα inhibits D2 activity by enhancing the degradation of D2 by proteasome in TCO-1 cells.

Recent studies have shown that ubiquitin-proteasome expression in muscle is increased in malignancy and sepsis [40, 41], which are thought to have high plasma TNFα level. Furthermore, in rat soleus muscle, the increase in the expression of ubiquitin, which plays major roles in the proteasomal degradation of D2 [42-44] by TNFα was postulated [45]. These data suggest plasma TNFα level is related to the ubiquitin-proteasome expression which contributes to the degradation of D2. Taken together, it is likely that TNFα, of which concentration is elevated in critical illness, inhibits $(Bu)_2$cAMP-stimulated D2 activity through the degradation of D2 at posttranslational level through the enhancement of the ubiquitin-proteasome expression.

Although our previous observation demonstrated the suppression of D2 mRNA expression by TNFα in cultured human skeletal muscle cells [7], in the present study, TNFα did not inhibit $(Bu)_2$cAMP-induced D2 mRNA expression, suggesting that a pretranslational mechanism might not be implicated in the TNFα suppression of D2 expression in TCO-1 cells. Further, TNFα remained effective in inhibiting $(Bu)_2$cAMP-stimulated D2 activity in the presence of BAY11-7082 or 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline. Since BAY11-7082 suppressed $(Bu)_2$cAMP-stimulated D2 activity, it might be possible that the constitutive activation of NF-κB is involved in the regulation of D2 activity in TCO-1 cells. However, 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline, another NF-κB inhibitor, did not affect $(Bu)_2$cAMP-stimulated D2 activity and these inhibitors failed to abolish the suppression of D2 activity by TNFα, which could induce the NF-κB activation. Although it has been shown that TNFα stimulates ubiquitin dependent proteasomal degradation through NF-κB mediated pathway [46], these data suggest that in TCO-1 cells, other mechanisms apart from NF-κB activation may be involved in the inhibitory effect of TNFα on $(Bu)_2$cAMP-stimulated D2 activity by proteasomal degradation. On the other hand, it has been shown that the expression of p65 subunit of NF-κB induces human D2 promoter activity in HC11 cells and HEK cells [24], and NF-κB binding site has been identified in HC11 cells [25], These observations suggest that the involvement of NF-κB in the pretranslational activation of D2 expression, whereas the present results suggest that NF-κB activation by TNFα did not affect D2 mRNA expression in TCO-1 cells. Taken together, it is likely that although the regulatory role of TNFα stimulation or NF-κB activation in D2

mRNA expression is tissue or cell specific, the mechanisms activated by TNFα including NF-κB related pathway may not be involved in the regulation of D2 expression in TCO-1 cells. Since it has been shown that proteasome is involved in TNFα induced decline in Akt protein through a caspase dependent posttranslational mechanism in adipocytes [47], caspase might be one of possible pathways through which TNFα inhibits its D2 activity in TCO-1 cells. In addition, the mechanism involved in the inhibitory effect of BAY11-7082 on $(Bu)_2$cAMP-stimulated D2 activity has not been clarified in the present study, further studies may be required to figure out the regulatory mechanism of TNFα or BAY11-7082 on D2 activity in TCO-1 cells.

Although recent studies have postulated that increased type 3 iodothyronine deiodinase (D3) activity, which catalyze $T_4$ to $rT_3$ in skeletal muscle may play a major role in nonthyroidal illness [48], it has been suggested that the impaired thyroidal production of $T_3$ may be involved in the rapid fall in serum $T_3$ [49]. The results in the present study suggest that D2 in thyroid gland may also contribute to the low $T_3$ level in nonthyroidal illness.

In summary, the present study clearly demonstrated the presence of D2 in TCO-1 cells derived from human anaplastic thyroid carcinoma. D2 activity is positively regulated by cAMP-mediated pathway and is negatively regulated by TNFα that plays a pivotal role in nonthyroidal illness. TNFα inhibits D2 activity through the enhancement of the degradation of D2 activity. MG132 sensitive proteasomal degradation pathway may be involved in the inhibitory mechanism of D2 activity by TNFα (Fig. 7).

