Thyroid hormone can increase estrogen-mediated transcription from a consensus estrogen response element in neuroblastoma cells

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Thyroid hormones (T) and estrogens (E) are nuclear receptor ligands with at least two molecular mechanisms of action: (i) relatively slow genomic effects, such as the regulation of transcription by cognate T receptors (TR) and E receptors (ER); and (ii) relatively rapid nongenomic effects, such as kinase activation and calcium release initiated at the membrane by putative membrane receptors. Genomic and nongenomic effects were thought to be disparate and independent. However, in a previous study using a two-pulse paradigm in neuroblastoma cells, we showed that E acting at the membrane could potentiate transcription from an E-driven reporter gene in the nucleus. Because both T and E can have important effects on mood and cognition, it is possible that the two hormones can act synergistically. In this study, we demonstrated that early actions of T via TRα1 and TRβ1 can potentiate E-mediated transcription (genomic effects) from a consensus E response element (ERE)-driver reporter gene in transiently transfected neuroblastoma cells. Such potentiation was reduced by inhibition of mitogen-activated protein kinase. Using phosphomutants of ERα, we also show that probable mitogen-activated protein kinase phosphorylation sites on the ERα, the serines at position 167 and 118, are important in TRβ1-mediated potentiation of ERE-induced transactivation. We suggest that crosstalk between T and E includes potential interactions through both nuclear and membrane-initiated molecular mechanisms of hormone signaling.

crosstalk | nuclear receptors | phosphorylation | synergy

Thyroid hormones (T) are important in the regulation of neural differentiation, neural development, and mood modulation. Most of the effects of T are mediated by T receptors (TRs). TRs belong to the nuclear receptor superfamily, whose members act as ligand-dependent transcription factors (1). Such genomic transcriptional effects usually require 10–15 min for T to be manifest. Different isoforms of the TR, TRα1 and TRα2, are derived from the TRα gene, whereas TRβ1 and TRβ2 are from a separate TRβ gene (2, 3). In contrast, T also have rapid effects (seconds to minutes), which are mediated through putative membrane receptors (ref. 4 and references therein). Previously, we have investigated the contribution of rapid nongenomic actions of estrogen (E) to slower genomic transcriptional actions of T in neuroblastoma cells. Cells transiently transfected with E receptor (ER)α and an E response element (ERE)-based reporter gene were exposed to two pulses of hormones. The first pulse of 20 min used a membrane limited estradiol conjugate [17β-estradiol linked to BSA (E-BSA)] to promote the nongenomic actions of E. The second pulse (1-h duration), separated from the first pulse by an interpulse hormone-free interval of 2 h, used 10−9 M 17β-estradiol and was designed to promote transcriptional actions from a consensus ERE. Using this two-pulse paradigm, we demonstrated that initial membrane actions of E were essential and sufficient to potentiate transcription from the consensus ERE in neuroblastoma cells (5).

In MCF-7 breast carcinoma cells, the membrane impermeant agarose-T4 applied for 15 min increased cell proliferation (6), suggesting that nongenomic actions of T can mimic 17β-estradiol in breast cancer cells (6). Triiodothyronine (T3)-liganded TRβ1 could also enhance liganded ERα-mediated transcription from an oxytocin receptor promoter in the neuroblastoma cell line, SK-N-BE(2)C (7). Could this transcriptional potentiation be due to nongenomic actions of T? Among the different TR isoforms, we investigated the role of TRα1 and TRβ1, because these are (i) ligand-binding TR isoforms unlike the TRα2 isoform and (ii) more ubiquitously distributed in different tissues, unlike TRβ2, which has a restricted distribution in the pituitary (8). In this study, we have adopted this two-pulse paradigm to test the hypothesis that early actions of 10−9 M T3 can potentiate transcription by 10−8 M 17β-estradiol from a consensus ERE in neuroblastoma cells. We demonstrate that either TRβ1 or TRα1 increases 17β-estradiol-mediated transcription upon short administration of 10−8 M T3. TRβ1-mediated potentiation requires mitogen-activated protein–extracellular signal-regulated kinase (MEK) activation and ERα phosphorylation.

Materials and Methods

Plasmids, Constructs, and Chemicals. The pGL2-TATA-Inr-Luc construct, a gift of Donald McDonnell (Duke University, Durham, NC), has three consensus tandem EREs upstream of the luciferease reporter (9). The pSG-hERα, a gift of Pierre Chambon (Institut National de la Sante et de la Recherche Medicales) (10, 11), and the TRα1 and TRβ1 plasmids in pCDNAI/Amp, a gift of William Chin (Lilly Research Laboratories, Indianapolis) have been described (12). The ERα phosphorylation mutants in the pCMV vector have been described (13). The 17β-estradiol 6-(carboxymethyl) oxime/BSA (E2-6-BSA; E-BSA) (Sigma) was dissolved in distilled water and filtered according to Stevis et al. (14) to remove free 17β-estradiol. The final concentration of E in the E-BSA (corrected for the molar ratio of 17β-estradiol/BSA) and 17β-estradiol (E2) (Sigma) (in ethanol) in the experiments was 10−9 M. To achieve physiologic free concentrations of T3 in a complex cell culture system, we have used a slightly higher concentration of total T3, i.e., 10−8 M T3, in experiments unless otherwise detailed. The inhibitors, obtained from Calbiochem, were dissolved either in water or DMSO, as appropriate.

