Activation of estrogen response element dependent transcription by thyroid hormone with increase in estrogen receptor levels in a rat pituitary cell line, GH3

N Fujimoto, N Jinno1 and S Kitamura1

Department of Developmental Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

1Department of Xenobiotic Metabolism and Molecular Toxicology, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

(Requests for offprints should be addressed to N Fujimoto; Email: nfjm@hiroshima-u.ac.jp)

Abstract

Interrelationships between thyroid hormone and estrogen actions have been documented with regard to a variety of physiological functions. Both hormones stimulate transcription of target genes by binding to their nuclear receptors that interact with specific responsive elements (estrogen and thyroid hormone response elements, i.e ERE and TRE, respectively) in the regulatory regions of the gene. In vitro studies have suggested that interplay between the two hormones might be due to cross-talk at hormone responsive elements, with the respective hormone receptors and ligands able to interact, although physiological relevance has yet to be proved. We have proposed a simpler mechanism for thyroid hormone effects on estrogen responses via increase in estrogen receptor α (ERα) with resultant increase in progesterone receptors, prolactin production and tumor growth. A pituitary cell line, GH3, has been widely used to investigate the function of mammom-somatotropic cells, especially regarding regulation of GH and prolactin production. In the present study, an ERE-luc reporter was transfected into GH3 cells and the responses to endogenous ERα were examined. We demonstrated that: (1) 3,5,3′-triiodothyronine (T3) induces mRNA expression of ERα; (2) T3 alone is able to induce ERE-luc activity and this is inhibited by OH-tamoxifen; (3) T3 synergistically acts on estradiol (E2)-induced ERE responses; and (4) ERE-luc activity is enhanced by co-transfection of an ERα expression vector. These results support the hypothesis that estrogen responses are potentiated by T3 through up-regulation of ERα levels.


Introduction

Interrelationships between thyroid hormones and estrogen actions have been documented with regard to a variety of physiological functions (Vasudevan et al. 2002) and elevation in thyroid hormone levels has been shown to have adverse effects on reproduction in rodents (Dellowade et al. 1996). Stimulation of mitosis in the uterine epithelium by estrogen is reduced in hypothyroid rats (Franklyn et al. 1994), while hypothyroidism is known to decrease bone density by depressing estrogenic action. Regulation of GH by thyroid hormones depends on the thyroid hormone response element (TRE) in the promoter region of the growth hormone (GH) gene and the estrogen receptor (ER) also binds to this TRE and promotes transcription in vitro (Graupner et al. 1991). Although the thyroid hormone receptor (TR) has a higher affinity for TRE than the ER, estrogen could result in interference with thyroid hormone dependent transcription.

Conversely, TR is also able to interact with estrogen response elements (EREs) of both the progesterone receptor and the vitellogenin A2 promoters which influence transcription (Scott et al. 1997). However, no evidence has yet been provided regarding any physiological significance for such cross-talk demonstrated in in vitro experiments. We have proposed a simple enhancing mechanism of thyroid hormones on estrogenic actions via an increase in ERα levels; we previously found this is to be the case in a transplantable pituitary tumor line, MtT/F84, with potentiation of estrogen responses such as prolactin production and the induction of progesterone receptors (Fujimoto et al. 1988, 1991, 1996, Ito et al. 1985).

GH1 and GH3 are widely used rat pituitary cell lines, originally isolated from the MtT/W5 pituitary tumor, whose growth and prolactin/GH synthesis are stimulated by both thyroid hormone and estrogen (Sorrentino et al. 1976). They express high amounts of ERα and also TR (Haug et al. 1978). In the literature, there is a large

0022–0795/04/0181–077 © 2004 Society for Endocrinology Printed in Great Britain

Downloaded from Bioscientifica.com at 12/06/2018 11:25:48PM via free access
variation in the reported estrogen-responsive growth of this cell line, from insensitive to very sensitive (Haug & Gautvik 1976, Kino & Dannies 1981, Lieberman et al. 1981, Scammell et al. 1986, Amara et al. 1987, Rhode & Gorski 1991). The Health Science Research Resources Bank in Osaka, Japan, which carries a collection of cell lines available to the research community, have two strains of GH3 cells in stock, both of which are very sensitive to estrogen as well as thyroid hormone with reference to promotion of cell growth.

In the present study, we further examined, in GH3 cells, our hypothesis of enhancement of estrogenic action by thyroid hormones via an increase in ERα levels. For this purpose, ERα mRNA expression and ERα-mediated responses were examined in vitro by mRNA quantification using the real-time PCR method and an estrogen-dependent transcription assay with an ERE-luciferase reporter.

