Estrogen receptor alpha mediates neuronal differentiation and neuroprotection in PC12 cells: critical role of the A/B domain of the receptor

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Abstract

Numerous studies, both *in vivo* and *in vitro*, have reported neuronal differentiating and neuroprotective actions of estrogens. Most of these estrogenic effects are mediated through specific receptors termed estrogen receptors. The aim of this study was to assess the importance of the N-terminal A/B domain of the estrogen receptor- α (ER α) in its neuronal aspects. Consequently, estrogen effects on (i) the transcriptional activity of target genes, (ii) neuronal differentiation and (iii) neuroprotection in PC12 cells transfected with either a full length form of ER α or an A/B domain truncated form (ER α CF), have been studied. We demonstrate that the maximal estrogen-induced transcriptional activity of reporter genes requires a full length ER α , especially when cells are differentiated. Precisely, the transcriptional activity of ER α in differentiated cells relies, predominantly, on the activation function AF-1, located in the A/B domain. Furthermore, in PC12 cells stably expressing ER α , 17 β -estradiol markedly enhances the neurite outgrowth triggered by treatment with nerve growth factor and protects cells from oxidative shocks induced by depletion of glutathione. These estrogenic effects are not observed in non-transfected cells and in cells transfected with the truncated ER, devoid of the A/B domain. Altogether, these results underline the importance of the A/B domain of ER α in both the differentiating and the neuroprotective effects of estrogens.

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Introduction

Estrogens control the development, differentiation and functioning of male and female reproductive organs. They also exhibit pleiotropic effects on non reproductive functions such as metabolism of bone, adipose or hepatic tissues, neuroendocrine and cardiovascular functions. Numerous reports suggest a role for estrogens in fetal and adult brain (Toran-Allerand *et al.* 1999, Wise *et al.* 2001, McEwen 2002, Maggi *et al.* 2004). The development and organization of neural circuits controlling a large spectrum of sexual functions is permanently influenced by gonadal steroids (McEwen 2002). Thus, estrogens have been reported to be involved in several aspects of neuronal differentiation such as neurite outgrowth, arborization and synapse formation. Estrogens also exert neuroprotective effects. Experiments carried out in animal models of focal ischemia demonstrate that estrogenic impregnation markedly reduces the incidence and importance of stroke events whereas some retrospective epidemiological studies suggest that substitutive hormonal therapy may reduce or delay the occurrence of Alzheimer's disease in elderly

women (Wise 2003). *In vitro*, estrogens reduce cell death following different neuronal insults, including serum deprivation, glutathione depletion and treatment with L-glutamate, and decrease the synthesis and toxicity of amyloid- β peptides in neuronal targets (Behl 2002).

Neuroprotective or differentiating effects of estrogens have been reported to be mediated, in part, by estrogen receptor alpha (ER α), this receptor subtype being found in several brain structures such as the hypothalamus and hippocampus (Behl 2002). ER α is a ligand-inducible transcription factor that belongs to the nuclear receptor family. Like the other members of this family, this protein presents a modular structure which can be subdivided into six distinct regions, from A to F (Evans 1988, Beato 1989). The C and E domains are responsible for DNA and ligand binding respectively. The B domain contains a ligand-independent transactivation function, AF-1, whereas a hormone-inducible transactivation function, AF-2, is present in the hormone binding domain (E).

Several data have recently emphasized the importance of the A/B domain in the cellular and physiological functions of ER α . Some transgenic ER α – / – mice

(ER α -Neo KO mice), which do not express the wild-type ER α but express an N-terminal A/B truncated form of the receptor (referred to as ER α 46) present critical physiological deficiencies, especially regarding reproductive function, and are thus infertile (Couse *et al.* 1995, Pendaries *et al.* 2002). Full activity of the transactivation function, AF-1, located in the A/B domain is required for the estradiol-dependent proliferation of the ER α -positive breast cancer cell line, MCF7 (Fujita *et al.* 2003). Moreover, the respective contributions that AF-1 and AF-2 make towards ER α activity have been demonstrated to vary in a cell differentiation stage-dependent manner (Mérot *et al.* 2004). Specifically, AF-1 is the dominant AF involved in ER α transcriptional activity in differentiated cells, and this strongly suggests the importance of the N-terminal domains in ER α transcriptional activity in most estrogen target cell types.

