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# Thyroid hormones differentially regulate phosphorylation of ERK and Akt via integrin $\alpha v\beta 3$ receptor in undifferentiated and differentiated PC-12 cells

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The effects of 3,5,3′-triiodo-L-thyronine (T3) and L-thyroxine (T4) on the integrin $\alpha v\beta 3$ receptor of thyroid hormones (TH) were investigated in pheochromocytoma PC-12 cells. Differentiation was induced by treatment of PC-12 cells with fisetin and the levels of phosphorylated extracellular signal-regulated kinase (ERK) and Akt in cytoplasm, as well as the content of FoxO6 transcription factor in nuclei was analysed in undifferentiated and differentiated conditions. We have found that in undifferentiated PC-12 cells, tetraiodothyroacetic acid (TETRAC), a known inhibitor of binding of T4 and T3 to plasma membrane integrin $\alpha v\beta 3$ receptor inhibits T4-dependent phosphorylation of ERK, whereas in differentiated PC-12 cells, TETRAC abolishes the effect of T3. In undifferentiated PC-12 cells, both TH increase the level of p-Akt, and this enhancement is not sensitive to TETRAC. In differentiated PC-12 cells, both TH increase the level of p-Akt; however, only T3-dependent activation of Akt is sensitive to the TETRAC. Furthermore, our results have shown that in differentiated PC-12 cells, the expression of FoxO6 was higher than in undifferentiated PC-12 cells, and this elevation has not changed under the action of TH. Only in undifferentiated PC-12 cells the T3-dependent expression of FoxO6 was sensitive to the TETRAC. We propose that PC-12 cells contain integrin $\alpha v\beta 3$ receptor, which T3 and T3/T4 sites are differentially regulated by TH in undifferentiated and differentiated conditions. Copyright © 2013 John Wiley & Sons, Ltd.

KEY WORDS—PC-12 cells; p-Akt; p-ERK; FoxO6; fisetin; TETRAC

## INTRODUCTION

The thyroid hormones (TH) are important regulators of growth, development and metabolism. The actions of 3,5,3′-triiodo-L-thyronine (T3) and L-thyroxine (T4) occur through its binding to the thyroid hormone receptor, which after translocation to the nuclei regulates various gene expressions. However, because the genomic effects of TR cannot explain many influence of THs, non-nuclear action of these hormones have been proposed.[1] Apparently, these nongenomic effects of thyroid hormones are mediated by the activation of integrin $\alpha v\beta 3$ receptor of THs, which deliver an antiapoptotic survival signal by several mechanisms, including the stimulation of ERK-dependent and Akt-dependent pathways.[2] It is proposed that integrin $\alpha v\beta 3$ receptor contains T3-specific site in the hormone-binding domain, as well as a site at which both T4 and T3 bind.[3] The T3-specific site activates PI3-kinase (PI3K)/Akt pathway and is linked to trafficking of certain intracellular proteins to the transcription machinery. T4 is unable to activate PI3K but causes ERK-dependent cell proliferation.[1] A deaminated derivative of T4, tetraiodothyroacetic acid (TETRAC), is an antagonist at the integrin receptor domain and blocks actions of THs at both the T4/T3 site and the T3-specific site. Interestingly, that inhibition of Src-kinase activity prevents THs activation of PI3-kinase and consequent cellular actions.[3] Binding sites for thyroid hormones in plasma membranes have been described in various tissues, including neurons suggesting that THs could stimulate the development of specialized nervous structures in embryogenesis.[4]

Integrins are heterodimeric structural proteins of the plasma membrane that interact with a large number of extracellular matrix proteins, growth factors, and hormones.[5,6] There are more than 20 integrins known, and integrin $\alpha v\beta 3$ is one of the most thoroughly investigated because of its angiogenic and tumorogenic activity.[7] $\alpha v\beta 3$ integrin receptor is expressed in native forms in PC-12 cells and stimulates by the neural cell adhesion molecule L1. This interaction induces neurite outgrowth in PC-12 cells by the mechanism distinct from other growth factors.[8]

FoxO6 is the most recently cloned FoxO family member with demonstrated expression in the brain.[9] The Akt site in the forkhead domain (Ser184) of FoxO6 regulates the