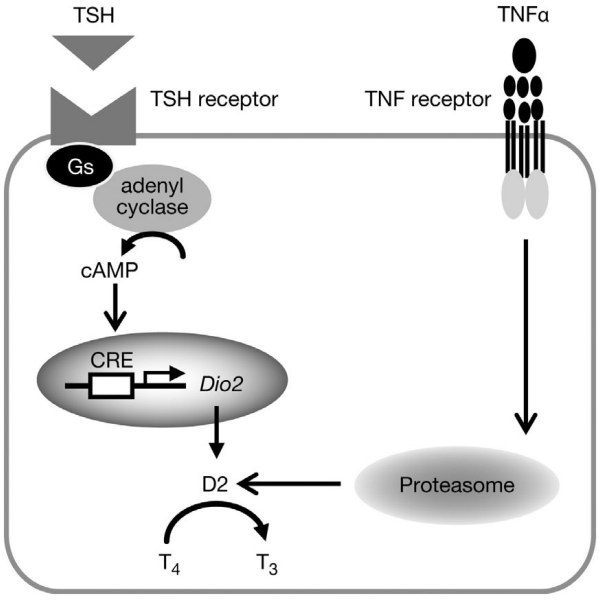


**Fig. 7** TNFα suppression of D2 expression in TCO-1 cells
D2 activity is positively regulated by cAMP-mediated pathway and is negatively regulated by TNFα. TNFα inhibits D2 activity through proteasomal degradation in TCO-1 cells.

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## References

1. Larsen PR (1996) Thyroid hormone transport, cellular uptake, metabolism and molecular action. In DeGroot LJ, Larsen PR and Hennermann G (ed) The thyroid and its disease. Churchill Livingstone, New York: 61-111.
2. Leonard JL, Koehrle J (1996) Intracellular pathways of iodothyronine metabolism. In: LE Braverman & RD Utiger (ed) Werner and Ingbar's the thyroid: a fundamental and clinical text, edn 7. Lippincott-Raven, Philadelphia: 125-161.
3. Larsen PR (1996) Mammalian type 2 deiodinase sequences: finally, the end of the beginning. *J Clin Invest* 98: 242-243.
4. Murakami M, Araki O, Hosoi Y, Kamiya Y, Morimura T, Ogiwara T, Mizuma H, Mori M (2001) Expression and regulation of type II iodothyronine deiodinase in human thyroid gland. *Endocrinology* 142: 2961-2967.
5. Croteau W, Davey JC, Galton VA, St Germain DL (1996) Cloning of the mammalian type II iodothyronine deiodinase: A selenoprotein differentially expressed and regulated in human and rat brain and other tissues. *J Clin Invest* 98: 405-417.
6. Salvatore D, Tibor B, Harney JW, Larsen PR (1996) Molecular biology and biochemical characterization of the human type 2 selenodeiodinase. *Endocrinology* 137: 3308-3315.
7. Hosoi Y, Murakami M, Mizuma H, Ogiwara T, Imamura

