Inhibition of GATA2-dependent transactivation of the TSHβ gene by ligand-bound estrogen receptor α

Koji Nagayama, Shigekazu Sasaki, Akio Matsushita, Kenji Ohba, Hiroyuki Iwaki, Hideyuki Matsunaga, Shingo Suzuki, Hiroko Misawa, Keiko Ishizuka, Yutaka Oki, Jaeduk Yoshimura Noh and Hirotoshi Nakamura

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan

1Ito Hospital, 4-3-6 Jingumae, Shibuya-ku, Tokyo, 150-8308, Japan

(Correspondence should be addressed to S Sasaki; Email: sasakis@hama-med.ac.jp)

Abstract

Transcriptional repression of the TSH-specific β subunit (TSHβ) gene has been regarded to be specific to thyroid hormone (tri-iodothyronine, T3) and its receptors (TRs) in physiological conditions. However, TSHβ mRNA levels in the pituitary were reported to decrease in the administration of pharmacologic doses of estrogen (17β-estradiol, E2) and increase in E2 receptor (ER)-α null mice. Here, we investigated the molecular mechanism of inhibition of the TSHβ gene expression by E2-bound E2-estrogen receptor 1 (E2-ERα). In kidney-derived CV1 cells, transcriptional activity of the TSHβ promoter was stimulated by GATA2 and suppressed by THRBs and ERα in a ligand-dependent fashion. Overexpression of PIT1 diminished the E2-ERα-induced inhibition, suggesting that PIT1 may protect GATA2 from E2-ERα targeting by forming a stable complex with GATA2. Interacting surfaces between ERα and GATA2 were mapped to the DNA-binding domain (DBD) of ERα and the Zn finger domain of GATA2. E2-dependent inhibition requires the ERα amino-terminal domain but not the Zn finger motif in E2-ERα-DBD. In the thyrotroph cell line, TαT1, E2 treatment reduced TSHβ mRNA levels measured by the reverse transcription PCR. In the human study, despite similar free thyroxine levels, the serum TSH level was small but significantly higher in post- than premenopausal women who possessed no anti-thyroid antibodies (1.90 U/ml ± 0.12 s.e.m. vs 1.47 U/ml ± 0.12 s.e.m., P < 0.05). Our findings indicate redundancy between T3-TR and E2-ERα signaling exists in negative regulation of the TSHβ gene.


Introduction

Thyrotropin (thyroid-stimulating hormone, TSH) is a heterodimer consisting of the TSH-specific β subunit (TSHβ) and the choric gonadotropin α chain (CGA) that is common to luteinizing hormone (LH), follicle-stimulating hormone (FSH), and chorionic gonadotropin. Thyroid hormone (tri-iodothyronine, T3)-mediated negative feedback of TSH production in the pituitary is a central mechanism of the pituitary–thyroid axis and is believed to be specific to T3 at physiological concentrations. However, mechanism of the pituitary–thyroid axis and is believed to be feedback of TSH production in the pituitary is a central mechanism of the pituitary–thyroid axis and is believed to be feedback of TSH production in the pituitary is a central mechanism of the pituitary–thyroid axis and is believed to be feedback of TSH production in the pituitary is a central mechanism of the pituitary–thyroid axis and is believed to be feedback of TSH production in the pituitary is a central mechanism of the pituitary–thyroid axis and is believed to be feedback of TSH production in the pituitary is a central mechanism of the pituitary–thyroid axis and is believed to be feedback of TSH production in the pituitary is a central mechanism of the pituitary–thyroid axis and is believed to be feedback of TSH production in the pituitary is a central mechanism of the pituitary–thyroid axis and is believed to be feedback of TSH production in the pituitary is a central •

Boado et al. (1983) showed that E2 benzoate induced a marked depression of intrapituitary TSH. Sekulic et al. (1998) demonstrated that E2 treatment reduced the number of TSHβ-positive cells in the rat pituitary.

There are two types of estrogen receptors (ERs), ERα and ERβ. In the anterior lobe of the pituitary, ERα is the major receptor (Kuiper et al. 1996, Pelletier et al. 2000, Liu & Cui 2005) and is expressed in thyrotrhops as well as gonadotrophs (Stefaneau et al. 1994, Gittoes et al. 1997). Interestingly, Scully et al. (1997) reported that, in the pituitary of ERα-deficient mice, mRNA levels for not only LHβ and FSHβ but also TSHβ and CGA were dramatically elevated. Compared with wild-type mice, the amount of mRNA for Tshβ and Cga in Esrα null mice increased by 3-20- and 4-36-fold respectively. Immunostaining using specific antibodies revealed that the number of TSHβ- and CGA-positive cells also increased (Scully et al. 1997). These findings are similar to observations in mice devoid of all known T3 receptors (TRs; Gothe et al. 1999). Although these observations suggest that E2 suppresses TSHβ expression via ERα, the molecular mechanism has not been clarified.


