

Agonist Activity of the 3-Hydroxy Metabolites of Tibolone Through the Oestrogen Receptor in the Mouse N20.1 Oligodendrocyte Cell Line and Normal Human Astrocytes

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17 β -oestradiol (E₂) may have a beneficial impact on the development of age-related diseases, in part through α and β oestrogen receptors (ER) in glia. Tibolone, a synthetic steroid, could influence glial-mediated neuroprotection if agonist oestrogenic activity is demonstrable. We used the N20.1 mouse oligodendrocyte cell line as a glial cell model to evaluate the response of ER α and ER β through oestrogen-response element (ERE) and AP-1-driven reporters to E₂, 4-hydroxytamoxifen (4OHT) and to two tibolone metabolites, 3 α -hydroxytibolone (3 α -OH-Tib) and 3 β -hydroxytibolone (3 β -OH-Tib). In addition, we tested the activity of these same ligands through the endogenous ER α in human normal astrocytes. Because endogenous ER was not detected in the N20.1 cells, we tested the ability of exogenous ER to activate transcription in response to ligands (100 nM) using a transient cotransfection assay with an ER α expression vector. To test the antagonist activity of 3 α -OH-Tib and 3 β -OH-Tib, we used them in combination with E₂ (10⁻⁸ M), at concentrations of 10⁻⁷ M and 10⁻⁶ M. The human normal astrocytes were treated similarly, with the exception that no ER-encoding DNA was used. Specific ER ligand mediated activity was shown using the E₂ antagonist ICI 162 780 and the pSG5 empty vector. E₂, 3 α -OH-Tib, and 3 β -OH-Tib stimulated ER α on an ERE-promoter at each concentration (P < 0.001) but not at an AP-1-driven promoter. 4OHT was an effective antagonist, but did not exhibit agonist activity on the ERE-driven promoter. 4OHT was an effective agonist through ER α on an AP-1-driven promoter. 3 α -OH-Tib and 3 β -OH-Tib were not effective antagonists of E₂. Both metabolites acted through the ER because the addition of an E₂ antagonist blocked their activity. These results show that 3 α -OH-Tib and 3 β -OH-Tib exert agonist activity, yet lack antagonist or additive activity, through the ER α and ER β on an ERE-driven but not on an AP-1-driven promoter in a glial cell model and in normal human astrocytes. This contrasts with the effects of 4OHT, which exerted little or no agonist activity, but reduced E₂-stimulated activity through ER α on the ERE, in the same cells.

Key words: glia, estrogen, tibolone, estrogen receptor, oligodendrocytes, astrocytes.

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It is well recognised that the main cause of dementia, affecting 2–4 million of Americans, is Alzheimer's disease (AD). Specific medical interventions for those with this disorder remain limited, and it is projected that in the year 2040, 14 million people will suffer from AD (1). Postmenopausal women may have a greater risk of developing AD than men, perhaps due to the decrease in the levels of 17 β -oestradiol (E₂) following menopause (2). Oestrogen (17 β -oestradiol, E₂) is reported to exert protective effects on the brain,

including promoting cholinergic activity, reducing neuronal loss, and stimulating development, plasticity, and survival of neurones (3–5). These multiple actions of E₂ in the central nervous system (CNS) may be associated with a decreased risk, delayed onset and progression, or enhanced recovery from numerous traumatic or chronic neurological and mental diseases. Of particular interest is the potential beneficial role of E₂ in AD and Parkinson's disease that has been suggested based on results from human and animal

studies (6–9). However, recent data from the Women's Health Initiative, a randomised controlled trial, failed to demonstrate a beneficial effect of supplemental E₂ in postmenopausal women, contradicting many epidemiological studies and contributing to the lack of consensus on the use of hormone replacement therapy to decrease or delay the onset of AD in this population (10). Therefore, it is important to know whether other menopausal therapies have the potential to protect against neuronal degeneration in the brain.

Tibolone, a novel hormone replacement drug used in over 90 countries, is known to protect postmenopausal women against climacteric symptoms and osteoporosis without inducing adverse effects on the endometrium and breast (11, 12). *In vivo*, tibolone is rapidly metabolised to two oestrogenic metabolites, 3 α -OH-tibolone (3 α -OH-Tib) and 3 β -OH-tibolone (3 β -OH-Tib), and Δ 4-tibolone, a metabolite with both progestogenic and androgenic properties (11). Tibolone itself is biologically inactive and its mixed hormonal activities are attributed to its oestrogenic and progestogenic/androgenic metabolites (11). Tibolone is classified as a tissue-selective oestrogen activity regulator (13) because it mediates its protective effects on bone via the oestrogen receptor (ER), while blocking growth-promoting effects in breast and endometrium by regulating the amount of active oestrogenic metabolites for the ER via steroid metabolising enzymes (13) rather than acting as a receptor antagonist, as do selective ER modulators (SERMs). Although tibolone mimics the effects of E₂ in several tissues, its mechanisms of action in the CNS have not been previously described.

E₂ exerts a wide array of actions in the CNS through the ERs, and recent data suggest that E₂ may influence diverse processes such as the regulation of synaptic plasticity and neuroprotection (14–17). Although potentially beneficial, E₂ possesses distinct limitations, including an increased risk of breast and uterine cancers, which may influence its clinical use. Because of such potential side-effects, there is growing interest in the development and potential therapeutic use of SERMs. In the present study, we investigated the activity of the oestrogenic metabolites of tibolone via the α - and β -ER (18–20).