Information is poor concerning differentiating or protective effects of ER α in the brain. Nevertheless, the ER α subtype is a critical mechanistic link in mediating the protective effects of physiological levels of estradiol in brain injury (Dubal *et al.* 1999, 2001), because the deletion of ER α by ER α knock-out in mouse abolishes the protective actions of estradiol in different brain regions (Dubal *et al.* 2001).

Therefore, the aim of our study was to investigate the precise role of the A/B domain of ER α in estrogen-mediated neuronal functions, using the well characterized pheochromocytoma PC12 cell line. PC12 cells have a special place among the different *in vitro* neuronal models. Indeed, they have been extensively used for studying mechanisms of both neuronal differentiation and survival because of their ability to differentiate into sympathetic-like neurons and to extend neurite outgrowth when treated with nerve growth factor (NGF), and because of their ability to undergo cell death following serum and NGF withdrawal (Gollapudi & Oblinger 1999a,b) or following some oxidative shocks, such as depletion of glutathione (Le Foll & Duval 2001). Besides, PC12 cells do not express endogenous ER α in their native state and this has allowed us to study directly the impact of different forms of ER α (full-length ER α or truncated ER α in the A/B domain) on the fate of PC12 cells after transient or stable transfections of the corresponding expression vectors. Using this approach, we demonstrated that ER α -mediated effects of estrogens on the transcriptional activity of estrogen target reporter genes, on the NGF-induced neurite sprouting or on cell survival to oxidative stress require the A/B domain of ER α .

Materials and methods

Chemicals

17 β -estradiol (17 β E2), 17 α -estradiol (17 α E2), 4-hydroxy-tamoxifen (4-OHT), and buthionine sulfoximine (BSO)

were purchased from Sigma (St Louis, MO, USA). ICI 182,780 and NGF were purchased from Tocris (Bristol, UK) and ICN (Irvine, CA, USA) respectively.

Cell culture

Wild-type PC12 cells were grown routinely at 37 °C in 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium with F12 (DMEM/F12; Sigma) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich), 5% horse serum (HS; Life Technologies, Pontoise, France), 1% of a solution containing 10 μ g/ml penicillin G, 10 mg/ml streptomycin, and 25 μ g/ml amphotericin B in 0.9% NaCl and 2 mM L-glutamine (Sigma).

Transcriptional activity of ER in PC12 cells and the activity of AFs

PC12 cells were transfected with FuGEN 6 (Roche). One hour prior to transfection, the routine medium was replaced with phenol red-free DMEM-F12 containing 1% charcoal-stripped HS. Transfection was carried out with a DNA mixture composed of the expression vector pSG5, pSG hER α or pSG hER α CF (truncated ER) (Green *et al.* 1988, Flouriot *et al.* 2000), the reporter gene, and the CMV- β Gal internal control (respectively 200, 400 and 400 ng per well).

The transcriptional activity of ER α was determined using two luciferase reporter plasmids: (i) an artificial promoter containing an estrogen responsive element (ERE) upstream of the thymidine kinase (TK) promoter (ERE-TK-LUC) (Flouriot *et al.* 2000) or (ii) the promoter of the human complement C3 (C3-LUC) (Métivier *et al.* 2001), responsive to 4-OHT (Norris *et al.* 1997). The luciferase activity was determined after 36 h transient transfection (Métivier *et al.* 2001) and was normalized with β -galactosidase activities.

Generation of stable transfectants expressing the full-length ER α or the truncated isoform ER α CF

Wild-type PC12 cells were grown to 50% confluence in phenol red-free DMEM/F12 containing 7.5% charcoal-stripped FCS and 2.5% charcoal-stripped HS and antibiotics (as above). They were stably transfected with FuGEN6 (Roche) using 1 μ g of each expression vector. The expression vectors pCR3.1 ER α and pCR3.1 ER α CF were obtained by cloning respectively the full length ER α coding region and the ER α coding region from +727 to +2030 of the human sequence (Flouriot *et al.* 2000) into the BamHI site of the parental expression vector pCR3.1. Stably transfected PC12 cells were selected by the addition of G418 (a neomycin analog, 800 μ g/ml) to the media for

1 month and were routinely maintained in phenol red-free DMEM containing 7.5% charcoal-stripped FCS and 2.5% charcoal-stripped HS, antibiotics (as above) and G418 (400 μ g/ml).