0022–0795/08/0199–113 © 2008 Society for Endocrinology Printed in Great Britain

DOI: 10.1677/JOE-08-0128

Online version via http://www.endocrinology-journals.org

Downloaded from Bioscientifica.com at 12/08/2018 02:49:24AM via free access
ER and TR belong to the nuclear hormone receptor superfamily and share a basic structure consisting of a receptor-specific amino-terminal domain (NTD), central DNA-binding domain (DBD), and carboxyl (C)-terminal ligand-binding domain (LBD; Mangelsdorf et al. 1995). In the promoter region of target genes whose transcription is enhanced by treatment with E₂ or T₃, ER homodimers and TR heterodimers formed with the retinoid X receptor (RXR) recognize the E₂-response element (ERE) and the T₃-response element (TRE) respectively. The tertiary structure of DBDs is maintained by two Zn finger motifs, and the amino acid sequences of the P- and D-boxes of DBD play critical roles in the differential recognition of ERE and TRE (Umesono & Evans 1989). E₂-bound ER (E₂-ER) and T₃-bound TR (T₃-TR) recruit coactivators such as members of the p160 family or CREB-binding protein (CREB)/p300, whose histone acetyl-transferase activities relax the chromatin structure and result in the enhancement of transcription (Perissi & Rosenfeld 2005). In addition, T₃-TR (Ito & Roeder 2001) and E₂-ER (Zhang et al. 2005) recruit TRAP220, a component of the TRAP/SRB/MED-containing cofactor complex of the RNA polymerase II holo-enzyme. In the absence of T₃, TR recruits co-repressors such as nuclear receptor co-repressor or the silencing mediator of retinoic acid and thyroid hormone receptors (SMRT). These co-repressors associate with histone deacetylases (HDACs), resulting in repression of transcription (Perissi & Rosenfeld 2005).

Of note, TR and ER have the potential to bind an identical half-site sequence, AGGTCA, although the number of spacing nucleotides between the half-sites and their orientations are different between TRE and ERE (Mangelsdorf et al. 1995); TRE can be recognized by the TR monomer, in addition to homodimer and heterodimer with RXR (Klinge et al. 1997). T₃-TR can also directly bind to ERE of the promoter for the progesterone receptor gene and stimulate its transcription (Scott et al. 1997). On the other hand, when TR and ER coexist, T₃-TR was reported to inhibit E₂-ER-mediated transactivation of the promoters for the preproenkephalin gene (Zhu et al. 1996, Vasudevan et al. 2001b) and the prolactin gene (Pernasetti et al. 1997), probably via squelching of coactivators common to both receptors. Similar mutual inhibition has been reported between T₃-TR and other ligand-bound steroid hormone receptors (Zhang et al. 1996). Thus, the redundancy of DNA recognition and the common utilization of cofactors have been postulated to mediate crosstalk between the E₂-ER and T₃-TR signaling pathways (Vasudevan et al. 2001a, 2002).

In the mouse TSHβ promoter, the DNA sequence between nt −271/−80 (corresponding to the sequence between nt −269/−78 in the human TSHB gene) was reported to be sufficient for maximal promoter activity in thyrotrops (Wood et al. 1990). In this promoter region, there are binding sites for two transcription factors, PIT1 and GATA2 (Haugen et al. 1996, Gordon et al. 1997, 2002, Dasen et al. 1999, Charles et al. 2006). Co-existence of PIT1 and GATA2 in thyrotrops is essential for the expression of the TSHβ gene (Gordon et al. 1997, Dasen et al. 1999). PIT1 is a pituitary-specific transcription factor expressed in somatotrophs, lactotrophs, and thyrotrops. GATA2 is a subtype of the GATA family of transcription factors and binds with the GATA-responsive element (GATA–RE) through its Zn finger domain, which has high homology among all GATA family members (Ferreira et al. 2005). It was recently reported that TRAP220, which is a coactivator for liganded TR and ER, also functions as a coactivator for GATA2 and PIT1 (Gordon et al. 2006) and plays an important role in the transactivation of the TSHβ gene (Ito et al. 2000). In addition, TRAP220 was reported to be a coactivator for GATA1 (Stumpf et al. 2006), transactivation by which is known to be inhibited by E₂-ERα (Blobel et al. 1995, Blobel & Orkin 1996).