The neuroprotective effects of E₂ may in part be due to its anti-inflammatory activity in glial cells (21, 22). Astrocytes, one of the glial cell types, have recently emerged as candidates for the mediation of the neuroprotective effects of oestradiol. Once assigned a solely supportive role in the CNS, astrocytes are now regarded as being active participants in brain function, with functions that include the regulation of synaptic plasticity and the protection or survival of neurones in the brain because the ablation of astrocytes *in vivo* results in a significant decrease in neuronal survival (23). We selected the N20.1 mouse cell line because it has several features of immature oligodendrocytes and expresses markers of both oligodendrocytes and astrocytes. N20.1 glial cells not only express high levels of message for glial fibrillary acidic protein (GFAP), a classical astroglial marker (24–27), but also they provide a reproducible source of glial cells.

Tibolone, a synthetic steroid, could influence glial-mediated neuroprotective pathways if agonist oestrogenic activity is demonstrable by its two oestrogenic metabolites. The mechanism of action by which 3 α -OH-Tib and 3 β -OH-Tib may exert their agonist or antago-

nist activity in the glial cell has not been previously described. Therefore, any potential effect of these oestrogenic compounds in preventing or protecting against neuronal degeneration remains to be elucidated. We addressed the role of ER subtypes and the responses to E₂, 3 α -OH-Tib, 3 β -OH-Tib and 4-hydroxytamoxifen (4OHT), in the N20.1 glial cell line via two different pathways, the classical, oestrogen-response element (ERE)-driven pathway, and the AP-1-mediated pathways. In addition, we tested the activity of the same ligands on the ER α , using reporter constructs driven by two different response elements in normal human astrocytes (NHAs).

Materials and methods

Plasmids hER α

HEGO in pSG5, was obtained from Drs Pierre Chambon and Hinrich Gronemeyer (INSERM, Illkirch, France). hER β (amino acids 1–530) in pSG5 as described by Ogawa *et al.* (28), was obtained from Dr J. A. Gustafsson (Karolinska Institutet, Stockholm, Sweden).

Culture conditions

The N20.1 cell line, originally provided by Dr Anthony Campagnoni (Yale University, New Haven, CT, USA), was derived from glial precursors immortalised with SV40 T antigen (24). It was passaged and maintained at a permissive temperature of 34 °C or at 37 °C for comparison, in Dulbecco's modified Eagle's medium/F-12/supplemented with 10% fetal bovine serum (FBS) 4.0 g/l glucose, and 2.4 g/l sodium bicarbonate, supplemented with 10% NuSerum (Collaborative Biomedical Products, Bedford, MA, USA) 100 μ g/ml G418 (geneticin) and 20 μ g/ml gentamicin were added after filtration. The cells have similar phenotypes at the two temperatures, with a slightly flattened appearance and irregular shape, unlike the more oligodendroglia-like rounded birefringent process-bearing cells seen under different growth conditions or with elevation of cyclic AMP (26). Expression of message for GFAP is not significantly changed at 34 °C compared to 39 °C (24). For experiments, cells were grown almost to confluence at 37 °C in an atmosphere of 5% CO₂. The cells were then seeded and cultured for an additional 2 days prior to transfection. One day before transfection, charcoal-stripped FBS (Hyclone, Salt Lake City, UT, USA) was substituted for NuSerum FBS at 10% for oestradiol-free media; all other components remained constant.

N20.1 cell transfection

Forty-eight hours prior to transfection in 2 ml of growth medium, the cells were washed with 1 \times phosphate-buffered saline and incubated with medium containing charcoal-stripped FBS. At 20 h prior to transfection, N20.1 cells were seeded at a density of 2.5×10^5 cells per 35-mm well (six-well dishes; Costar, Cambridge, MA, USA). Cells were transfected with 2 μ g of total plasmid DNA and 2.5 μ l/ μ g of SuperFect (Qiagen Inc., Valencia, CA, USA) per 1 μ g of DNA according to the manufacturer's specifications. Cells were transfected with 0.66 μ g of either ER α wild-type or ER β wild-type vector DNA, 1.32 μ g of 2ERE-luciferase reporter DNA and 0.013 μ g of *Renilla* luciferase. pRL-SV40 was used to control for differences in transfection efficiency. Each construct was transfected in triplicates. Cells were incubated with the DNA/SuperFect cocktail for 4 h. After that, medium containing vehicle (ethanol) with or without ligands at the indicated concentration was added. Lysates were prepared approximately 40 h post DNA addition. Aliquots of lysate were evaluated for reporter gene expression using the Dual Luciferase Assay Kit (Promega, Madison, WI, USA) for deter-

mination of luciferase activities. Luminescence was measured as relative light units (RLU, firefly luciferase activity/*Renilla* luciferase activity) using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

Western blotting

We prepared whole normal human astrocytes (NHAs) and MCF-7 cell extracts in Laemmli buffer (29). The cell protein extracts were separated on a 10% SDS/PAGE gel (50 µg protein/lane). We determined protein concentration using the Bradford method. Proteins were transferred to nitrocellulose. The membranes were blocked in 5% dry milk in T-TBS (20 mM Tris, pH 7.6; 1.5% NaCl; 0.05% Tween) and incubated with either the mouse anti-ER monoclonal antibody Ab-15 or ERβ Ab-24 (Neomarkers, Fremont, CA, USA) (2.0 µg/ml) for 3 h, washed in T-TBS, incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (1 : 2000) for 1 h, and washed in T-TBS. Bands were detected using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Bucks, UK).