**Materials and Methods**

**Chemicals**

17β-estradiol (E2), 3,5,3′-triiodothyronine (T3) and an estrogen antagonist, 4-hydroxytamoxifen (OH-tamoxifen), were purchased from Sigma. Each was dissolved in ethanol to give stock solutions.

**Cell culture**

The pituitary cell line, GH3, was obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 mixed medium (Sigma) containing penicillin and streptomycin with 10% horse serum (HS, Gibco/Invitrogen) and 2.5% fetal bovine serum (FBS, Gibco/Invitrogen). The MCF-7 cell line was maintained in DMEM containing penicillin and streptomycin with 5% FBS. Before transfection experiments and cell growth assays, cells were maintained for a week in phenol-red-free medium (Sigma) containing the same antibiotics along with dextran-charcoal-treated serum. For cell growth assays, GH3 cells were seeded in 24-well plates at 1 × 10⁴ cells/well, and hormones were added the next day. Growth was measured after 5 days by means of a modified MTT assay with WST-1 (Dojindo Chemicals, Kumamoto, Japan).

**RNA isolation and reverse transcription**

GH3 cells were seeded in 60 mm dishes at 1 × 10⁶ per dish, treated with hormones the next day and harvested after 24 h. RNA preparation was carried out with an SV-total RNA isolation kit from Promega, following the supplied protocol. One microgram of total RNA was reverse transcribed with 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen) and 2.5 pmol oligo-dT primer (Invitrogen) in 25 µl buffer containing 1 mM dNTP, 100 mM Tris-HCl (pH 8.3), 150 mM KCl, 6 mM MgCl₂, 60 mM dithiothreitol and 5 U/µl RNasin with incubation at 37°C for 60 min.

**Measurement of ERα mRNA by quantitative real-time PCR**

The real-time PCR method with a QuantiTect Sybr Green PCR kit (Qiagen) and an ABI Prism 7700 (PerkinElmer Life Sciences, Boston, MA, USA) was employed for quantitative measurement of ERα and glyceraldehyde-3-phosphate dehydrogenase (G3 PDH) mRNAs following the supplied protocol (Woo et al. 1998). The primers for ERα were 5′-CCAATTCCTGAC AATCGACGC (+470/+489, located in exon 2) and 5′-TCTTTATCGATGGTGCATTGGTT (+688/+710, located in exon 3) giving a PCR product of 241 bp. The primer pairs for G3 PDH were GGTTGATGCTGGTG CTGAGT (+255/+274) and TGGCATGGAATG TGTCATG (+516/+515) resulting in a 262 bp product. The PCR conditions were a 15 min initial activation step followed by 45 cycles of 15 s at 94°C, and 30 s at 50°C and 60 s at 72°C. Prior to the quantitative analysis, PCR products were prepared separately and purified by gel-electrophoresis. Fragments extracted from the gels were used as standards for quantification. ERα mRNA contents were normalized with reference to the G3 PDH mRNA level.

**ERE-luc reporter gene assay**

The estrogen-responsive reporter plasmid, (ERE)₃-SV40-luc, contains three consensus ERE motifs from the Xenopus vitellogenin A2 gene (Kudoh et al. 1996). A rat ERα expression plasmid was constructed by inserting the EcoRI fragment of pRcER6 (Kojima et al. 1987) into an EcoRI site of the pSG5 vector. phRL-CMV was a renilla luciferase expression plasmid from Promega.

GH3 cells were plated at 4 × 10⁴ cells/well in 48-well plates and transiently transfected with 0.4 µg (ERE)₃-SV40-luc along with 0.01 µg phRL-CMV (Promega) with TransFast transfection reagent containing a synthetic cationic lipid (Promega), following the supplier’s protocol. The weight ratio of TransFast reagent to DNA was 1:1. After 24 h incubation with hormones, cells were harvested with 30 µl cell lysis buffer (Promega) and the firefly and renilla luciferase activities were determined with a dual luciferase assay kit (Promega) by measuring luminescence with a Wallac Micro-Beta scintillation counter (PerkinElmer Life Sciences). Firefly luciferase reporter activity was normalized to the renilla luciferase activity from phRL-CMV.

**Statistical analysis**

Statistical comparisons were made using Student’s t-test.
Results

Induction of ERα mRNA expression by T3

Treatment with T3 for 24 h significantly increased the ERα mRNA level in GH3 cells (Fig. 1). The response was dose dependent between $10^{-9}$ and $10^{-7}$ M. A time-course study revealed that the mRNA level was already significantly increased after 1 h of treatment with T3 at $10^{-7}$ M.