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DNA-binding characteristics, and the N-terminal Akt site acts as a growth factor sensor. FoxO6 is not a constitutively active transcription factor and can be regulated by growth factors in a Thr26-dependent and Ser184-dependent manner, independent of shuttling to the cytosol.[10] This member of FoxO family is highly enriched in the adult hippocampus, and its deficiency results in decreased dendritic spine density in hippocampal neurons *in vitro* and *in vivo*.[11] The FoxO6 plays an essential role in the specification of undifferentiated neurites into axons and dendrites in postmitotic neurons. Like other FoxO family members, FoxO6 is negatively regulated by the insulin/insulin-like growth factor (IGF) signalling pathway in cells.[10] During development, the FoxO family is required to establish neuronal polarity, and the ectopic expression of FoxO6 can rescue the neuronal polarity defects due to the deficiency of the FoxO family.[12] Interestingly, that expression of FoxO6 in various brain regions fluctuates depending on the age, sex and food.[13]

To further evaluate the role of FoxO6, ERK and Akt in the mechanism involved in TH integrin receptor-induced neurite outgrowth, we have chosen the rat pheochromocytoma (PC-12) cell line as a model system. In response to various growth factors and plant-derived compounds, these cells differentiate into sympathetic-like neurons and extend long neurites.[14,15] Thyroid hormones control the differentiation and neuritogenic activity of PC-12 cells,[16] but participation of integrin receptor in these processes, as well as FoxO6, is unknown. To evaluate the possible role of TH integrin $\alpha v\beta 3$ receptor in the expression of FoxO6, we examined the effects of T3 and T4 on the nuclear content of FoxO6 in undifferentiated and differentiated PC-12 cells. In parallel, we investigated the level of phosphorylated ERK and Akt in the cytoplasm of differentiated and undifferentiated PC-12 cells and found that the content of both protein kinases differentially changed after stimulation of integrin $\alpha v\beta 3$ receptor by T3 and T4. We suppose that in undifferentiated PC-12 cells, integrin receptor $\alpha v\beta 3$ is regulated mainly through T3/T4 site and operates via ERK pathway, whereas in differentiated PC-12 cells, this receptor is regulated predominantly by T3 site through Akt protein kinase pathway.

## MATERIALS AND METHODS

### *Cell line*

PC-12—pheochromocytoma cells (PC-12) were a generous gift from Dr J. Szeberenyi (Pech University, Hungary). Cells were grown on 24-plates in a humidified atmosphere containing 5% CO2 at 37 °C in an RPMI-1640 (Sigma-Aldrich) medium supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich) and 5% fetal bovine serum (Sigma-Aldrich). For the induction of differentiation, PC-12 cells were incubated with the 5 μM fisetin for 48 h. The cells were scored as differentiated if one or more neurites were longer than the diameter of the cell body. Differentiated and undifferentiated cells were treated with 20 nM T3 or 20 nM T4 for 48 h. In the corresponding experiments, 100 nM TETRAC was added to the incubation medium with the thyroid hormones. Preliminary experiments showed that thyroid hormones have no effect on the proliferation and differentiation of PC-cells in this time interval, and TETRAC alone does not change the content of p-Akt or p-ERK in differentiated or in undifferentiated PC-12 cells (data not shown). This data agrees with the observations of Muñoz *et al*.,[17] and Lin *et al*.[3]

### *Preparation of nuclear and cytoplasmic fractions from PC-12 cell line*

After 48 h incubation, PC-12 cells were removed from 24-plates and were pelleted by centrifugation at 300 × g. After centrifugation, PC-12 cells were washed with Ringer solution twice. The last incubated PC-12 cells were suspended in lysis buffer containing 1.5 mM $MgCl_2$, 10 mM KCl, 10 mM HEPES, 1 mM DTT, 1 mM phenylmethylsulphonylfluoride and 15 mM soybean trypsin inhibitor, pH 7.9. After 30 min incubation at 4 °C temperature, the cells were homogenized, and nuclei were sedimented at 800 × g for 10 min. The pellets were resuspended in the 0.05 M Tris–HCl buffer, pH 7.5 and used as the nuclear fraction for the following analysis of FoxO6. Post-nuclear fraction was centrifuged at 14 000 × g for 20 min, and supernatants were used as the cytoplasmic fraction for the following analysis of p-Akt and p-ERK protein kinases.