Culture of NHAs

The human normal astrocytes of the second generation (HNA) were purchased from Cambrex. Cells were cultured with a specific astrocyte medium (Cambrex, Walkersville, MD, USA); 500 ml of the medium contains 0.5 ml of hrEGF, 1.25 ml of insulin, 0.5 ml ascorbic acid, 0.5 ml of GA-1000, 5 ml of L-glutamine and 5 ml of FBS. The cells of the fourth generation were used for experiments. Three days before the experiments, the NHAs were cultured in phenol red free DMEM/F12 with 10% charcoal/dextran treated FBS (Hyclone).

Transfection of normal human astrocytes

2.5×10^4 NHAs were seeded in each well of a 24-well plate in phenol-red free DMEM/F12 with 10% charcoal/dextran treated FBS. After 24 h, the NHAs in each well were supplied with 0.5 ml fresh medium; to the NHA in each well was then added a transfection complex that contained 0.19 µg of p2ERE luciferase reporter, 0.05 µg pRL-SV40 reporter vector (Promega) and 0.5 µl of FuGene6 (Roche, Basel, Switzerland). The transfection complex was prepared according to the manufacturer's protocol. After a 6-h treatment, the medium was removed and replaced with medium containing vehicle with or without ligands. After 24 h of incubation, the cells were harvested and subjected to dual luciferase assay.

Data analysis

The activity (RLU) in the presence of ligands (mean ± SEM) was compared with the activity in the absence of ligand. Comparisons were analysed by an ANOVA (Dunnett's test).

Results

Activity through ERα at an ERE-driven promoter in mouse N20.1 cells

The genomic effects of E₂ are mediated by its binding to two receptor subtypes, ERα and ERβ. Although glia have been reported to express both ERα and ERβ (18–20), we have previously shown that the mouse N20.1 cell line does not express endogenous ER (30). Therefore, all subsequent experiments using these cells were carried out using ERα and ERβ that were introduced into the cells by transient transfection.

The classical pathway of ER action involves binding of the ligand-bound ER to a consensus DNA sequence, the ERE, near the promoter of E₂-stimulated genes. With this in mind, we compared the activity of E₂ with the activity of 3α-OH-Tib, and 3β-OH-Tib, through the ERα using an ERE-driven promoter. In the ERα transfected cells, E₂ at 10^{−7} M and 10^{−8} M showed significant activity through the ERE driven promoter ($P < 0.01$ compared to no ligand, ANOVA; Dunnett's test), with an activity of E₂ in the ERα expressing cells of 8.60 ± 0.60 at 10^{−7} M, and 8.44 ± 0.59 at 10^{−8} M. Both tibolone metabolites exhibited significant activity on the ERE-driven promoter ($P < 0.01$). The metabolite 3-α-OH-Tib increased transcription to 6.25 ± 0.30 RLU at a concentration of 10^{−6} M, and 8.40 ± 0.683 RLU at 10^{−7} M, whereas 3β-OH-Tib increased transcription to 7.38 ± 0.45 RLU at 10^{−6} M, and 8.72 ± 0.23 RLU at 10^{−7} M, in the ERα cells through the ERE-driven promoter. The lack of stimulation observed using the empty vector pSG5, and the ability of the anti-oestrogen ICI 182 780 to block ligand-stimulated reporter activity (Fig. 1a) supports the observation that the transcription stimulation by hormone was mediated through the transfected ERα.

We also tested the ability of 3α-OH-Tib and 3β-OH-Tib, at 10^{−6} M and 10^{−7} M, and the SERM 4OHT at the same concentrations, to block the activity of E₂ through the ERα on the ERE-driven reporter (Fig. 1b). 3α-OH-Tib, 10^{−6} M and 10^{−7} M, in combination with E₂, 10^{−8} M, did not significantly decrease the E₂-stimulated activity; the activity with two different concentrations of 3α-OH-Tib was 6.81 ± 0.41 and 6.93 ± 0.55 RLU, respectively, compared with 8.44 ± 0.59 RLU in the presence of E₂ alone. Similarly, 3β-OH-Tib, 10^{−6} M and 10^{−7} M, in combination with E₂, 10^{−8} M, did not significantly decrease the activity of E₂; activity was 8.44 ± 0.59 RLU in the presence of E₂ alone, compared with 6.55 ± 0.54 and 8.01 ± 0.54 fold, respectively, in the presence of two different concentrations of 3β-OH-Tib (Fig. 1b). In addition, no additive effect when combining E₂ with the tibolone metabolites was detected. By contrast, the transcription stimulation by 4OHT via ERα was not statistically significant ($P > 0.05$) (Fig. 1b), and it reduced the stimulatory effect of oestradiol on transcription (Fig. 1b).

Overall, these data show that the 3-OH metabolites of tibolone exert agonist activity through the ERα, ERE-mediated pathway, while lacking antagonist activity.