Using non-pituitary CV1 cells, we recently reported that T₃-TRβ2 represses the expression of the TSHβ gene driven by GATA2 and PIT1 through interference of GATA2-induced transactivation, and that, contrary to the previous reports, the negative TRE (nTRE; Wondisford et al. 1989) and negative regulatory elements (NRE; Sasaki et al. 1999) were not required (Matsushita et al. 2007). The reporter assay using CV1 cells provides an ideal experimental platform to investigate the difference between positive and negative regulation by T₃, since this cell line is one of those most frequently used for the study of positive regulation (Nakano et al. 2004). We found that THRDB-DBD directly interacts with the Zn finger domain of GATA2 (GATA2-Zf) and that T₃-TR targets TRAP220, which functions as a coactivator for GATA2 in the context of the TSHβ promoter (Matsushita et al. 2007). Reflecting T₃-specificity in vivo, ligand-bound retinoic acid receptor, vitamin D receptor, RXR, or peroxisome proliferator-activated receptor γ2 all did not exhibit negative regulation of the TSHβ promoter in our experimental system (Nakano et al. 2004). Unexpectedly, however, E₂-ERα exhibited significant suppression of the TSHβ promoter although the magnitude of suppression (39.4% ± 4.5 s.e.m.) was less than that of T₃-TRβ2 (64.1% ± 2.6 s.e.m.; Nakano et al. 2004). Here, we report that, in the negative regulation of the TSHβ gene, there is a redundancy between E₂-ERα- and T₃-TR-mediated signaling pathways.

Materials and Methods

Plasmid construction

We recently found that not only T₃-TR (Tillman et al. 1993, Maia et al. 1996) but also E₂-ERα have a tendency to suppress firefly luciferase-based reporter gene (data not shown). Thus, we employed the CAT-based reporter system. The TSHβ-CAT reporter gene was constructed by fusing the human TSHβ promoter (nt. −128/+37) with the CAT reporter gene, whose backbone lacks the pUC-derived AP-1 site (Sasaki et al. 1999, Nakano et al. 2004). TSHβ-D4-CAT was reported previously (Matsushita et al. 2007). The DNA fragment encompassing the promoter region for TSHβ gene

Downloaded from Bioscientifica.com at 12/08/2018 02:49:24AM via free access
Cell culture and transient transfection

CV1 cells were grown in monolayers and cultured at 37 °C under CO2/air (1:19) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% dextran–charcoal-stripped fetal bovine serum (10% DCC serum), penicillin G (100 units/ml), and streptomycin (100 μg/ml). TzT1 cells, a mouse thyrotroph cell line (Yuasa et al. 1998), were seeded on Matrigel-coated plates (Becton Dickinson Labware, Bedford, MA, USA). The cells were maintained under the same conditions as CV1 cells. The CV1 cells were trypsinized and plated in 60 mm diameter dishes for 24 h before transient transfection using the calcium phosphate technique (Sasaki et al. 1995). The cells at a density of 106 cells/plate were transfected with 0.6 μg of the expression plasmids for Eσα or Thβ along with 4.0 μg of the TSH-Cat reporter gene, 1.8 μg β-galactosidase expression vector pCH111 (a modified version of pCH110; Pharmacia LKB Biotechnology), 0.1 μg human PIT1 expression vector (pCB6-hPIT1), and 0.4 μg mouse Gata2 expression vector (pCDNA3-mGATA2), and the pCMX empty vector as carrier DNA (7.2 μg DNA per dish in total). After the cells were exposed to the calcium phosphate/DNA precipitates for 20 h, the medium was replaced with phenol red-free DMEM containing 5% (v/v) and the cells were allowed to grow for an additional 24 h without or with 1 μM E2 or T3. After incubation for an additional 24 h, the cells were harvested and CAT activity was measured as described previously (Sasaki et al. 1995). The transfection efficiency was normalized by β-galactosidase assay. For each CAT reporter assay, we performed transfection with CAT reporter gene driven by cytomegalovirus promoter (CMV-CAT; 40 ng/dish), the magnitude of which was adjusted to the value of 100. Immunoblotting with anti-ERα antibody (1:200, cat. sc-8002, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed using a standard method described previously (Matsushita et al. 2007).

Gel shift assay

Oligonucleotides for vit-A2 consensus ERE (Scott et al. 1997; sense, 5′-AATTCGTCAGCTGACAGTGACGCTGATCAAAGTT-3′; antisense, 5′-AACCTTGATCGCTACGTGACCTGACTTGGACCAAGTT-3′) were labeled with γ32P-ATP using thyroxine (T4)-polynucleotide kinase (Toyobo, Tokyo, Japan). The receptor proteins were translated in vitro using a TNT T7 quick-coupled transcription/translation system (Promega Corp). The radio-labeled probes and in vitro-translated receptors were incubated for 20 min at room temperature in 20 μl binding buffer containing 10 mM HEPES–NaOH (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, and 0.1 mg/ml poly (dl-dC). The DNA–protein complexes were resolved on 5% polyacrylamide gels at 150 V for 1.5 h. Gels were dried and visualized using the FLA-3000 autoradiography system.

