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

Koji Nagayama, Shigekazu Sasaki, Akio Matsushima, Kenji Ohba, Hiroyuki Iwaki, Hideyuki Matsunaga, Shingo Suzuki, Hiroko Misawa, Keiko Ishizuka, Yutaka Oki, Jaeduk Yoshimura Noh\(^1\) and Hirotoshi Nakamura

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

\(^1\)Ito 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 THRβs 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 tertiary structure of the second 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 μU/ml ± 0.13 S.E.M. vs 1.47 μU/ml ± 0.12 S.E.M., P<0.05). Our findings indicate redundancy between Tα-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 chorionic 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, administration of pharmacologic doses of estrogen (17β-estradiol, E2) was known to augment the effect of thyroid hormone replacement to suppress TSHβ and CGA mRNA (Wondisford 1996 and references therein). Indeed, E2 inhibits the upregulation of Tshβ and Cga mRNA in the pituitary of hypothyroid rats (Franklyn et al. 1987). Bottner & Wudtke (2005) and Bottner et al. (2006) reported that ovariectomy increased pituitary TSHβ mRNA levels and that this elevation was abolished by 17β-E2-3-benzoate treatment. 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 thyrotrophs 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 Lhb and Fshb 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.
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 (Umesson & 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); ERE 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 prepro enkephalin gene (Zhu et al. 1996, Vasudevan et al. 2001b) and the pro lactin 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 TSHβ gene) was reported to be sufficient for maximal promoter activity in thyrotrophs (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, Danse et al. 1999, Charles et al. 2006). Co-existence of PIT1 and GATA2 in thyrotrophs 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 thyrotrophs. 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 (Feireira 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 THRβ-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
was normalized by b expression vector (pCB6mGATA2), human P1 (pCB6h-P1), wild-type human TRβ 1 (pCMX-hTRβ1), TRβ 1-deletion mutants (pCl-1 and C2), wild-type rat TRβ 2 (pCMX-rTRβ2), and human ERα were described previously (Nakano et al. 2004). The deletion mutants of human ERα (Δ170 and Δ247) and mutant ERα such as C205S, C221G, and C240S were generated from wild-type ERα using standard subcloning techniques or a site-directed mutagenesis kit (Stratagene). The plasmid expressing the recombinant protein of the GATA2 Zn finger domain (GATA2-Zf) combined with glutathione-S-transferase (GST), pGEX-4T-1-GATA2-Zf, was described elsewhere (Matsushita et al. 2007). All constructs were confirmed by DNA sequencing.

**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 (Yusta 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 Estα or Thrb along with 4·0 µg of the Tshβ-Cat reporter gene, 1·8 µg β-galactosidase expression vector pCH111 (a modified version of pCH110; Pharmacia LKB Biotechnology), 0·1 µg human P1 expression vector (pCB6h-P1), 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 with or without 1 mM 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).

**GST pull-down assay**

E. coli (DH5α) transformed with pGEX-4T-1-GATA2-Zf were induced with 0·1 mM isopropyl-1-thio-β-galactopyranoside for 4 h. The E. coli pellet was sonicated and the fusion proteins were mixed with glutathione-sepharose beads (Amersham Pharmacia Biotech) for purification. Receptor proteins were translated in vitro using rabbit reticulocyte lysate (Promega Corp.) in the presence of 35S-methionine. Radio-labeled receptors were incubated with GST protein fused to GATA2-Zf (GST-Zf) in the binding buffer (150 mM NaCl, 20 mM Tris–HCl (pH 7·5), 0·3% Nonidet P-40, 1 mM dithiothreitol, 0·5 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin and leupeptin) for 3 h at 4°C. The precipitants were washed three times with the binding buffer. Bound protein was analyzed by 10% SDS-PAGE and visualized using the FLA-3000 autoradiography system (Fujifilm, Tokyo, Japan).

**Oligonucleotides for vit-A2 consensus ERE**

Oligonucleotides for vit-A2 consensus ERE (Scott et al. 1997; sense, 5′-AATCTGCTCAAGTCAAAGTGCTCAGTCTGACTTGATCAAGATT-3′; antisense, 5′-AATCTTGTACAGCTCAGCTGACATTTGGGACGAATT-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 guanidium 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′-GGCAATCTGTTTTTTCCCAA-3′) and the reverse primer (5′-TCTGTGGCTTGGTGCAG-3′). The cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with the forward primer (5′-TGAACGGGAAGCTCAGTG-3′) and the reverse primer (5′-TCCACCACCTGTGCTGTA-3′). PCR amplification was carried out using a DNA thermal cycler (Takara Bio Inc., Shiga, Japan) under the following conditions...
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 years old (47.47±9.30 years old) and their serum E₂ levels were 20 pg/ml. TSH and free T₄ were measured with Roche 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 (Matsushita 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 (Matsushita 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. 3 A), 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