Cell Culture and Transfections. SK-N-BE(2) C cells were plated in Ham’s F12/MEM (1:1) (Cellgro, Kansas City, MO) supple-

Abbreviations: T, thyroid hormone; TR, T receptor; E, estrogen; ER, E receptor; E-BSA, 17β-estradiol linked to BSA; T3, triiodothyronine; MEK, mitogen-activated protein–extracellular signal-regulated kinase; ERE, E response element; In, inhibitor n; MAPK, mitogen-activated protein kinase.

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To achieve complete control of ER and TR expression, we transfected ERα and TR into SK-N-Be(2)C cells, a neuroblastoma cell line, with no endogenous ER and TR. This transfected cell line has been used as a model for E action in the brain (15–17). We have previously designed (5) a two-pulse paradigm to demonstrate that rapid nongenomic effects of E can potentiate the transcriptional effects of E. Using that paradigm, we investigated whether a pulsatile application of T3 can potentiate liganded ERα-mediated transcription from a consensus ERE in the presence of transfected TRα or TRβ. T3 was added in the first pulse (20 min), and 10⁻³ M 17β-estradiol was added in the 1-h second pulse, with the pulses separated by a 2-h hormone-free interval.

T3 (10⁻⁷ M), via TRα1 (Fig. 4A) or TRβ1 (Fig. 4B), potentiated ERα-mediated transcription from a consensus ERE-driven luciferase gene compared with a single 17β-estradiol pulse, similar to the potentiation caused by E-BSA and 17β-estradiol added in the first pulse. Neither E-BSA nor T3 given in a single pulse had any effect on TRα1 or TRβ1 (Fig. 4A and 4B). Because both TRα1 and TRβ1 potentiated transcription at 10⁻⁸ M T3, this concentration was chosen for future experiments.

Because E-BSA appears to potentiate transcription via a rapid signal transduction cascade, we hypothesized that T5 may act similarly. Hence, we used inhibitors to PKA [R-p-cAMPS, inhibitor (I)], PKC (chelerythrine, I2), MEK (U0126, I4), and a catalytic inhibitor of calcium [1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetate– acetoxymethyl ester, I3] either in the first pulse along with T3 or in the second pulse with 17β-estradiol (Fig. 3) in cells transfected with either TRα1 or TRβ1. Neither T3 given in a single pulse had any effect on transcription. Potentiation was greatest at 10⁻⁷ M for TRα1 and TRβ1 (Fig. 3A and 3B). Because both TRα1 and TRβ1 potentiation at 10⁻⁸ M T3, this concentration was chosen for future experiments.

Results
To achieve complete control of ER and TR expression, we transfected ERα and TR into SK-N-Be(2)C cells, a neuroblastoma cell line, with no endogenous ER and TR. This transfected cell line has been used as a model for E action in the brain (15–17). We have previously designed (5) a two-pulse paradigm to demonstrate that rapid nongenomic effects of E can potentiate the transcriptional effects of E. Using that paradigm, we investigated whether a pulsatile application of T3 can potentiate liganded ERα-mediated transcription from a consensus ERE in the presence of transfected TRα or TRβ. T3 was added in the first pulse (20 min), and 10⁻³ M 17β-estradiol was added in the 1-h second pulse, with the pulses separated by a 2-h hormone-free interval.

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pulse along with $10^{-8}$ M T3 inhibited ERα-mediated transcription. Hence, we chose to examine the role of mitogen-activated protein kinase (MAPK) and TRβ1 in T3 potentiation of E-driven ERα-mediated transcription.