RNA isolation and real-time reverse transcription-PCR (RT-PCR)

TzT1 cells cultured in 10% DCC serum were incubated in the presence of 1 μM E2 or T3 for 48 h. The cells were harvested and total RNAs were purified by the acid guanidinium thiocyanate–phenol–chloroform extraction method (Kawai et al. 2004). For the first-strand cDNA synthesis, 2 μg total RNA were mixed with random hexanucleotides and 200 units Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp). The cDNA for TSHβ was amplified with the forward primer (5′-GGCAAACTGTCTTCTTCCCAA-3′) and the reverse primer (5′-TGCTGTGCTTGGTCCAGTAG-3′). The cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with the forward primer (5′-TGAACGGGAAGTCAGTGGAGAAGCTGTGGGTA-3′) and the reverse primer (5′-TCCACCAACCGCTGTGGTAGA-3′). PCR amplification was carried out using a DNA thermal cycler (Takara Bio Inc., Shiga, Japan) under the following conditions: 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The amplified products were resolved on 5% polyacrylamide gels at 150 V for 30 min. Gels were stained with ethidium bromide and visualized under ultraviolet light.
conditions: denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min, extension at 72 °C for 2 min, and the final extension at 72 °C for 4 min. We determined the cycle number for each primer set so that the specific product was amplified during the exponential phase of the amplification. Based on preliminary studies (data not shown), 27 cycles were employed for the amplification of TSHβ and GAPDH cDNA. The PCR products were subjected to electrophoresis on a 1.4% agarose gel and stained with ethidium bromide. Using the SYBR Green I kit and Light Cycler (Roche Diagnostics), precipitated DNA was quantified by real-time PCR using the primers mentioned above. The thermal cycling conditions were 10 min at 95 °C, followed by 27 cycles of 10 s at 95 °C for denaturing, 10 s at 62 °C for annealing, and 7 s at 72 °C for extension. PCR signals were analyzed using Light Cycler software Ver. 3.5 (Roche Diagnostics).

Measurement of serum TSH, free T₄, and E₂ in pre- and postmenopausal women

Among female patients who visited Ito Hospital in May 2004, 134 subjects with simple goiter or thyroid nodule(s) and no thyroid autoantibodies against thyroglobulin, thyroid peroxidase, or TSH receptor were extracted. They were divided into 62 premenopausal women and 72 postmenopausal women. The premenopausal group consisted of women between 30 and 50 years old (37.35 ± 5.40 years old) and their serum E₂ levels were all higher than 20 pg/ml. The postmenopausal group included women between 50 and 70 (59-29 ± 4.70) years old with serum E₂ levels lower than 20 pg/ml. TSH and free T₄ were measured with Roche ECLusys kit (Roche) and E₂ with electro chemiluminescence immunoassay (SRL, Tachikawa, Japan).

Statistical analysis

Each experiment was performed in duplicate more than three different times and each result is expressed as the mean ± S.E.M. Using StatView 4.0 software (Abacus Concepts Inc., Berkeley, CA, USA), we examined statistical significance in Figs 6 and 7 by Mann–Whitney U test. All other statistical analyses were performed using one-way ANOVA followed by Fisher’s protected least significant difference test. P<0.05 was considered significant.

Results

TSHβ promoter activity is inhibited by not only T₃-TRβ2 but also E₂-ERα

To explore the molecular mechanism underlying the E₂-dependent inhibition of the TSHβ gene expression, we carried out a reporter assay using TSHβ-CAT (Fig. 1A), in which the human TSHβ promoter (nt. 128/+37) is fused to the CAT reporter gene. In accordance with previous reports (Gordon et al. 1997, Dasen et al. 1999), co-expression of PIT1 and GATA2 transactivated the TSHβ promoter in CV1 cells (Fig. 1B, lane 2). This activity was significantly repressed by not only T₃-TRβ2 but also E₂-ERα (lanes 2, 4, and 6). When cognate receptors were co-expressed with PIT1 and GATA2, T₃ and E₂ inhibited transcription by 64.1% ± 2.6 S.E.M. (lanes 3 and 4) and 39.4% ± 4.5 S.E.M. (lanes 5 and 6) respectively. E₂-ERα-induced repression depended on the E₂ concentration (Fig. 1C) and the expression level of ERα (Fig. 1D). The inhibition of the TSHβ promoter by E₂-ERα was observed in Hela and 293T cells (data not shown). We also examined the effect of E₂-ERα on the TSHβ promoter encompassing nt. −615/+37, and found again that the transcriptional activity in CV1 cells was stimulated by PIT1 and GATA2, and significantly decreased by 1 μM E₂ (Fig. 1E).