P- and D-boxes and the zinc finger structure in DBD are critical constituents that determine receptor specificity in ligand-dependent positive regulation (Mangelsdorf et al. 1995). To investigate the involvement of these motifs in the negative regulation of the TSHβ gene, we generated three mutant ERαs: C205S in the P-box and C221G and C240S in the stem of the second Zn finger (Fig. 4A, upper panel). Western blot analysis with an anti-ERα antibody indicated that expression levels of these mutants in CV1 cells were comparable (lower panel). As predicted, these mutants lost DNA-binding affinity as demonstrated by gel shift assay with vitellogenin A2-derived ERE (vit-A2-ERE; Scott et al. 1997; Fig. 4B), and failed to activate the ERE-tk-CAT reporter gene, in which vit-A2 ERE is fused to the CAT reporter gene driven by the thymidine kinase (tk) promoter (Fig. 4C, left panel). As shown in the right panel of Fig. 4C, negative regulation of the TSHβ promoter induced by Pit1 and GATA2 was abolished in C205S and C221G. The findings that C205S and C221G directly interacted with GATA2-Zf (Fig. 4D) suggest that physical interaction of ERα-DBD with GATA2 is not sufficient for inhibition by E2-ERα. Unexpectedly, another mutant ERα, C240S (Fig. 4A) preserved E2-induced negative regulation of the TSHβ gene (Fig. 4C) as well as in vitro binding with GATA2-Zf (Fig. 4D), suggesting that the tertiary structure of the second Zn finger motif, which is necessary for ERE recognition, is dispensable for inhibition of the TSHβ gene.

Effect of E2 on the expression of the TSHβ gene in thyrotroph cell line Tα1T1

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 thyrotroph cell line Tα1T1 (Yusta et al. 1998), 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
TSHβ gene was mediated via direct binding of ERα with the putative nTRE (Wondisford 1996). However, this is unlikely, because E2-induced inhibition is preserved in the ERα mutant C240S, which fails to bind or activate canonical vit-A2-ERE (Fig. 4C). In agreement with this, E2-ERα inhibited GATA2-induced transactivation of TSHβ-D4-CAT, which lacked a DNA sequence homologous to the half-site. Moreover, E2-ERα repressed the activity of TSHβ-M1-CAT (Fig. 2B) and CGA-CAT stimulated by GATA2 alone (data not shown), suggesting that interference of GATA2-induced transactivation by E2-ERα is the mechanism for inhibition of the TSHβ gene. This notion is further supported by the observation that transactivation by the mutant GATA2-C295A was resistant to E2-ERα-induced suppression (Fig. 3C). ERα 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, Schaufele 1999), whose most important PIT1-binding site is located adjacent to the ERE (Nowakowski & Maurer 1994). However, current results indicate that E2-ERα-dependent inhibition of the TSHβ 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 E2, the overall results were in accordance with our recent findings regarding the T3-dependent inhibition of the TSHβ gene (Matsushita et al. 2007). The repression of GATA2-induced activation by E2-ERα and T3-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

Figure 4  Mutation analysis of ERα-DBD. (A) Schematic of ERα-DBD (upper panel). Cysteines at codon 205 in the P-box and 221 and 240 in the stem of second Zn finger were substituted to serine (C205S), glycine (C221G), and serine (C240S). The expression levels of wild-type and mutant ERαs were comparable (lower panel). CV1 cells were transfected with the expression plasmids for wild-type or mutant ERαs. Whole cell extracts were fractionated by SDS-PAGE and subjected to western blot (WB) with anti-ERα antibody against the C-terminal region of ERα. (B) 32P-radio-labeled vit-A2 ERE was incubated with in vitro-translated protein for wild-type or mutant ERαs. ERE, specific cold competitor. NS, non-specific cold oligo DNA. Arrow, specific binding of ERE; arrowhead, nonspecific binding. (C) ERE-tk-CAT (left panel) was transfected into CV1 cells together with the expression plasmid for ERα in the presence or absence of 1 μM E2. CAT activity with 1 μM E2 was divided by that without E2 to calculate fold activation. TSHβ-CAT (right panel) was transfected into CV1 cells together with the expression plasmids for PIT1, GATA2, and wild or mutant ERαs as shown in Fig. 1B. CAT activity without E2 was divided by that with 1 μM E2 to calculate fold repression. (D) 35S-radio-labeled receptors were incubated with GST protein fused to GST-Zf and binding fractions were subjected to SDS-PAGE.
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-kB 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,

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 ERzs 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.