Activity through ERβ at an ERE-driven promoter in mouse N20.1 cells

The ERβ, which was discovered in the late 1990s and which is encoded by a different gene than the ERα, has a DNA-binding domain nearly identical to that of the ERα; however, the two receptor subtypes differ substantially in their ligand-binding domains (LBDs, approximately 64% homology) and N-terminal domains (< 25% homology; 28). Although E₂ binds with high affinity to both the ERα and the ERβ, other oestrogenic ligands bind preferentially to one or the other subtype (28, 31, 32). In addition, the ERβ is generally less transcriptionally active than the ERα (33). Because some glial cell populations have been reported to contain ERβ, we tested the activity of the 3-OH metabolites of tibolone via the ERβ on an ERE-driven promoter in the mouse N20.1 cell line.

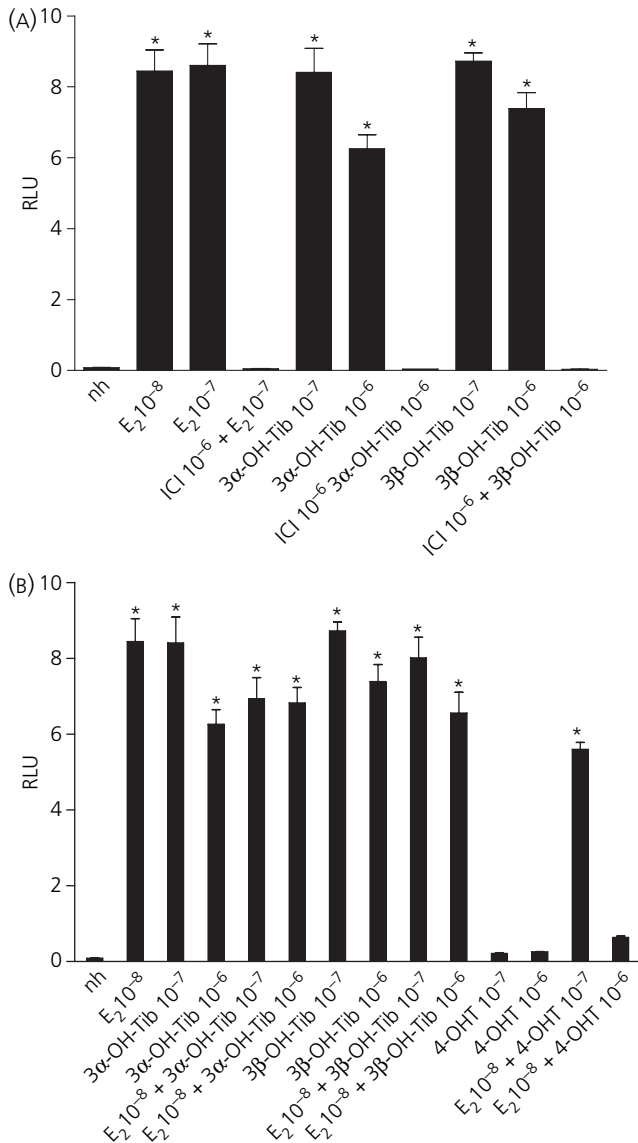


Fig. 1. Activity of oestrogen (E₂), 3 α -hydroxytibolone (3 α -OH-Tib), 3 β -hydroxytibolone (3 β -OH-Tib) and 4-hydroxytamoxifen (4OHT) via the ER α -oestrogen-response element (ERE) pathway in N20.1 glial cells. Transcription activation was measured using a transient transfection assay with wt ER α (HEGO), an ERE-driven reporter (pERE-luciferase), and a *Renilla* luciferase transfection control (pRL-SV40) in N20.1 glial cells as described in the Materials and Methods. The activity of ER is measured by the relative luciferase activity (RLU), which is the ratio of firefly luciferase activity to *Renilla* luciferase activity. The RLU was measured in the absence of hormone (nh) and in response to E₂ (10⁻⁷ M and 10⁻⁸ M), 3 α -OH-Tib (10⁻⁶ M and 10⁻⁷ M) and 3 β -OH-Tib (10⁻⁶ M and 10⁻⁷ M) in the absence and presence of the antagonist ICI 182 780 (10⁻⁶ M) (A), as well as in the presence of 4OHT (10⁻⁶ M and 10⁻⁷ M) (B); and the combination of 3 α -OH-Tib, 3 β -OH-Tib, 4OHT (10⁻⁶ M and 10⁻⁷ M) and E₂ (10⁻⁸ M) (B). The values are the mean \pm SEM of at least three independent experiments, each carried out in triplicate. *P \leq 0.05 compared to nh (no hormone).

In the N20.1 cells transfected with ER β -expressing cDNA, E₂ at 10⁻⁷ M and 10⁻⁸ M exhibited significant stimulatory activity through the ERE-driven promoter (P < 0.01 compared to no ligand,