Western blot analysis

Whole cell extracts from different PC12 subclones were fractionated on polyacrylamide-SDS gels and transferred on nitrocellulose membranes (Amersham, Bucks, UK). The blots were then blocked with a 5% milk PBS/Tween 0.01%, and incubated for 90 min with a rabbit polyclonal anti-human ER α (HC-20; TEBU, Le Perray en Yvelines, France; 1/500 dilution). After washing, the blots were incubated with a peroxidase-conjugated goat anti-rabbit IgG for 1 h (TEBU; 1/5000 dilution). Secondary antibodies were detected using the SuperSignal West Dura kit (Pierce, Rockford, IL, USA).

Cell death and neuroprotective effects of sex steroids

The neuroprotective actions of steroids (17 β E2, 17 α E2) were investigated following cell death caused by a buthionine sulfoximine (BSO)-induced depletion of intracellular glutathione (Froissard *et al.* 1997, Le Foll & Duval 2001). Cells were incubated for 24 h with steroids and then treated with 300 μ M BSO for 24 h. Dead cells were counted in the culture supernatant using the trypan blue exclusion procedure. In separate experiments, 17 β E2 was given at the same time as treatment with BSO (lack of a pretreatment) or 24 h before BSO in association with the estrogen antagonist ICI 182,780.

The amount of intracellular glutathione (reduced glutathione (GSG)+GSSG oxidized glutathione) was measured using the method of Tietze (1969). The protein concentration was determined using the BCA protein assay kit (Pierce).

Cell differentiation

Cells (4×10^3 cells/cm²) were plated in Petri dishes, in phenol red-free DMEM/F12 containing 1% inactivated HS and antibiotics (as above). Twenty-four hours after seeding, the media were replaced and a recombinant NGF (5 ng/ml) was added for 48 or 96 h. Cells were scored as differentiated or undifferentiated cells.

Statistical analysis

Comparisons used ANOVA, followed by a *post hoc* analysis (Fisher test) of individual group differences.

Figure 1 Transcriptional activity of ER α in relation to the differentiation stage of PC12 cells. Undifferentiated and differentiated PC12 cells (primed with 5 ng/ml NGF) were transiently transfected with the expression vectors pSG5 (control), pSG ER α (full length ER) and pSG ER α CF (truncated ER). Cells were then treated with 10 nM 17 β E2 or ethanol (control). The transcriptional activity was determined using an ERE-TK synthetic estrogen-sensitive promoter coupled to luciferase (ERE-TK-LUC). Histograms show the means $\pm$ S.E.M. of values normalized to the activity of the reporter gene measured in the presence of pSG5 without E2, obtained in at least three separate experiments.

Results

The mechanisms underlying the transcriptional activity of ER α in PC12 cells depend upon the differentiation status and the presence of the A/B domain

The transcriptional activity of ER α was studied, focusing on the role of the A/B domain in differentiated and undifferentiated cells. Treatment with NGF leads to differentiation of PC12 cells into sympathetic-like neurons characterized by neurite outgrowths as shown in Fig. 1A. To assess the impact of the physiological stage of PC12 cells on ER α transcriptional activity,

undifferentiated and differentiated cells were transiently co-transfected with the ERE-TK-LUC reporter gene and the expression vectors pSG5, pSG ER α (full length ER α) or pSG ER α CF (truncated ER α) and then treated or not with 17 β E2. As expected, no estrogenic induction of the reporter gene was observed in either undifferentiated or differentiated cells transfected with the empty expression vector pSG5, confirming the absence of expression of endogenous ER. In undifferentiated cells, the activity of ER α CF on the ERE-TK-LUC represented approximately 80% of that of the full-length receptor ER α (Fig. 1B). By contrast, the ER α CF transactivation activity represented only 30% of that of the full-length ER α after differentiation of PC12 cells (Fig. 1B). These data suggest that the transcriptional activity of ER α depends mainly upon the A/B domain in differentiated cells.