Activation of ERE-luc transcription by E2 and T3

The ERE-luc reporter plasmid transfected into GH3 cells responded well to E2 at concentrations between $10^{-11}$ and $10^{-9}$ M. Interestingly, T3 was also able to induce ERE-directed luciferase activity at $10^{-9}$ M, albeit with lower activity than E2 (Fig. 2A). An anti-estrogen, OH-tamoxifen (TAM), inhibited both T3- and E2-induced ERE-luc responses. When the same reporter was transfected into MCF-7 cells, ERE-luc activity was again dependent on the concentration of E2. T3, on the other hand, did not induce any ERE-luc activity in this cell line (Fig. 2B).

Enhancement of E2-induced ERE-luc responses by T3 in GH3 cells

E2-induced ERE-luc responses were enhanced from 4- to 6-fold with E2 alone to 6- to 11- fold with E2 plus...
10⁻⁸M T₃, while the elevation due to T₃ alone was only 2- to 2.5-fold (Fig. 3). Similar findings were obtained with T₃ at 10⁻⁷ M.

Effects of rERα expression vector transfection on ERE responses

Co-transfection of the rERα expression vector, pSG5-rERα, significantly increased the basal level of ERE-luc activity 1.6-fold (Fig. 4). When 10⁻⁹ M E₂ was administered, significantly higher luciferase activities were noted in ERα-transfected cells.

Effects of E₂ and T₃ on GH3 cell proliferation

Data for the effects of E₂ and T₃ on cell proliferation of GH3 are summarized in Fig. 5. The growth of the cells almost stopped when the medium was replaced with phenol-red-free DMEM with dextran-charcoal-stripped serum. The cell growth was very sensitive to estrogen, with significant growth noted at 10⁻¹² M and reaching the maximum at 10⁻⁹ M. With T₃, significant growth was noted at 10⁻¹⁰ M and was maximal at 10⁻⁸ M.

OH-tamoxifen at 10⁻⁷ M inhibited both E₂- and T₃-induced cell proliferation, while not exerting any influence when given alone.

Discussion

MtT/F84 is a transplantable mammo-somatotropic tumor, which was originally induced by chronic E₂ administration and subsequent transplantation in estrogenized F344 rats. It is characterized by the production of GH and prolactin and by the presence of ER and TR. We have previously described that growth of MtT/F84 correlates well with the administered dose of estrogen (Fujimoto et al. 1991). Under hypothyroidal conditions, interestingly, estrogen responses (including tumor growth and progesterone receptor induction) are suppressed, accompanied by a decrease in ER levels. On isolation of a series of pituitary cell lines from MtT/F84, we proved regulation of ERα expression by thyroid hormones in vitro (Fujimoto et al. 1997), suggesting that this is the mechanism underlying the modification of estrogen responses. Long before our cell lines were established, GH1 and GH3 cell lines were isolated from the rat pituitary tumor MtT/W5. They
have been widely used to investigate the function of mammo-somatotrophic cells, since the regulation of GH and prolactin production in this cell line appears to be physiologically relevant with dependence on hormones such as T3, E2 and dexamethasone. In the present study of GH3 cells, we further tested and confirmed our hypothesis that T3 stimulates estrogenic actions by increasing the ER level using an ERE-reporter gene assay and ER mRNA measurement by real-time PCR.

We have demonstrated that the mRNA of ERα is up-regulated by T3 in GH3 cells. Since ERα is the dominant type of the receptor in GH3 (ERβ mRNA level was only about 1/60 of ERα mRNA according to measurements by real-time PCR, data not shown), only ERα was investigated in the present study. In the pituitary gland, it has been reported that the ER concentration is decreased in hypothyroid and increased in hyperthyroid rats (Andre et al. 1982). T3 has been shown to increase the ER level in rat liver (Freysschuss et al. 1994) and in Xenopus liver cells, playing a crucial role in preparing the latter to respond to estrogen by switching-on ER expression (Ulisse & Tata 1994). The cells thus become able to produce E2-dependent vitellogenin protein only after T3 treatment. In the mammary gland, one of the primary targets for estrogen and which generally expresses high levels of ERα, there are no reports indicating an effect of thyroid hormones on ER expression either in vivo or in vitro. Indeed T3 did not influence ER mRNA expression in MCF-7 cells in the present study (data not shown). Promoter regions of the ERα gene have been reported and well characterized. In humans, transcription is from three different promoters, while only two promoters are involved in the rat (Grandien et al. 1995, 1997, Freysschuss & Grandien 1996). However, there seem to be no thyroid hormone responsive transcriptional motifs in the known promoter structures. Although the induction of ERα mRNA by T3 occurred within 1 h, the transcription may not be regulated directly by T3 through the TR–TRE mechanism.