### *Western blotting of p-Akt and p-ERK*

About 50 μg of protein from cytoplasmic fractions (for p-ERK and p-Akt analysis) or nuclear fractions (for FoxO6 analysis) was denatured at 90 °C for 5 min, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5–12% gradient gels and transferred to nitrocellulose membranes (Bio-Rad protocol). Equal loading and transfer of the samples were confirmed by staining the nitrocellulose with Ponceau-S. After blocking with blocking buffer (5% non-fat dry milk and 0.05% Tween 20 in Tris–HCl-buffered saline), the sheets were incubated with primary antibodies for 1 h (rabbit anti-p-Akt, rabbit anti-p-ERK, rabbit anti-FoxO6 or rabbit anti-actin at dilution of 1:20 000; Santa Cruz Biotechnology, Inc.) in the blocking solution on the shaker. After washing, the sheets were incubated with secondary antibodies (anti-rabbit horseradish peroxidase conjugated antibody at dilution of 1:5000; Santa Cruz Biotechnology, Inc.) for 40 min in blocking solution on the shaker. Labelled bands were visualized using enhanced chemiluminescence (Amersham, California, USA) and X-ray film for chemiluminescent blotting substrates. The content of proteins was quantified from the intensity of the bands, which is linear to the quantity of samples applied to the gel.

## STATISTICAL ANALYSIS

Statistical analyses were performed by either an unpaired *t*-test or a one-way ANOVA and Scheffe's *post hoc* analysis

when appropriate. Results were considered significant when $p < 0.05$. The results are expressed as the groups mean ± SEM from at least three independent experiments.

## RESULTS

PC-12 cell line is widely used as a cellular model for studies of neurotrophic action.[18] To investigate the effect of thyroid hormones on the plasma membrane receptor integrin $\alpha v\beta 3$, we used two types of PC-12 cells: cells maintained in the normal RPMI-1640 medium and differentiated cells after treatment with fisetin. To evaluate the neuritogenic action, adherent PC-12 cells were treated with 5 μM fisetin for 48 h for the analysis of cell morphology change. Quantification of neurite outgrowth data showed that treatment of PC-12 cells with fisetin significantly increased neurite outgrowth, and the percentage of neurite-bearing cells was 34.8 ± 3.2% that completely agreed with the observation of Sagara *et al.*[15] Thus, we induced the differentiation of PC-12 cells with fisetin and examined the effects of thyroid hormones on the content of p-Akt, p-ERK and FoxO6. We have found that in undifferentiated cells, both thyroid hormones (T3 and T4) increase the content of p-Akt; however, this elevation is not sensitive to the antagonist of integrin $\alpha v\beta 3$ receptor—TETRAC (Figure 1). In differentiated PC-12 cells, TH also increases the level of p-Akt; however, in this case, only T3-dependent activation of Akt is sensitive to the TETRAC.

Next, we determined the content of p-ERK in the differentiated and undifferentiated PC-12 cells. We have found that only T4 increases the expression of p-ERK in the undifferentiated PC-12 cells, whereas T3 has no effect. And vice-versa, in the differentiated cells, elevation of expression of p-ERK was observed in the presence of T3. In both cases, the effects of thyroid hormones were sensitive to TETRAC, suggesting that surface expressed integrin receptors is involved in the activation of ERK (Figure 2). These data suggest that the main ligand acting through integrin $\alpha v\beta 3$ receptor in undifferentiated PC-12 cells is a T4, which apparently operated through the activation of ERK protein kinase.

In the next series of experiments, we determined the content of FoxO6 in nuclear fraction of PC-12 cells after incubation of undifferentiated cells with T3 or T4. We have found that both T3 and T4 induce FoxO6 expression, but only the effect of T3 is suppressed by TETRAC (Figure 3). Furthermore, we examined the effects of thyroid hormones on the differentiated cells. We have found that fisetin, *per se*, induces the expression of FoxO6, but this elevation was insensitive to thyroid hormones and TETRAC. These data suggest that in undifferentiated PC-12 cells, only T3-operated site of integrin receptor could change the expression of FoxO6.

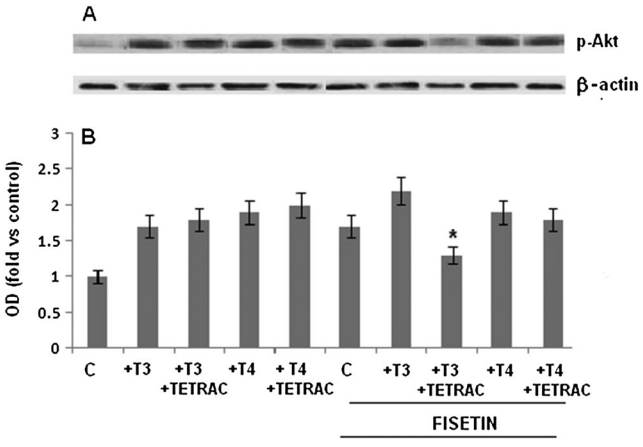


Figure 1. Effect of T3, T4, tetraiodothyroacetic acid and fisetin on the content of p-Akt protein kinase in cytoplasmic fraction of PC-12 cells. For immunoblots, 50 μg of total protein from each fraction was loaded per lane, resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-p-Akt and anti-actin as described in 'Material and Methods' section. (A) Western blots, blot is representative of three similar experiments. (B) The average densitometric quantification of the results of three independent experiments. Immunoreactive bands were scanned, and the results are expressed as fold changes versus control. Optical density (OD) is presented as means ± SEM from three independent experiments. *$p < 0.05$, *t*-test compared with corresponding control simples. (compared without antagonist, i.e. TETRAC action)