Because of the importance of MEK activation, we hypothesized that a MAPK target that increases transcription might be the ERα itself. To test this, cells were transfected with either mutant phospho-deficient ERα or wild-type ERα and subjected to a two-pulse paradigm, as noted (Fig. 5). ERα proteins from the wild-type and mutant plasmid promoters were at equivalent levels. The serine at position 118 is phosphorylated by MAPK both in vivo and in vitro (ref. 19 and references therein). Fig. 5A shows that when the serine at position 118 is mutated to alanine (S118A), potentiation by T3-liganded TRβ1 was reduced. However, when E-BSA was used in the first pulse, such a mutant did not abrogate E-BSA-mediated potentiation, demonstrating that phosphorylation of this amino acid is specifically important in liganded TRβ1 signaling to ERα-mediated transcription. On the other hand, the S167A mutant blocked both E-BSA and T3-mediated potentiation (Fig. 5B) and serves as a common functional phosphorylation site in the ERα for cognate (E) ligands and for crosstalk with T3. Finally, although the S294A mutant abrogates E-BSA-mediated potentiation, it does not affect the T3 potentiation of ERα-mediated transcription (Fig. 5C), showing specificity for the signal from the cognate membrane-impermeant ligand. The S118A and S294A mutants also decrease basal transcription from the consensus ERE initiated by T3 (bar 6 in Fig. 5A) and E-BSA, respectively (bar 8 in Fig. 5C). These three different phosphorylation mutants demonstrate that T3
specifically modulates transcription from a consensus ERE via phosphorylation at the MAPK sites at serines 118 and 167 in ERα.

Discussion
Nongenomic effects of T have been demonstrated by using both T3 and T4 (4, 20, 21). In glial cells, T4 and reverse T3, but not T3 itself, cause a transition from soluble actin to F-actin (22). T4 increases actin polymerization and subsequent integrin anchorage in astrocytes, increasing granule cell migration toward the cerebellum (23, 24). T4 is more potent than T3 in PKC activation in erythroid cells (25) and MAPK activation in HeLa and CV-1 cells (26). Membrane-impermeant T4-agarose could replicate this T4-mediated MAPK activation, suggesting that T4 initiated a signal transduction cascade at the membrane. On the other hand, T3 was more active than T4 in stimulating the Na+/H antiporter via the PKC pathway in rat myoblasts (27) and in evoking action potentials (28), suggesting that the potency of T varies according to cell type. In this study, we have used the genomically more active form of T, T3. Although the unavailability of the membrane-limited T compound from commercial sources for this study makes it difficult to unequivocally assert that the T3 actions in this study are membrane-limited, limited exposure (20 min) of the cells to hormone and the importance of MAPK activation to transcription make it probable. Recently, an integrin receptor at the membrane has been shown to function in MAPK activation by T. However, it is also conceivable that T3 may activate MAPK transcriptionally.

On a reporter gene driven by a single ERE in CV-1 cells, transiently transfected with both ERα and TRβ1, coapplication of 10−7 M 17β-estradiol and 10−6 M T3 for 48 h did not affect the level of transactivation seen with 17β-estradiol alone (29). Compared with continuous administration of both these hormones, preliminary administration of T3 followed by 17β-estradiol resulted in increased E transcription in neuroblastoma cells. This could be because of differences in cell lines or enhancers driving the reporter gene (three EREs vs. a single ERE in the previous study) or the method of hormonal administration. Pulsatile administration of estradiol has also been shown to be as effective as continuous administration in promoting cell proliferation (30) and gene transactivation (31) in MCF-7 cells and sex behavior in female rodents (32, 33). Both TRα1 and TRβ1 potentiate ERα-mediated transcription in response to T3, suggesting that these isoforms could function in a parallel manner in the CNS.
shown that this mutant is effective in decreasing ER (42–44). In a previous study (unpublished work), we have demonstrated that these sites (13). In vitro, S167 is phosphorylated by AKT and casein kinase II (42–44). In a previous study (unpublished work), we have shown that this mutant is effective in decreasing ERα transactivation mediated by E-BSA, and hence this regimen (EBSA and T3) was used as a positive control. In this study, we show that the S167A ERα phosphomutant could also decrease the T3 potentiation of transcription by ERα. Migliaccio et al. (45–47) have reported that specifically in breast and prostate cell lines, estradiol activates MAPK via c-src, which in turn phosphorylates the ERα at S118, although this has been disputed by other studies (48, 49). Similar to the results on the S167A mutant, this S118A mutant was selectively effective in abrogating T3-mediated potentiation of ERα transcription. These studies demonstrate that both sites (serine at positions 118 and 167) that are MAPK targets in other cells are also functionally significant in ERα transactivation in this neuroblastoma cell line. We used a third phosphomutant, the ERα S294A, as a negative control, because it was shown to be unimportant in 17β-estradiol-mediated ERα phosphorylation in COS cells (13), despite being a consensus site for proline-directed protein kinase. Although this site was not important in T3 potentiation of ERα transcription, it was important for E-BSA-mediated potentiation, suggesting that the E-mediated phosphorylation of this site in the ERα may be cell-specific.

**Physiological Relevance of T and E Synergy in the Brain.** In male Sprague–Dawley rats, hypothyroidism produces a general inattention to the environment (50). Supporting the role of TRs in affective disorders, mice lacking TRα1 show symptoms of increased anxiety (51). Postmenopausal women show heightened irritability and depression (52–54). Both T and E can therefore synergize to affect mood, anxiety, and cognition (55–59).

This study demonstrates the interaction not only between two hormones but also between two different mechanisms of hormone action, which are relevant to neuroendocrine action.

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