M, Mori M (1999) Expression and regulation of type II iodothyronine deiodinase in cultured human skeletal muscle cells. *J Clin Endocrinol Metab* 84: 3293-3300.
8. Mizuma H, Murakami M, Mori M (2001) Thyroid hormone activation in human vascular smooth muscle cells Expression of type II iodothyronine deiodinase. *Circ Res* 88: 313-318.
9. Mooradian AD, Reed RL, Osterweil D, Schiffman R, Scuderi P (1990) Decreased serum triiodothyronine is associated with increased concentrations of tumor necrosis factor. *J Clin Endocrinol Metab* 71: 1239-1242.
10. De Groot LJ (1999) Dangerous dogmas in medicine: the nonthyroidal illness syndrome. *J Clin Endocrinol Metab* 84: 151-164.
11. Yu J, Koenig RJ (2000) Regulation of hepatocyte thyroxine 5'-deiodinase by T3 and nuclear receptor coactivators as a model of the sick euthyroid syndrome. *J Biol Chem* 275: 38296-38301.
12. van der Poll T, Romijn JA, Wiersinga WM, Sauerwein HP (1990) Tumor necrosis factor: a putative mediator of the euthyroid syndrome in man. *J Clin Endocrinol Metab* 71: 1567-1572.
13. Pang XP, Hershman JM, Mirell CJ, Pekary AE (1989) Impairment of hypothalamic-pituitary-thyroid function in rats treated with human recombinant tumor necrosis factor-alpha (cachectin). *Endocrinology* 125: 76-84.
14. Takano T, Ito Y, Matuzaka F, Miya A, Kobayashi K, Yoshida H, Miyauchi A (2007) Quantitative measurement of telomerase reverse transcriptase, thyroglobilin and thyroid transcription factor 1 mRNAs in anaplastic thyroid carcinoma tissues and cell lines. *Oncol Rep* 18; 715-720.
15. Samuels HH, Stanley F, Casanova J (1979) Depletion of L-3,5,3'-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. *Endocrinology* 105: 80-85.
16. Leonard JL, Rosenberg IN (1980) Iodothyronine 5'-deiodinase from rat kidney: substrate specificity and the 5'-deiodination of reverse triiodothyronine. *Endocrinology* 107: 1376-1383.
17. Murakami M, Tanaka K, Greer MA, Mori M (1988) Anterior pituitary type II thyroxine 5' deiodinase activity is not affected by lesions of the hypothalamic paraventricular nucleus which profoundly depress pituitary thyrotropin secretion. *Endocrinology* 123: 1676-1681.
18. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254.
19. Richard K, Hume R, Kaptein E, Sanders JP, van Toor H, De Herder WW, den Hollander JC, Krenning EP, Visser TJ (1998) Ontogeny of iodothyronine deiodinases in human liver. *J Clin Endocrinol Metab* 83: 2868-2874.
20. Murakami M, Hosoi Y, Negishi T, Kamiya Y, Miyashita K, Yamada M, Iriuchijima T, Yokoo H, Yoshida I, Tsushima Y, Mori M (1996) Thymic hyperplasia in patients with Graves' disease. Identification of thyrotropin receptors in human thymus. *J Clin Invest* 98: 2228-2234.
21. Steinsapir J, Harney J, Larsen PR (1998) Type 2 iodothyronine deiodinase in rat pituitary tumor cells is inactivated in proteasomes. *J Clin Invest* 102: 1895-1899.
22. Steinsapir J, Bianco AC, Buettner C, Harney J, Larsen PR (2000) Substrate-induced down-regulation of human type 2 deiodinase (hD2) is mediated through proteasomal degradation and requires interaction with the enzyme's active center. *Endocrinology* 141: 1127-1135.
23. Nagaya T, Fujieda M, Otsuka G, Yang JP, Okamoto T, Seo H (2000) A potential role of activated NF-κB in the pathogenesis of euthyroid sick syndrome. *J Clin Invest* 106: 393-402.
24. Fekete C, Gereben B, Doleschall M, Harney JW, Dora JM, Bianco AC, Sarkar S, Liposits Z, Rand W, Emerson C, Kacskovics I, Larsen PR, Lechan RM (2004) Lipopolysaccharide induces type 2 iodothyronine deiodinase in the mediobasal hypothalamus: implications for the nonthyroidal illness syndrome. *Endocrinology* 145: 1649-1655.
25. Zeöld A, Doleschall M, Haffner MC, Capelo LP, Menyhért J, Liposits Z, da Silva WS, Bianco AC, Kacskovics I, Fekete C, Gereben B (2006) Characterization of the nuclear factor-κB responsiveness of the human *dio2* gene. *Endocrinology* 147: 4419-4429.
26. Martin AG, San-Antonio B, Fresno M (2001) Regulation of nuclear factor κB transactivation. *J Biol Chem* 276: 15840-15849.
27. Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME (1997) Novel inhibitors of cytokine-induced IκBα phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem* 272: 21096-21103.
28. Armstrong SJ, Wiberg M, Terenghi G, Kingham PJ (2008) Laminin activates NF-κB in Schwann cells to enhance neurite outgrowth. *Neurosci Lett* 439: 42-46.
29. Bartha T, Kim SW, Salvatore D, Gereben B, Tu HM, Harney JW, Rudas P, Larsen PR (2000) Characterization of the 5'-flanking and 5'-untranslated regions of the cyclic adenosine 3',5'-monophosphate-responsive human type 2 iodo-thyronine deiodinase gene. *Endocrinology* 141: 229-237.
30. Beech SG, Walker SW, Dorrance AM, Arthur JR, Nicol F, Lee D, Beckett GJ (1993) The role of thyroidal type-I iodothyronine deiodinase in tri-iodothyronine production by human and sheep thyrocytes in primary culture. *J Endocrinol* 136: 361-370.
31. Miyashita K, Murakami M, Iriuchijima T, Takeuchi T, Mori M (1995) Regulation of rat liver type 1 iodothyro-

nine deiodinase mRNA levels by testosterone. *Mol Cell Endocrinol* 115: 161-167.