## DISCUSSION

The nongenomic actions of thyroid hormone involve activation of signalling pathways through plasma membrane $\alpha v\beta 3$-integrin receptor. It has been proposed that this surface-exposed receptor has distinct binding sites for T3 and T4. One binding site binds only T3 and activates the PI3K/Akt pathway, whereas other binding site binds both T3 and T4 and activates the ERK1/2 MAP kinase pathway.[3] Thus, T3 binds to both sites and activates both the ERK and PI3K/Akt pathways, whereas T4 activates ERK after binding to only one of the two surface integrin sites. The stimulation of T3/T4 binding site induces cell proliferation, whereas activation of T3 site involves transcription factors translocation and specific gene expression. In the PC-12 cells, the activation of surface-exposed $\alpha v\beta 3$-integrin receptor stimulates neurite outgrowth through the mitogen-activated protein kinase cascade;[8] however, the effects of thyroid hormones on the $\alpha v\beta 3$-integrin-mediated signalling pathway and down-stream protein kinases are unknown.

We study the action of thyroid hormones on the two types of PC-12 cells: on the undifferentiated, proliferated PC-12 cells and on the fisetin-induced neurite-bearing differentiated PC-12 cells. We have found that in undifferentiated PC-12 cells, TETRAC, a known inhibitor of binding of T4 and T3 to plasma membrane integrins,[3] inhibits T4-dependent phosphorylation of ERK, whereas in differentiated PC-12 cells, TETRAC does not change the effect of T4 on the p-ERK. In this condition, only T3 increases the content of phosphorylated ERK, which is decreased in the presence of TETRAC. Moreover, both thyroid hormones increase the content of p-Akt in undifferentiated

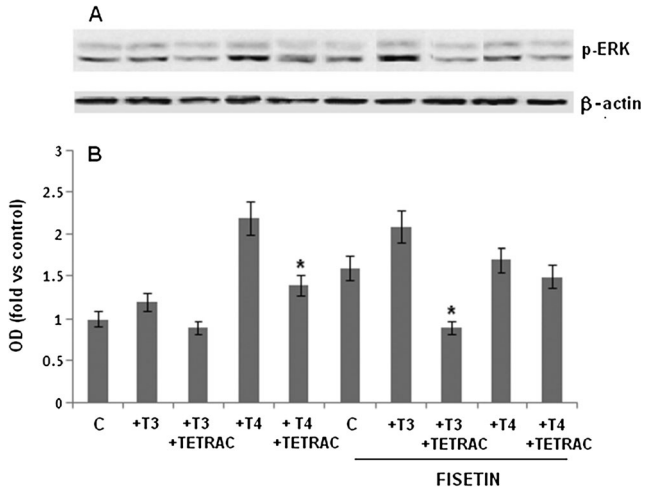


Figure 2. Effect of T3, T4, tetraiodothyroacetic acid and fisetin on the content of p-extracellular signal-regulated protein kinase in cytoplasmic fraction of PC-12 cells. For immunoblots, 50 μg of total protein from each fraction was loaded per lane, resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-extracellular signal-regulated kinase and anti-actin as described in 'Material and Methods' section. (A) Western blots, blot is representative of three similar experiments. (B) The average densitometric quantification of the results of three independent experiments. Immunoreactive bands were scanned, and the results are expressed as fold changes versus control. OD is presented as means ± SEM from three independent experiments. $*p < 0.05$, *t*-test compared with corresponding control simples. (compared without antagonist, i.e. TETRAC action)