Overexpression of PIT1 relieves E₂-dependent inhibition of the TSHβ gene and E₂-ERα targets GATA2-induced transactivation

We tested the effect of PIT1 overexpression on the negative regulation of the TSHβ promoter by E₂-ERα. As shown in Fig. 2A, inhibition by E₂-ERα was abolished by increased levels of PIT1, suggesting that a large amount of PIT1 antagonizes the E₂-ERα-dependent inhibition of the TSHβ gene. We recently found that deletion of a short sequence between GATA-REs and the TATA-box in the TSHβ promoter (nt. 82/52; Fig. 1A) enabled GATA2 alone to transactivate without PIT1 (Matushita et al. 2007), and we designated this deleted sequence as the suppressor region (SR). The construct TSHβ-M1-CAT (Fig. 2B, left panel) in which SR was deleted was activated by GATA2 alone, and this activity was inhibited by E₂-ERα (Fig. 2B, right panel). GATA2 alone without PIT1 transactivated two other reporter constructs, TSHβ-D4-CAT, in which nTRE/NRE in TSHβ-M1-CAT was mutated (Matushita et al. 2007), and CGA-CAT, which possesses a functional GATA-RE (Steger et al. 1994). Again, E₂-ERα repressed the GATA2-induced transactivation of TSHβ-D4-CAT and CGA-CAT by 53.1% ± 1.9 S.E.M. and 30.5% ± 6.2 S.E.M. respectively (data not shown). Collectively, transactivation by GATA2 alone was sufficient to mediate negative regulation by E₂-ERα and PIT1 antagonized this inhibition.

The Zn finger domain of GATA2 directly interacts with TR and ERα in vitro

The amino acid sequence of the Zn finger domain of GATA2 (GATA2-Zf), but not the N- or C-terminal region, has high homology with that of GATA1 (Fig. 3A), and both GATAs are known to transactivate the same GATA-RE. In addition, E₂-ERα was reported to inhibit GATA1-induced transactivation (Blobel et al. 1995). As predicted, GATA1-induced transactivation of TSHβ-CAT was also repressed by
E2-ERα (Fig. 3B). While C-terminal finger of GATA1 directly recognizes the GATA-RE, the N-terminal finger is known to interact with Friend of GATA (FOG) 1 and 2 that are the strong co-repressor for the GATA family transcription factors. We reported (Matsushita et al. 2007) that transactivation by a mutant GATA2, GATA2-C295A (Fig. 3A), which has an amino acid substitution from cysteine to alanine at codon 295 of the N-terminal Zn finger, was resistant to the inhibition by T3-TRβ2. The finding that the basal transcription by C295A was significantly higher than that by empty vector (Fig. 3C) indicates that the overall structure as functional GATA2 is partly preserved in this mutant. However, transactivation induced by GATA2-C295A was not inhibited by E2-ERα. These findings indicate that interaction between GATA2-Zf and ERα is essential to E2-ERα-mediated inhibition. Using the GST fusion protein with GATA2-Zf (GST-Zf, Fig. 3A), we tested the direct interaction between ERα and GATA2-Zf in vitro. As reported previously (Matsushita et al. 2007), GST-Zf interacted with TRβ1 (Fig. 3D, upper panel) but not the luciferase protein (lower panel). In an E2-independent manner, GST-Zf also bound to radio-labeled full-length ERα (Fig. 3D, middle panel). As shown in Fig. 3E, the mutant ERα N2 and Δ247, which lacked DBD, did not interact with GST-Zf, suggesting that ERα-DBD binds to GATA2-Zf as in the case of THRβ-DBD.
The E₂-ERα-induced inhibition of the TSHβ promoter does not require the second Zn finger motif

The E₂-ERβ-induced inhibition of the TSHβ promoter requires ERα-NTD

We previously reported that TRβ1-NTD is dispensable for the negative regulation of the TSHβ promoter (Nakano et al. 2004). We wanted to compare the function of NTDs of ERα. In contrast to TRβ1, the ERα-NTD truncation mutant Δ170 (Fig. 5A, middle upper panel) did not inhibit but rather stimulated the transcription (right panel), although it mediated positive regulation (left panel). These data suggest that ERα-NTD is critical for inhibition by E₂. As predicted, ERα (Δ247), which lacks the DBD, did not exhibit ligand-dependent transactivation or inhibition. The expression levels of mutant ERαs were comparable (middle lower panel).

As shown in Fig. 5B, the amino acid sequences between ERα and ERβ were conserved in their DBD (96%) and LBD (58%) but not in NTDs (McInerney et al. 1998). We found that repression of the TSHβ promoter by E₂-ERβ was impaired and the effect of E₂ was not statistically significant (Fig. 5C). To exclude the possibility that ERα-LBD may communicate with the NTD of the same ERα molecule, we tested the function of the chimeric ERα, βNαC, in which the NTD was substituted with that of ERα (Yi et al. 2002; Fig. 5B). Again, βNαC failed to mediate E₂-dependent repression (Fig. 5D). Western blotting with an antibody against the C-terminal region of ERα indicated that expression levels of wild-type ERα and βNαC were comparable (Fig. 5E). Together, these findings indicate that the ERα-NTD has an important role in E₂-dependent inhibition.