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CBP/p300 (Blobel conclusions were made after analysis of mutant GR using the surface of DBD may be functionally critical. Similar dependent inhibition, and subtle changes of amino acids on Physical binding alone is not sufficient to mediate ligand-

Figure 6 Effect of E\textsubscript{2} on the expression of TSH\textbeta mRNA in the cultured thyrotroph cell line, TzT1. (A) TSH\textbeta mRNA isolated from TzT1 cells treated with 1 \mu M T\textsubscript{3} or 1 \mu M E\textsubscript{2} for 48 h and evaluated with RT-PCR. RT (--), PCR without reverse transcriptase. (B) TSH\textbeta mRNA expression evaluated by real-time quantitative RT-PCR. The experiments were repeated five times. The amount of the PCR products was normalized with that of the GAPDH gene. The data are shown as means \pm S.E.M. Statistical significance was determined by Mann–Whitney U test. *P<0.05.

TRAP220 (Crawford \textit{et al}. 2002, Gordon \textit{et al}. 2006), and transcriptional repressors such as HDAC3 (Ozawa \textit{et al}. 2001) and FOG 1 and 2 (Ferreira \textit{et al}. 2005). ER\textalpha 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 T\textsubscript{3}-THR\textbeta (Matsushita \textit{et al}. 2007), overexpression of SRC-1, CBP, or PCAF did not show reproducible effects on the negative regulation of the TSH\textbeta gene by E\textsubscript{2}-ER\textalpha (data not shown). TRAP220 is required for the expression of the TSH\textbeta gene (Ito \textit{et al}. 2000) and functions as a coactivator for GATA2 (Gordon \textit{et al}. 2006), GATA1 (Stumpf \textit{et al}. 2006), TR (Ito \& Roeder 2001), and ER\textalpha (Zhang \textit{et al}. 2005). TR (Ito \& Roeder 2001) and ER\textalpha (Zhang \textit{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 (Acevedo \& Kraus 2003, Coulthard \textit{et al}. 2003). We reported that dnTRAP220 (Yuan \textit{et al}. 1998) relieved the T\textsubscript{3}-TR\textbeta-dependent inhibition of the TSH\textbeta promoter and that TRAP220 dissociated from this promoter after T\textsubscript{3} treatment of TzT1 cells (Matsushita \textit{et al}. 2007). We found that the expression of the dnTRAP220 also abolished the E\textsubscript{2}-ER\textalpha-induced inhibition of the TSH\textbeta gene (data not shown). Thus, TRAP220 may play a role in the inhibition of the TSH\textbeta gene by E\textsubscript{2}-ER\textalpha and T\textsubscript{3}-TR.

We found that ER\textalpha-NTD plays an essential role in the repression of the TSH\textbeta gene by E\textsubscript{2} (Fig. 5). This domain interacts directly with the p160 family (Lavinsky \textit{et al}. 1998, Webb \textit{et al}. 1998), p300 (Kobayashi \textit{et al}. 2000), HDAC4 (Leong \textit{et al}. 2005), and repressor of tamoxifen transcriptional activity (Norris \textit{et al}. 2002). Although ER\textalpha-NTD may bind directly with LBD (Kraus \textit{et al}. 1995, Metivier \textit{et al}. 2000, \textit{et al}. 2001) or regulate the interaction between the antagonist bound LBD and its co-repressor (Lavinsky \textit{et al}. 1998), the \betaN\textalphaC data indicate that ER\textalpha-NTD has an intrinsic function to control the E\textsubscript{2}-dependent inhibition of the TSH\textbeta gene. TR\textbeta2-NTD was reported to have a strong T\textsubscript{3}-independent transactivation effect on TRE (Sjoberg \& Vennstrom 1995) and also to regulate the interaction between the antagonist bound LBD and its co-repressor (Lavinsky \textit{et al}. 1998). A function unique to TR\textbeta2-NTD may account for its stronger repression of the TSH\textbeta gene than TR\textbeta1 (Nakano \textit{et al}. 2004). Importance of GR-NTD was also reported for ligand-dependent inhibition of AP-1 activity (Heck \textit{et al}. 1994).

E\textsubscript{2} is known to have profound influence on the thyroid hormone system, including cell proliferation (Furlanetto \textit{et al}. 1999), iodine uptake (Furlanetto \textit{et al}. 1999, Atoai\textit{b}i \textit{et al}. 2006, Lima \textit{et al}. 2006), thyroid peroxidase activity (Lima \textit{et al}. 2006), de-iodinases (Lisboa \textit{et al}. 2001, Wasco \textit{et al}. 2003), or TBG (Gross \textit{et al}. 1971, Surks \& Sievert 1995, Araf\textalpha 2001). Altered thyroid hormone production and metabolism may mask the direct effect of E\textsubscript{2} on TSH\textbeta expression. To exclude the influences of E\textsubscript{2} on T\textsubscript{3} and T\textsubscript{4} levels in the circulating blood, we measured TSH\textbeta mRNA in TzT1 cells and found that E\textsubscript{2} significantly repressed the expression of TSH\textbeta mRNA (Fig. 6). We studied serum
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 showed the progressive shift toward higher concentrations of TSH with age irrespective of thyroid antibodies. 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.

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