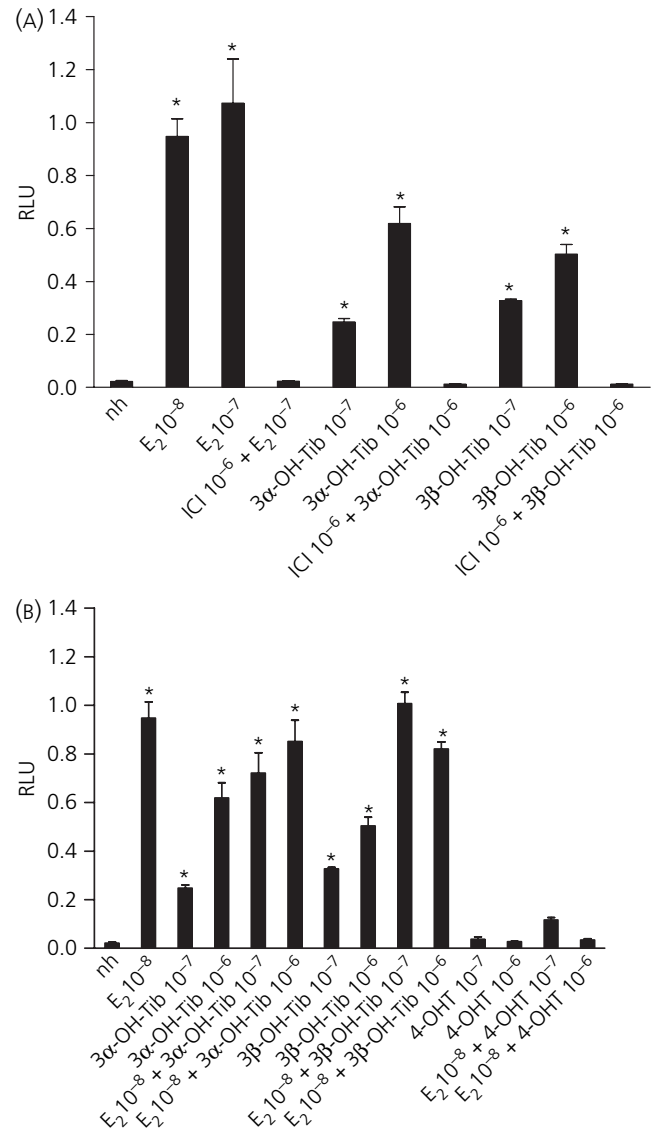


Fig. 2. Activity of oestrogen (E₂), 3 α -hydroxytibolone (3 α -OH-Tib), 3 β -hydroxytibolone (3 β -OH-Tib) and 4-hydroxytamoxifen (4OHT) via the ER β -oestrogen-response element (ERE) pathway in N20.1 glial cells. Transcription activation was measured using a transient transfection assay with wt ER β , an ERE-driven reporter (pERE-luciferase), and a *Renilla* luciferase transfection control (pRL-SV40) in N20.1 glial cells as described in the Materials and Methods and the legend to Fig. 1, with the exception that a plasmid expressing the ER β , rather than ER α , was used. The activity of ER is measured by the relative luciferase activity (RLU), which is the ratio of firefly luciferase activity to *Renilla* luciferase activity. The values are the mean \pm SEM of at least three independent experiments, each carried out in triplicate. *P \leq 0.01 compared to nh (no hormone).

ANOVA; Dunnett's test) (Fig. 2A). Unlike activity via the ER α , the stimulation of transcription by the 3-OH metabolites of tibolone via the ER β was dose-dependent (Fig. 2A). The activity in the presence of E₂, 10⁻⁷ M, in the ER β -expressing cells was 1.07 \pm 0.16. The activity in the presence of E₂, at 10⁻⁸ M, in the ER β -expressing cells was 0.94 \pm 0.06. 3 β -OH-Tib showed significant activity through the ERE driven promoter in the ER β transfected cells at both con-

centrations used, 10^{-6} M and 10^{-7} M ($P < 0.01$). In comparison, 3α -OH-Tib exhibited significant stimulation only at the higher concentration, 10^{-6} M ($P < 0.01$). The activity in the presence of 3α -OH-Tib was 0.61 ± 0.06 at 10^{-6} M, and 0.24 ± 0.01 at 10^{-7} M, while the activity of 3β -OH-Tib was 0.50 ± 0.03 at 10^{-6} M, and 0.32 ± 0.01 at 10^{-7} M, in the ER β cells through the ERE-driven promoter (Fig. 2A). Again, to confirm that the activity of both metabolites of tibolone was ER-mediated, we used the E₂ antagonist ICI 182 780, 10^{-6} M, in combination with 3α -OH-Tib or 3β -OH-Tib at 10^{-6} M, and in combination with E₂ at 10^{-7} M (Fig. 2A).

We tested the ability of 3α -OH-Tib and 3β -OH-Tib, at 10^{-6} M and 10^{-7} M, to block the activity of E₂ through the ER β . 3α -OH-Tib, 10^{-6} M and 10^{-7} M, in combination with E₂, 10^{-8} M, did not significantly decrease oestrogen-stimulated activity (0.94 ± 0.06 RLU versus 0.85 ± 0.41 and 0.72 ± 0.08 RLU, respectively). Similarly, 3β -OH-Tib, 10^{-6} M and 10^{-7} M, in combination with E₂, 10^{-8} M, did not decrease the activity of E₂ (0.94 ± 0.06 RLU versus 0.82 ± 0.02 and 1.00 ± 0.04 RLU, respectively) (Fig. 2B). Like its activity via the ER α , transcription stimulation by 4OHT via ER β was not statistically significant ($P > 0.05$); however, it effectively blocked the activity of E₂ (Fig. 2B).

These results show that E₂, 3α -OH-Tib, and 3β -OH-Tib, but not 4OHT, exert agonist activity through the ER α and ER β receptors on ERE-driven promoter in the mouse N20.1 cell line (Figs 1 and 2). Neither ten- nor 100-fold molar excesses of 3α -OH-Tib and 3β -OH-Tib significantly reduced the E₂-stimulated activity of both ER α and ER β (Figs 1 and 2). These results show that 3α -OH-Tib and 3β -OH-Tib are effective oestrogenic agonists, but not antagonists, through both ER α and ER β on an ERE-driven promoter in the N20.1 cell line.