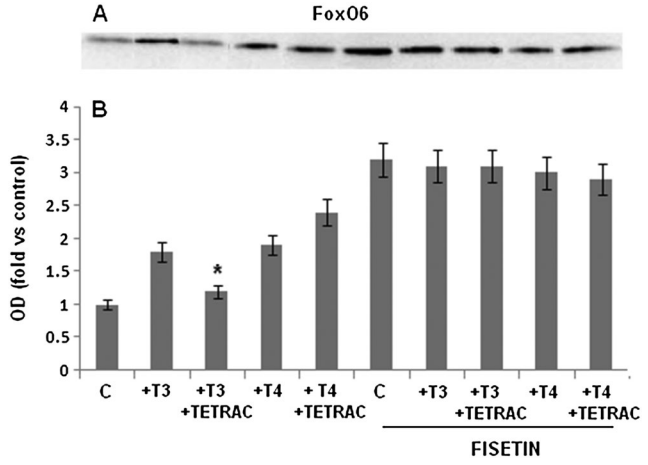


Figure 3. Effect of T3, T4, TETRAC and fisetin on the content of FoxO6 in nuclear fraction of PC-12 cells. (A) Western blots, western blot was performed using anti-FoxO6 antibodies as described in 'Material and Methods' section. Blot is representative of three similar experiments. (B) Immunoreactive bands were scanned, and the results are expressed as fold changes versus control. OD is presented as means ± SEM from tree independent experiments. $*p < 0.05$, *t*-test compared with corresponding control samples (compared without antagonist, i.e. TETRAC action)

PC-12 cells, but this elevation is not sensitive to the TETRAC. Only in differentiated PC-12 cells T3-dependent phosphorylation of Akt is sensitive to this antagonist. Because in differentiated PC-12 cells, T3 could increase the phosphorylation of both protein kinases—ERK and Akt, it can be concluded that in differentiated conditions, T3-response was dominated through T3 sites of integrin receptor. Because ERK phosphorylation has not changed under the action of T3 in undifferentiated PC-12 cells, T4 can probably operates via T3/T4 sites. These data suggest that in differentiated PC-12 cells, the specificity of integrin $\alpha v\beta 3$ receptor may be switched from T4-dependent ERK pathway to T3-dependent P13K/Akt pathway.

FoxO family member, FoxO6, is unique in that its mRNA is expressed predominantly in the CNS in mammals.[19,9] Like other FoxO family members, FoxO6 is negatively regulated by the insulin/IGF signalling pathway in neurons. Phosphorylation of FoxO6 by Akt attenuates FoxO6-dependent transcription; although, it does not affect FoxO6 nuclear localization.[19,10] We have found that both thyroid hormones increase the levels of FoxO6 in undifferentiated PC-12 cells in nuclei; however, only the action of T3 is sensitive to the TETRAC. The effect of T3, in this case, is not mediated either Akt or ERK, because phosphorylation of any of these protein kinases did not correlate with the amount of expressed FoxO6. This discrepancy may be explained by the TH-dependent activation of other intracellular systems. It has been shown that the stimulation of TETRAC-sensitive T3 site of integrin receptor by T3 activates Src family of tyrosine kinases.[1] These tyrosine kinases have a broad substrate specificity[20,21] and can phosphorylate many regulatory proteins, including proteins involved in the insulin/IGF pathway,[22] as well as enzymes participating in stability, relocation and transcriptional activity of Fox proteins.[10] In addition, T3-dependent and TETRAC-sensitive $\alpha v\beta 3$ integrin receptor participates in the effects of IGF,[22] epidermal growth factor, vascular endothelial growth factor and basic fibroblast growth factor suggesting on the existence of cross-talk between the integrin receptor for THs and growth factor receptors.[7] Thus, it is possible that the effects of T3 on FOXO6 relocation are mediated by regulatory proteins participating in growth factors-dependent pathways. Furthermore, our results have shown that expression of FoxO6 was increased in differentiated PC-12 cells, but in this case, thyroid hormones do not change the levels of FoxO6. These results suggest that thyroid hormones, via integrin receptor, can induce the expression of FoxO6 only in undifferentiated PC-12 cell whereas increased expression of the FoxO6 in differentiated PC-12 cells due to the action of other growth factors or is the result of cell differentiation.

In summary, the present work has shown that PC-12 cells contain $\alpha v\beta 3$ integrin receptor, which activity is differentially regulated by thyroid hormones in undifferentiated and differentiated conditions. In the undifferentiated, proliferating state $\alpha v\beta 3$ integrin receptor is regulated primarily through T3/T4 site and operates via ERK pathway, whereas in the differentiated state, PC-12 cells are regulated predominantly by T3 site via P13K/Akt pathway. In the undifferentiated state, activation of T3/T4 site could induce the elevation of nuclear FoxO6, whereas in differentiated state, such response disappears. Further study is needed to evaluate the role of TH integrin receptors in the transcriptional activity of FoxO6.

## CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

## ACKNOWLEDGEMENT

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