Effect of E₂ on the expression of the TSHβ gene in thyrotrroph cell line TαT1

Serum E₂ concentration is known to influence not only the level of T₄-binding globulin (TBG; Surks & Sievert 1995) but also the production of T₃ and T₄ from the thyroid gland (Furlanetto et al. 1999, Sosic-Jurjevic et al. 2005, Alotaibi et al. 2006, Lima et al. 2006). Using cultured thyrotrroph cell line TαT1 (Yusta et al. 1996), we studied the direct effect of E₂ on the TSHβ mRNA level. When TSHβ mRNA was detected by conventional RT-PCR, not only T₃ but also E₂ reduced the intensity of the TSHβ mRNA band (Fig. 6A). We further measured TSHβ mRNA expression by real-time quantitative RT-PCR (Fig. 6B). Although the magnitude was smaller than that for T₃, E₂ treatment also significantly reduced the level of TSHβ mRNA (67.2% ± 12.1% S.E.M. vs 49.5% ± 14.9% S.E.M., P < 0.05). The magnitudes of suppression were correlated to those from the reporter assay using TSHβ-CAT (Fig. 1B).

Comparison of serum TSH levels between pre- and postmenopausal women

To evaluate the clinical significance of E₂ effects on serum TSH, we compared the serum TSH level of 72 postmenopausal women with that of 62 premenopausal women.
who visited Ito Hospital because of simple goiter or thyroid nodule(s) in May 2004 and none had any thyroid autoantibodies. Because age-related decline in thyroid function is not noticeable until the eighth decade of life (Mariotti et al. 1993), we recruited female subjects with ages up to 70 years old as the postmenopausal population. As predicted, the serum E2 levels in postmenopausal females were very low (Fig. 7, left panel). Although there was no difference in the free T4 concentrations (middle panel), the serum TSH level of the postmenopausal group was significantly higher than that of the premenopausal group (right panel).

**Discussion**

In the present study, we demonstrated that E2-ERα inhibits transactivation of the TSHβ gene through a mechanism similar to inhibition by T3-TRs. We used reporter assay in the CV1 cell line, which is ideal for comparing the mechanism of ligand-dependent negative regulation of the TSHβ gene with that of positive regulation (Nakano et al. 2004). Our findings imply that redundancy in the recognition of target genes exists not only in the positive regulation (Klinge et al. 1997, Scott et al. 1997) but also in the negative regulation of the TSHβ gene. It was postulated that E2-dependent inhibition of the
$TSH\beta$ gene was mediated via direct binding of ER$\alpha$ with the putative nTRE (Wondisford 1996). However, this is unlikely, because E$2$-induced inhibition is preserved in the ER$\alpha$ mutant C240S, which fails to bind or activate canonical vit-A2-ERE (Fig. 4C). In agreement with this, E$2$-ER$\alpha$ inhibited GATA2-induced transactivation of $TSH\beta$-D4-CAT, which lacked a DNA sequence homologous to the half-site. Moreover, E$2$-ER$\alpha$ repressed the activity of $TSH\beta$-M1-CAT (Fig. 2B) and CGA-CAT stimulated by GATA2 alone (data not shown), suggesting that interference of GATA2-induced transactivation by E$2$-ER$\alpha$ is the mechanism for inhibition of the $TSH\beta$ gene. This notion is further supported by the observation that transactivation by the mutant GATA2-C295A was resistant to E$2$-ER$\alpha$-induced suppression (Fig. 3C). ER$\alpha$ interacts directly with PIT1 (Ying et al. 1999), and these two transcription factors synergize in the transactivation of the prolactin gene in lactotrophs (Chuang et al. 1997, Schaaffe 1999), whose most important PIT1-binding site is located adjacent to the ERE (Nowakowski & Maurer 1994). However, current results indicate that E$2$-ER$\alpha$-dependent inhibition of the $TSH\beta$ promoter does not require PIT1 (Fig. 2B) and is rather crippled by the overexpression of this transcription factor (Fig. 2A).

Although the results of the transfection experiments as well as the cell culture studies are obtained with high pharmacological doses of E$2$, the overall results were in accordance with our recent findings regarding the T$3$-dependent inhibition of the $TSH\beta$ gene (Matsushita et al. 2007). The repression of GATA2-induced activation by E$2$-ER$\alpha$ and T$3$-TR may occur by a ‘tethering mechanism’ (Nissen & Yamamoto 2000, Herrlich 2001), where liganded nuclear receptors interfere with the activity of DNA-binding transcription factors through protein–protein interactions. In this kind of inhibition, redundancy among liganded...
receptors has been reported. For example, E2-ERα (Kalaitzidis & Gilmore 2005) and glucocorticoid (GC)-bound GC receptor (GR; McKay & Cidlowski 1999, De Bosscher et al. 2003) repress transactivation by the proinflammatory transcription factor NF-κB by interacting with it. Similarly, the transcriptional activity of AP-1 (typically the Jun/Fos heterodimer) is impaired by T3-TR and GC-GR (De Bosscher et al. 2003).