Activity through ER α and ER β at an AP-1 driven promoter

In addition to the ERE-mediated pathway for activation, the ER can alter transcription through non-DNA binding mechanisms by means of protein-protein interactions. One such mechanism is the AP-1-mediated pathway, wherein the ER binds to the fos/jun complex which is itself bound to AP-1 sites. Different ligands elicit different responses when the ER binds to effector sites such as AP-1 and Sp1, rather than binding to EREs (31–34). Through receptor interactions with other transcription factors at their corresponding response elements, the same ligand can cause activation or repression of different sets of genes. Depending on the ER subtype present and cell type being studied, the SERM 4-OHT can stimulate transcription, while E₂ can inhibit transcription, through this mechanism (31–34). We therefore tested the activity of E₂, 3α -OH-Tib, 3β -OH-Tib and 4OHT through ER α and ER β via an AP-1-driven promoter. Because none of the ligands tested altered transcription through the ER β on an AP-1 driven promoter (data not shown), we focused on the activity of the ligands via the ER α .

In the cells expressing ER α , neither E₂ nor 3α -OH-Tib or 3β -OH-Tib stimulated transcription at concentrations of 10^{-7} M and 10^{-8} M ($P > 0.05$). Of all the ligands tested, only the micromolar concentration of 4OHT significantly stimulated transcription through the ER α on the AP-1 driven promoter ($P < 0.05$); stimulation by 10^{-7} M 4OHT, or by the other ligands, was not significant

($P > 0.05$) (Fig. 3A). In addition, although there is a trend towards inhibition of activity of the AP-1-driven promoter by E₂ and the 3OH-metabolites of tibolone, the differences are not statistically significant. These observations suggest that the activity of the ER through the AP1 pathway may be relatively low in this cell line.

We then measured the activity of 3α -OH-Tib and 3β -OH-Tib, at 10^{-6} M and 10^{-7} M, in combination with E₂ through the ER α in the AP-1-driven promoter. Again, no stimulatory activity was detected; neither was there any additive effect of 3α -OH-Tib and 3β -OH-Tib, 10^{-6} M and 10^{-7} M, when used in combination with E₂, 10^{-8} M ($P > 0.05$) (Fig. 3B).

The lack of agonist activity of 3α -OH-Tib and 3β -OH-Tib via the ER α and ER β through the AP-1 pathway in the N20.1 cell line, compared with their activity via the ERE-driven promoter, demonstrates that these ligands are receptor subtype- and promoter-specific.

Activity of E₂ and tibolone metabolites in NHAs

The previous experiments show that, in mouse N20.1 cells, E₂ and the 3-OH metabolites of tibolone can stimulate transcription of an ERE-driven pathway via the ER α and the ER β . Because this cell line has characteristics of both oligodendrocytes and astrocytes, we then tested the activity of these same ligands in NHAs. We first carried out western immunoblotting against the ER α and ER β to test whether the NHAs express either receptor subtype. Western immunoblotting showed that astrocytes contained a band that comigrates with the ER α in MCF-7 cells (Fig. 4, inset) but not with a nonspecific band found in the ER-negative MDA-MB-231 cells. No immunoreactivity against ER β was detected (anti-ER β Ab-24, Neo-Markers, not shown). In addition, preliminary studies showed that E₂ stimulated transcription activation of an ERE-driven reporter in the absence of plasmid expressing ER (not shown), supporting the idea that the NHAs contained endogenous ER. We then examined the response of the NHAs to E₂ and other ligands on an ERE-driven promoter via the endogenous ER α .

Oestradiol, 3α -OH-Tib and 3β -OH-Tib exerted agonist activity that was blocked by ICI 182 780 (Fig. 4A). The ability of the anti-oestrogen ICI 182 780 to block ligand-stimulated transcription of an ERE-driven reporter provides additional evidence that transcription was driven by endogenous ER. Combining the tibolone metabolites with E₂ had no effect, whereas the addition of 4OHT at micromolar concentration reduced the E₂-stimulated activity (Fig. 4B). These results show that the 3-OH metabolites of tibolone exert agonist activity, but not antagonist activity, in the NHAs as well as in the N20.1 cell line.

Discussion

To our knowledge there is no previous description of the activity of 3α -OH-Tib or 3β -OH-Tib through different ER-signalling pathways in a glial cell model. Our results show that in the oligodendrocyte/astrocyte N20.1 cell line, 3α -OH-Tib, 3β -OH-Tib, and E₂, stimulated both ER α and ER β on an ERE-driven promoter. Similarly, E₂ and the 3-OH metabolites of tibolone stimulated activity through the endogenous ER α via an ERE-driven promoter in human normal