The full-length ER α contains two major transactivation functions, one located in the A/B domain (AF-1), the other in the C-terminal part of the hormone binding domain (AF-2) (Parker 1995). Since ER α CF is devoid of the A/B domain, the above result could be related to changes in permissiveness of PC12 cells to both AFs, the differentiation process favoring AF-1. To confirm this assumption, the transcriptional activity of the full-length ER α was determined in the presence of the partial estrogen agonist 4-OHT. Indeed, the estrogenic activity of 4-OHT exclusively relies on the transactivation function, AF-1, and is observed only in cells sensitive to this AF (Berry *et al.* 1990). Therefore, PC12 cells were transiently transfected with the expression vectors pSG5, pSG ER α or pSG ER α CF and the C3-LUC reporter gene conducted by the human complement C3 promoter, a well characterized 4-OHT-responsive promoter (Norris *et al.* 1997). Treatment with 4-OHT weakly increased (fourfold induction) the transcriptional activity of the C3-LUC in undifferentiated PC12 cells transfected with pSG ER α (Fig. 2), confirming the weak permissiveness of these cells to AF-1. In contrast, in differentiated cells transfected with pSG ER α , 4-OHT strongly potentiated the transcriptional activity of the reporter gene (15-fold induction). As expected, 4-OHT had no effects in PC12 cells transfected with pSG ER α CF. These data demonstrate that the differentiation of PC12 into sympathetic-like neurons changes the relative contribution that both AFs exert on transcriptional activity by increasing cell sensitivity to AF-1.

An increase by 17 β E2 of the NGF-induced neurite outgrowth is observed in PC12 cells stably transfected with ER α and requires the presence of the A/B domain

Previous studies have reported an estrogenic potentiation of NGF-induced neurite outgrowths in PC12 cells stably expressing ER α (Gollapudi & Oblinger 2001).

Figure 2 Transcriptional activity of ER α : relation with differentiation and contribution of the AF-1 function. Undifferentiated and differentiated PC12 cells (primed with 5 ng/ml NGF) were transiently transfected with the expression vectors pSG5 (control), pSG ER α (full length ER) and pSG ER α CF (truncated ER). Cells were then treated with 1 μ M 4-hydroxytamoxifen (4-OHT) or ethanol (control). The transcriptional activity was determined using the human complement C3 promoter coupled to luciferase (hC3-LUC) as a reporter gene. Histograms show the means $\pm$ S.E.M. of values normalized to the activity of the reporter gene measured in the presence of pSG5 without E2, obtained in at least three separate experiments.

Therefore, the aim of the present experiment was to establish whether the A/B domain is involved in such an ER α -dependent potentiation. PC12 cells were stably transfected with the expression vectors pCR3-1, pCR3-1 ER α or pCR3-1 ER α CF and three subclones (PC12 control, PC12 ER α and PC12 ER α CF) were selected as described in the Materials and methods section. As expected, western blots revealed the expression of both full-length ER α and ER α CF proteins in the corresponding subclones (C1, ER α -4, ER α CF-1) (Fig. 3A). The absence of receptors in the PC12 cells transfected with the empty expression vector should be noted, confirming the ER α negative phenotype of parental PC12 cells. Moreover, no endogenous ER α or ER β signals were found following PCR experiments using specific primers amplifying cDNAs from parental PC12 cells (data not shown). Addition of NGF (5 ng/ml) to the culture medium significantly increased neurite outgrowths in the three subclones PC12 control, PC12 ER α and PC12 ER α CF, with some differences in the induction between clones (data not shown). This effect became apparent

within 24 h of treatment for both criteria of neurite outgrowth (neurites greater than one or two cell bodies), and reached a plateau after 4 days (data not shown).

17 β E2 (10^{-8} M) markedly potentiated the NGF-induced neurite extension in the PC12 ER α clone. After 2 days of treatment with NGF and estradiol, almost two times as many differentiated cells (cells having neurites greater than one or two cell bodies) were observed compared with treatment with NGF alone (Fig. 3B). This estradiol-induced potentiation of the effects of NGF in the PC12 ER α clone was not associated with changes in the density of the cell population (data not shown). By contrast, 17 β E2 (10^{-8} M) did not modify the NGF-induced neurite extension of PC12 ER α CF and PC12 control clones. 17 β E2 did not modify neurite formation in the absence of NGF in any of the three clones. Similar data have been found using other control, ER α and ER α CF subclones (data not shown).