Although the precise mechanism of how E2-ERα inhibits the function of GATA2 in transactivation of the TSHβ gene is uncertain, the present analysis provides several insights. First, the GST pull-down assay (Fig. 3E) indicated that DBD is essential for the interaction of ERα with GATA2-Zf as in the case of THRβs (Matsumori et al. 2007). Second, E2-dependent inhibition does not require the tertiary structure of the second Zn finger motif in ERα-DBD, since E2-dependent inhibition was preserved in the mutant ERαC240S (Fig. 4C), which contains a disrupted second Zn finger motif (Fig. 4A). Third, it should be noted that the mutant ERαs, C205S, and C221G, failed to inhibit E2-dependent inhibition of the TSHβ gene (Fig. 4C), although both mutants can physically bind GATA2-Zf in vitro.

Figure 5 Involvement of NTD in the negative regulation of TSHβ gene by E2-ERα. (A) In the presence of 1 μM E2, expression plasmids for ERα or its mutants, Δ170 and Δ247 (middle upper panel) were transfected into CV1 cells together with the ERE-tk-CAT reporter gene (left panel) or TSHβ-CAT and the expression plasmids for PIT1 and GATA2 (right panel). *P<0.05 versus empty vector for receptor expression plasmids. The CAT activity with the ligand was divided by that without ligand to calculate fold activation (left panels). The CAT activity without ligand was divided by that with 1 μM ligand to calculate fold repression (right panels). The expression levels of wild-type and mutant ERαs were comparable (middle lower panel). The CV1 cells were transfected with the expression plasmid for wild-type or mutant ERαΔ170 and Δ247. Western blot (WB) was performed as Fig. 4A. (B) Schematic of ERα, ERβ and chimeric receptor βNαC. In βNαC, NTD of ERα is replaced with that of ERβ. The homology of amino acids among ERβ and βNαC relative to ERα is indicated as a percentage in the boxes. (C) The expression plasmid for ERα or ERβ was transfected into CV1 cells together with TSHβ-CAT and the expression plasmids for PIT1 and GATA2. *P<0.05 versus PIT1 and GATA2. (D) In the presence or absence of 1 μM E2, the expression plasmid for ERα or βNαC was transfected into CV1 cells together with TSHβ-CAT and the expression plasmids for PIT1 and GATA2. CAT activity without E2 was divided by that with E2 (1 μM) to calculate the fold repression (right panel). The results are shown as means±S.E.M. for eight separate experiments. *P<0.05. (E) Expression levels of wild-type ERα and βNαC were comparable. The CV1 cells were transfected with the expression plasmid for wild-type or βNαC and western blot with antibody against the C-terminal region of ERα was performed as Fig. 4A.
TRAP220 (Crawford et al. 2002, Gordon et al. 2006), and transcriptional repressors such as HDAC3 (Ozawa et al. 2001) and FOG 1 and 2 (Ferreira et al. 2005). ERα and TR share common coactivators including the p160 family (SRC1, TIF2, GRIP1, and AIB1/ACTR), CBP/p300, p300/CBP-associating factor (PCAF), and TRAP220. As in the case of T3-THR-Bs (Matsushita et al. 2007), overexpression of SRC-1, CBP, or PCAF did not show reproducible effects on the negative regulation of the TSHβ gene by E2-ERα (data not shown). TRAP220 is required for the expression of the TSHβ gene (Ito et al. 2000) and functions as a coactivator for GATA2 (Gordon et al. 2006), GATA1 (Stumpf et al. 2006), TR (Ito & Roeder 2001), and ERα (Zhang et al. 2005). TR (Ito & Roeder 2001) and ERα (Zhang et al. 2005) recognize an extended amino acid sequence encompassing two LXXLL motifs in TRAP220 with a specificity distinct from that of coactivators in the p160 family (Acvedo & Kraus 2003, Coulthard et al. 2003). We reported that dnTRAP220 (Yuan et al. 1998) relieved the T3-TR-dependent inhibition of the TSHβ promoter and that TRAP220 dissociated from this promoter after T3 treatment of TzT1 cells (Matsushita et al. 2007). We found that the expression of the dnTRAP220 also abolished the E2-ERα-induced inhibition of the TSHβ gene (data not shown). Thus, TRAP220 may play a role in the inhibition of the TSHβ gene by E2-ERα and T3-TR.