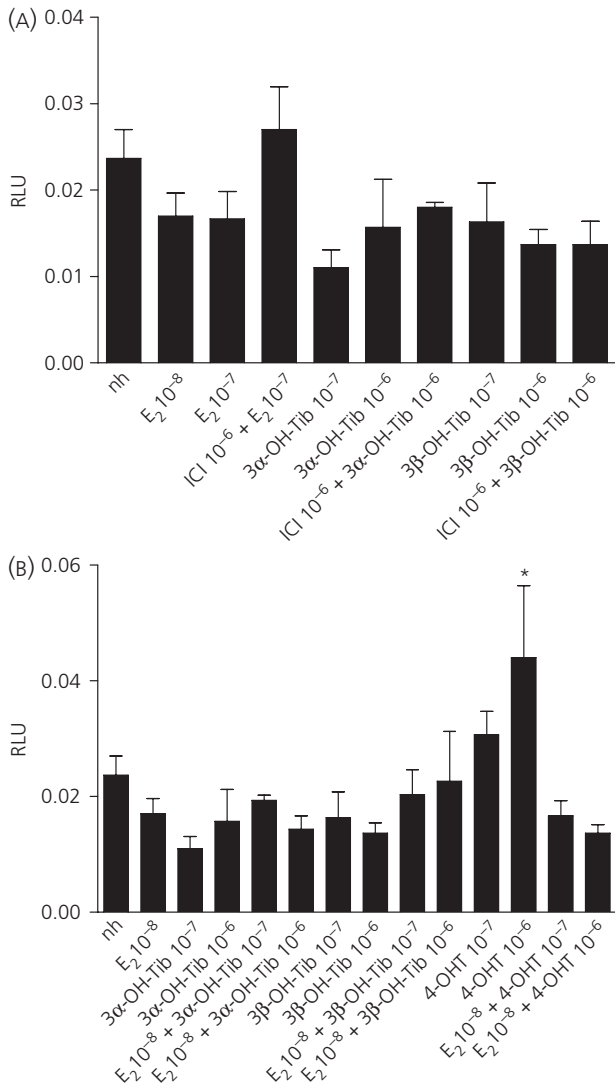


Fig. 3. Activity of oestrogen (E₂), 3 α -hydroxytibolone (3 α -OH-Tib), 3 β -hydroxytibolone (3 β -OH-Tib) and 4-hydroxytamoxifen (4OHT) via the ER α -oestrogen-response element (ERE) pathway in N20.1 glial cells. Transcription activation was measured using a transient transfection assay with wt ER α (HEGO), an AP-1-driven reporter (pAP-1-luciferase), and a *Renilla* luciferase transfection control (pRL-SV40) in N20.1 glial cells as described in the Materials and Methods and the legend to Fig. 1, with the exception that transcription of the luciferase reporter was under the control of an AP-1-driven promoter, rather than an ERE-driven promoter. The activity of ER is measured by the relative luciferase activity (RLU), which is the ratio of firefly luciferase activity to *Renilla* luciferase activity. The values are the mean ± SEM of at least three independent experiments, each carried out in triplicate. *P ≤ 0.05, **P ≤ 0.01 compared to nh (no hormone).

astrocytes. In addition, specific mechanisms of tibolone's action have been well described in other systems (13); our results are consistent with previous observations that the 3-OH metabolites of tibolone preferentially activate ER α and mimic at least some of the effects of E₂ (35). These and other observations provide additional evidence that the N20.1 cell line is a valid tool for the study of the activity of oestrogenic ligands in a glial cell model (30).