It is evident that thyroid hormones and estrogen actions are interrelated in a variety of physiological functions involving both hormones, such as growth of the uterine epithelium, change in bone density and determination of sexual behavior (Franklyn et al. 1994, Delovadie et al. 1996). It has been speculated that the interrelations might be the result of the competition and/or co-operation of their receptors in promoter regions, since over-expressed ER and TR can interfere with each other in vitro at TRE and ERE sites. In the present study, the ERE-luc reporter was transfected into GH3 cells endogenously expressing ER and TR, which makes our model more realistic. In the ERE-luc–transfected GH3 cells, we showed that: (1) T3 is able to induce ERE-luc activity; (2) this T3-dependent activity can be inhibited by an anti-estrogen; (3) T3 synergistically stimulates E2-induced ERE responses; and finally (4) ERE-luc activity could be enhanced by co-transfection of an ERα expression vector. The results support our previously proposed hypothesis that estrogen responses are potentiated by T3 through up-regulation of ER levels, rather than involving TR and ERE. T3 alone activated ERE-luc expression in the present study, possibly due to responses to endogenous estrogen in the culture serum remaining after the dextran–charcoal treatment.

Effects of T3 and E2 on pituitary cell growth have long drawn attention. Early work at Sirbasku’s laboratory indicated that in vivo growth of GH3 indeed depends on both estrogen and thyroid hormones (Kirkland et al. 1976, Sorrentino et al. 1976). However, only thyroid hormones are required for growth in cell culture. While findings for T3-responsive growth are consistent, reported sensitivity to estrogen has varied in the literature (Kiino & Harnies 1981, Scammell et al. 1986, Amara et al. 1987, Chun et al. 1998, Rhode & Gorski 1991). This inter-laboratory variation might be due to differences in strain, since GH3 has a rather old origin and has been widely used. However, technical problems with the charcoal treatment of serum to remove estrogenic substances could have had an impact (Riss & Sirbasku 1989). The estrogenic activity of phenol red or related contaminants in common culture medium was not recognized until Katzenellenbogen’s group provided convincing evidence (Berthois et al. 1986). With updated culture conditions, Sirbasku’s group successfully reconstructed estrogen-responsive growth in GH4 cells. Our previous studies with pituitary cell lines have repeatedly shown that as little as 10−12 M E2 is effective in inducing estrogen-dependent reporter gene activity as well as E2-responsive cell growth. The data thus suggest
that GH3 cells respond to both E2 and T3 in the culture condition with careful removal of estrogenic compounds.

The growth of GH3 cells responsive to both E2 and T3 has been found to be inhibited by OH-tamoxifen; this is consistent with a report concerning F4Z2 cells, another estrogen–dependent pituitary cell line (Zhou-Li et al. 1992). The fact that the required concentration of T3 for growth stimulation ($10^{-11}$ to $10^{-8}$ M) did not coincide with that for ER induction ($10^{-9}$ to $10^{-7}$ M) suggests direct effects of T3 on pituitary cell proliferation in this case.

Acknowledgements

We thank Mr Y Mizuno for his expert technical assistance and Dr M A Moore for reading the manuscript and suggesting English clarification.

Funding

This work was supported in part by Grant-in-Aid (H13-Seikatsu) from the Ministry of Health, Labor and Welfare, Japan and a Grant-in-Aid (14042241) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. There is no conflict of interest that would prejudice the impartiality of the research.

References

Amara JF, Van IC & Dannies PS 1987 Regulation of prolactin production and cell growth by estradiol: difference in sensitivity to estradiol occurs at level of messenger ribonucleic acid accumulation. Endocrinology 120 264–271.


Berthois Y, Katzenellenbogen JA & Katzenellenbogen BS 1986 Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. PNAS 83 2496–2500.

Chun TY, Gregg D, Sarker DK & Gorski J 1998 Differential regulation by estrogens of growth and prolactin synthesis in pituitary cells suggests that only a small pool of estrogen receptors is required for growth. PNAS 95 2325–2330.

Dellodecote TL, Zhu YS, Krey L & Pfaaff DW 1996 Thyroid hormone and estrogen interact to regulate behavior. PNAS 93 12581–12586.


Kino DR, & Dannes PS 1981 Insulin and $17$ beta-estradiol increase the intracellular prolactin content of GH4C1 cells. Endocrinology 109 1264–1269.


Scott RE, Wu-Peng XS, Yen PM, Chin WW & Pfaaff DW 1997 Interactions of estrogen- and thyroid hormone receptors on a progesterone receptor estrogen response element (ERE) sequence: a comparison with the vitellogenin A2 consensus ERE. Molecular Endocrinology 11 1581–1592.

Ulisse S & Tata JR 1994 Thyroid hormone and glucocorticoid independently regulate the expression of estrogen receptor in male *Xenopus* liver cells. *Molecular and Cellular Endocrinology* **105** 45–53.


Received 27 November 2003
Accepted 24 December 2003