We found that ERα-NTD plays an essential role in the repression of the TSHβ gene by E2 (Fig. 5). This domain interacts directly with the p160 family (Lavinsky et al. 1998, Webb et al. 1998), p300 (Kobayashi et al. 2000), HDAC4 (Leong et al. 2005), and repressor of tamoxifen transcriptional activity (Norris et al. 2002). Although ERα-NTD may bind directly with LBD (Kraus et al. 1995, Metivier et al. 2000, et al. 2001) or regulate the interaction between the antagonist bound LBD and its co-repressor (Lavinsky et al. 1998), the βNuc data indicate that ERα-NTD has an intrinsic function to control the E2-dependent inhibition of the TSHβ gene. TRβ2-NTD was reported to have a strong T3-independent transactivation effect on TRE (Sjoberg & Vennstrom 1995) and also to regulate the interaction between the antagonist bound LBD and its co-repressor (Lavinsky et al. 1998). A function unique to TRβ2-NTD may account for its stronger repression of the TSHβ gene than TRβ1 (Nakano et al. 2004). Importance of GR-NTD was also reported for ligand-dependent inhibition of AP-1 activity (Heck et al. 1994).

E2 is known to have profound influence on the thyroid hormone system, including cell proliferation (Furlanetto et al. 1999), iodine uptake (Furlanetto et al. 1999, Alotaibi et al. 2006, Lima et al. 2006), thyroid peroxidase activity (Lima et al. 2006), de-iodinases (Lisboa et al. 2001, Wasco et al. 2003), or TBG (Gross et al. 1971, Surks & Sievert 1995, Arafa 2001). Altered thyroid hormone production and metabolism may mask the direct effect of E2 on TSHβ expression. To exclude the influences of E2 on T3 and T4 levels in the circulating blood, we measured TSHβ mRNA in TzT1 cells and found that E2 significantly repressed the expression of TSHβ mRNA (Fig. 6). We studied serum

![Figure 6](image_url) Effect of E2 on the expression of TSHβ mRNA in the cultured thyrotrhop cell line, TzT1. (A) TSHβ mRNA isolated from TzT1 cells treated with 1 μM T3 or 1 μM E2 for 48 h and evaluated with RT-PCR. RT (−), PCR without reverse transcriptase. (B) TSHβ mRNA expression evaluated by real-time quantitative RT-PCR. The data are shown as means ± S.E.M. Statistical significance was determined by Mann–Whitney U test. *P<0.05.

![Figure 7](image_url) Elevation of serum TSH levels after menopause in women with simple goiter who possess no autoantibodies against thyroglobulin, thyroid peroxidase, or TSH receptor. The levels of E2 (left panel), free T4 (middle panel), and TSH (right panel) in 62 premenopausal women and 72 postmenopausal women are indicated. The data are shown as means ± S.E.M. Statistical significance was determined by Mann–Whitney U test. *P<0.05; **P<0.01.
TSH levels in women with simple goiter or thyroid nodule(s), who had no thyroid autoantibodies. The TSH level was significantly higher in the postmenopausal than premenopausal population (Fig. 7). Since the age of the postmenopause group was between 50 and 70 years old, thyroid deterioration due to aging is unlikely (Mariotti et al. 1993). A small but significant increase in the TSH level after menopause may be related to the decreased TSH suppression by E2. Although free T4 or serum E2 data were not available, the NHANES III report (Hollowell et al. 2002) also showed that the TSH concentration tended to increase with age in female populations without thyroid autoantibodies. Surks & Hollowell (2007) have recently analyzed TSH frequency distribution curves in NHANES III and NHANES IV and showed progressive shift toward higher concentrations of TSH with age irrespective of thyroid autoantibodies. They say that an explanation for this shift to higher TSH ranges in elderly people is not apparent, but we consider that our finding in this study is one of the main factors.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported, in part, by a Health Sciences Research Grant to H. N., and a Grant-in Aid for Scientific Research to S. S. and H. N. from the Ministry of Education, Culture, Sports, Science, and Technology in Japan.

Acknowledgements

We are grateful to the following researchers for providing the plasmids; Drs Kazuhiko Umestono (Kyoto University, Japan), Ronald M Evans (The Salk Institute, La Jolla, USA), Akihiro Sakurai (Shinshu University, Matsumoto, Japan), Masaaki Yamamoto (Tukuba University, Tukuba, Japan), Akira Kakuazuka (Kyoto University, Kyoto, Japan), Takashi Nagaya (Nagoya University, Nagoya, Japan), Keita Tatsumi (Osaka University, Osaka, Japan), Haruko Muyan (University of Rochester Medical Center, Rochester, USA), and Leonard P Freedman (Memorial Sloan-Kettering Cancer Center, New York, USA). We also thank Dr P.L. Mellon (University of California, San Diego, CA, USA) for providing TαT1 cells and Dr Keiichi Itoi (Tohoku University, Japan) for the sequence information of the RT-PCR primers.

References

Acevedo ML & Kraus WL 2003 Mediator and p300/CPB-steroid receptor coactivator complexes have distinct roles, but function synergistically, during estrogen receptor α-dependent transcription with chromatin templates. Molecular and Cellular Biology 23 335–348.


K Nagayama and others. E2 inhibits TSHβ gene expression


Maia AL, Harney JW & Larsen PR 1996 Is there a negative TRE in the luciferase reporter cDNA? Thyroid 6 325–328.


Li.