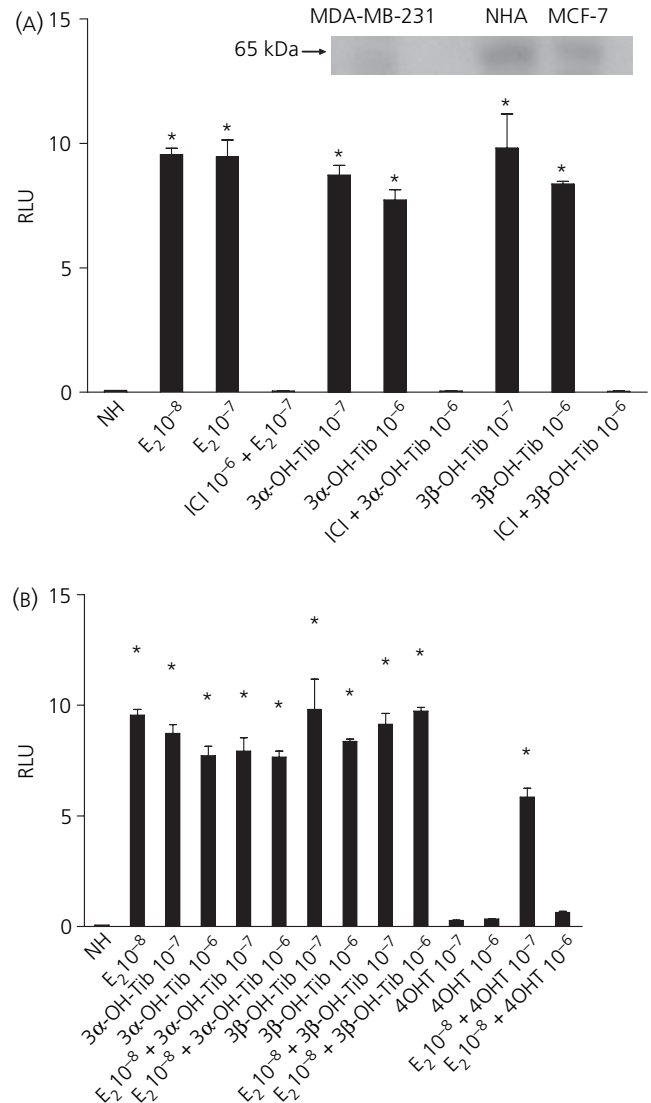


Fig. 4. Activity of oestrogen (E₂), 3 α -hydroxytibolone (3 α -OH-Tib), 3 β -hydroxytibolone (3 β -OH-Tib) and 4-hydroxytamoxifen (4OHT) via the ER α -oestrogen-response element (ERE) pathway in normal human astrocytes. Western immunoblotting (A, inset) was carried out using anti-ER α Ab-15 (Clone AER611, NeoMarkers) as described in the Materials and Methods. Transcription activation was measured using a transient transfection assay with an ERE-driven reporter (p2ERE-luciferase), and a *Renilla* luciferase transfection control (pRL-SV40) in HNAs as described in the Materials and Methods. The activity of ER is measured by the relative luciferase activity (RLU), which is the ratio of firefly luciferase activity to *Renilla* luciferase activity. The RLU was measured in the absence of hormone (vehicle control) and in response to E₂ (10⁻⁷ M and 10⁻⁸ M); 3 α -OH-Tib, and 3 β -OH-Tib (A), in the absence and presence of the antagonist ICI 162 780 (10⁻⁶ M) (top), as well as 4OHT (10⁻⁶ M and 10⁻⁷ M); and the combination of 3 α -OH-Tib (10⁻⁶ M and 10⁻⁷ M), 3 β -OH-Tib (10⁻⁶ M and 10⁻⁷ M) and E₂ (10⁻⁸ M) (b). The values are the mean ± SEM of at least three independent experiments, each carried out in triplicate. P ≤ 0.05.

The response to E₂ and anti-oestrogens at an AP-1 site depends on the subtype of the receptor as well as the cell type used (34); E₂ elicits transcriptional activation with ER α , but transcriptional

repression with ER β . In addition, the SERM 4OHT can stimulate transcription through a different ER α -AP-1-mediated pathway in uterine cells (31–34). We observed that 4OHT at micromolar concentration exhibited a weak stimulatory activity through ER α when tested on an AP-1-driven promoter; E₂ and the tibolone metabolites had no stimulatory activity.

One of the characteristics distinguishing tibolone from tamoxifen and raloxifene is that, unlike the SERMs, the oestrogenic metabolites of tibolone do not exhibit antagonist activity. When we tested the ability of 3 α -OH-Tib and 3 β -OH-Tib to block the activity of E₂ on the ER α and ER β through the ERE-driven promoter, neither ten- nor 100-fold higher concentrations of 3 α -OH-Tib and 3 β -OH-Tib were effective antagonists of E₂ through the ER α and ER β in the ERE- and AP-1 driven promoters in the N20.1 cell line. In addition, neither tibolone metabolite increased E₂-stimulated transcription activation. The activity of these metabolites in a glial model thus parallels observations made in other cells and cell lines (35). Our findings of differences in the transcriptional activity of E₂, 3 α -OH-Tib and 3 β -OH-Tib depending on the subtype of ER is consistent with the observations showing that E₂ can trigger a marked increase in tyrosine hydroxylase promoter-driven luciferase activity in the presence of ER α , whereas it elicits a modest, but consistently significant, inhibition with ER β (36).

Astroglia comprise an important target for E₂ in the brain; they are reported to express both ER α and ER β (37) and play a key role in oestrogen-induced developmental processes, such as synapse formation, plasticity and neuronal morphology (38, 39). Although previous classical studies examining effects of hormones on myelin differentiation and structure could be secondary phenomena, recent studies demonstrating the presence of ER support the idea that E₂ directly affects oligodendrocyte function *in vivo* (19). A role for astrocytes in oestrogen-induced neuroprotection has been suggested based on the ability of E₂ to modulate astrocyte activation that occurs after brain lesion (38) and also on the ability of astrocytes to produce trophic factors (40–42). It is particularly intriguing that an up-regulation of ER α and/or ER β occurs in astrocytes after neuronal damage, including AD, suggesting a prominent role for astrocytes in the oestrogen effects that occur in response to injury (43, 44). In addition, although ER α and ER β are predominantly expressed in neurones, their presence in glial cells of the spinal cord *in vivo* has been confirmed (45). As additional support for astrocytes as a candidate for the mediation of E₂ action in the brain, E₂ has been demonstrated to increase glial cell proliferation and enhance expression of the astrocyte specific marker, GFAP (46). However, we observed only ER α in normal human astrocytes (Fig. 4), whereas only ER β is reported to colocalise with the astrocyte marker GFAP (46, 47). Recently, direct evidence has been provided of the expression of both ER α and ER β in oligodendrocytes *in vitro* and the expression of ER β in oligodendrocytes *in vivo* (19). The presence of the ER subtypes is likely to be related to the specific population of the glia under study.

An interesting hypothesis has recently been raised suggesting that E₂-induced neuroprotection achieved with physiological doses of E₂ involves, at least in part, mediation by astrocytes (48–50).

Through an indirect protective mechanism, physiological levels of oestrogen stimulate the release of astrocyte-derived neuroprotective factors influencing protection of neurones from cell death. This possible parallel pathway of indirect and direct protection could explain how E₂ could achieve widespread protection of the cerebral cortex, striatum and hippocampus despite the fact that ER is not globally expressed in all neurones in these areas (48, 50).

To our knowledge there is no previous description of the activity of 3 α -OH-Tib or 3 β -OH-Tib through different ER-signalling pathways in a glial cell model. Our studies support the idea that oestrogens and related ligands can exert neuroprotective activity through their ability to direct transcription via ER in glia.

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