32. Chopra IJ, Sakane S, Teco GN (1991) A study of the serum concentration of tumor necrosis factor-alpha in thyroidal and nonthyroidal illnesses. *J Clin Endocrinol Metab* 72: 1113-1116.
33. Pekary AE, Berg L, Santini F, Chopra I, Hershman JM (1994) Cytokines modulate type I iodothyronine deiodinase mRNA levels and enzyme activity in FRTL-5 rat thyroid cells. *Mol Cell Endocrinol* 101: R31-35.
34. Hashimoto H, Igarashi N, Miyawaki T, Sato T (1995) Effects of tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-6 on type I iodothyronine 5'-deiodination in rat thyroid cell line, FRTL-5. *J Interferon Cytokine Res* 15: 367-375.
35. Tang KT, Braverman LE, DeVito WJ (1995) Tumor necrosis factor-alpha and interferon-gamma modulate gene expression of type I 5'-deiodinase, thyroid peroxidase, and thyroglobulin in FRTL-5 rat thyroid cells. *Endocrinology* 136: 881-888.
36. Fujii T, Sato K, Ozawa M, Kasono K, Imamura H, Kanaji Y, Tsushima T, Shizume K (1989) Effect of interleukin-1 (IL-1) on thyroid hormone metabolism in mice: stimulation by IL-1 of iodothyronine 5'-deiodinating activity (type I) in the liver. *Endocrinology* 124: 167-174.
37. Baur A, Bauer K, Jarry H, Kohrle J (2000) Effects of proinflammatory cytokines on anterior pituitary 5'-deiodinase type I and type II. *J Endocrinol* 167: 505-515.
38. Molnar I, Balazs C, Szegedi G, Sipka S (2002) Inhibition of type 2,5'-deiodinase by tumor necrosis factor alpha, interleukin-6 and interferon gamma in human thyroid tissue. *Immunol Lett* 80: 3-7.
39. St. Germain DL, Galton VA, Hernandez (2009) A Minireview: Defining the Roles of the Iodothyronine Deiodinases: Current Concepts and Challenges. *Endocrinology* 150: 1097-1107.
40. Khal J, Wyke SM, Russell ST, Hine AV, Tisdale MJ (2005) Expression of the ubiquitin-proteasome pathway and muscle loss in experimental cancer cachexia. *Br J Cancer* 93: 774-780.
41. Tiao G, Hobler S, Wang JJ, Meyer TA, Luchette FA, Fischer JE, Hasselgren PO (1997) Sepsis is associated with increased mRNAs of the ubiquitin-proteasome proteolytic pathway in human skeletal muscle. *J Clin Invest* 99: 163-168.
42. Gereben B, Goncalves C, Harney JW, Larsen PR, Bianco AC (2000) Selective proteolysis of human type 2 deiodinase: a novel ubiquitin-proteasomal mediated mechanism for regulation of hormone activation. *Mol Endocrinol* 14: 1697-1708.
43. Botero D, Gereben B, Goncalves C, De Jesus LA, Harney JW, Bianco AC (2002) Ubc6p and ubc7p are required for normal and substrate-induced endoplasmic reticulum-associated degradation of the human selenoprotein type 2 iodothyronine monodeiodinase. *Mol Endocrinol* 16: 1999-2007.
44. Kim BW, Zavacki AM, Curcio-Morelli C, Dentice M, Harney JW, Larsen PR, Bianco AC (2003) Endoplasmic reticulum-associated degradation of the human type 2 iodothyronine deiodinase (D2) is mediated via an association between mammalian UBC7 and the carboxyl region of D2. *Mol Endocrinol* 17: 2603-2612.
45. Llovera M, Garcia-Martinez C, Agell N (1997) TNF can directly induce the expression of ubiquitin-dependent proteolytic system in rat soleus muscles. *Biochem Biophys Res Commun* 230: 238-241.
46. Kikuchi H, Ozaki T, Furuya1 K, Hanamoto T, Nakanishi M, Yamamoto H, Yoshida K, Todo S, Nakagawara A (2006) NF-κB regulates the stability and activity of p73 by inducing its proteolytic degradation through a ubiquitin-dependent proteasome pathway. *Oncogene* 25: 7608-7617.
47. Medina EA, Afsari RR, Ravid T, CastilloSS, Erickson KL, Goldkorn T (2005) Tumor necrosis factor-α decreases Akt protein levels in 3T3-L1 adipocytes via the caspase-dependent ubiquitination of Akt. *Endocrinology* 146: 2726-2735.
48. Rodriguez-Perez A, Palos-Paz F, Kaptein E, Visser TJ, Dominguez-Gerpe L, Alvarez-Escudero J, Lado-Abeal J (2008) Identification of molecular mechanisms related to nonthyroidal illness syndrome in skeletal muscle and adipose tissue from patients with septic shock. *Clin Endocrinol (Oxf)* 68: 821-827.
49. Warner MH, Beckett GJ (2010) Mechanisms behind the non-thyroidal illness syndrome: an update. *J Endocrinol* 205: 1-13.