These results clearly demonstrated that the increase of NGF-induced neuronal differentiation by estrogen requires an ER α presenting the A/B domain.

17 β E2 offers protection from the toxic action of BSO by an ER α -dependent mechanism involving the A/B domain

Neuroprotective effects of estradiol have been reported in the literature, following different oxidative shocks. In the present report, a gradual death was caused by

Figure 3 (A) Generation of PC12 ER α and ER α CF stable transfectants. PC12 cells were stably transfected with the expression vectors pCR3-1, pCR3-1 ER α or pCR3-1 ER α CF. The respective harvested subconfluent subclones PC12 control (clone C1), PC12 ER α and PC12 ER α CF (clones ER α -4 and ER α CF-1) were lysed at 4 °C during 60 min in a RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Thirty micrograms whole cell extracts were fractionated on a polyacrylamide-SDS gel. Proteins of 66 kDa (full length ER) and 46 kDa (truncated ER) were revealed with a rabbit polyclonal IgG with an epitope mapping the carboxy-terminal region of the ER (HC-20). The *in vitro* expression of ER α and ER α CF using the rabbit reticulocyte-coupled transcription/translation kit (TNT, Promega, Madison, WI, USA) acted as a positive control. (B) NGF-induced differentiation of PC12 control, PC12 ER α and PC12 ER α CF (truncated ER α). Cells were treated by NGF (5 ng/ml) during two days with vehicle, 17 β E2 only (10 nM), NGF only (5 ng/ml) or NGF plus 17 β E2. Cells having at least a neurite greater than one (short neurites) or two (long neurites) cell bodies and total cells were counted for 20–30 fields (5–10 fields per dish in 3 separate experiments, at 200 $\times$ magnification) in light microscopy. The ratios of 'cells with short or long neurites/total cells' were determined (means $\pm$ S.E.M.) and used as criteria of differentiation after normalization with respective values of PC12 cells treated with NGF alone. Columns with different superscripts differ significantly ($P < 0.05$). A significant interaction between NGF and E2 was found by two-way ANOVA on day 2 ($P < 0.05$) for both the criteria (short and long neurites).

treatment with BSO within 24 h (Froissard *et al.* 1997, Le Foll & Duval 2001). As expected, BSO led to the death of almost 70–80% of cells after 24 h incubation (Fig. 4). The three clones (PC12 control, PC12 ERα and PC12 ERαCF) were equally sensitive to the toxicity of BSO (data not shown). Preincubation with 17βE2 for 24 h before treatment with BSO, in the physiological concentration range of 0.1–10 nM, significantly increased the viability of PC12 cells expressing the full length ERα (Fig. 4A). Moreover, 1 nM 17βE2 did not

modify proliferation of ERα PC12 cells during a 6–7 days incubation period and failed to induce any changes in cell morphology during this period (data not shown). By contrast, 17βE2 was unable to protect control cells and cells expressing ERαCF from the oxidative shock (Fig. 4B). Altogether, our data suggest that 17βE2 is neuroprotective and requires an ERα presenting the A/B domain. In addition, the ERα-dependent neuroprotection by 17βE2 was not observed for the PC12 ERα clone when (i) 17βE2 was given at the same time as BSO (i.e. without any preincubation with the steroid) (Fig. 4A), (ii) cells were pretreated with 17αE2 (1–100 nM), the transcriptional inactive isomer of 17βE2 (Fig. 4A), and (iii) 17βE2 was associated with the estrogen antagonist ICI 182,780 (Fig. 5A). As the neuroprotective effects of estradiol require a period of pretreatment and are antagonized by ICI 182,780, we suggest that ERα-mediated alteration of gene expression is required to afford neuroprotection. Moreover, 1 nM 17βE2 did not, by itself, change intracellular glutathione (GSH) content of PC12 cells expressing ERα and failed to block the GSH depletion (about 90%) in the presence of BSO (Fig. 5B).

Discussion

Although estrogens exert a wide variety of actions on the developing and the adult brain, by regulating both neuronal differentiation and survival, their mechanisms of action are still unclear. The identification of many putative estrogen receptors in the brain has increased the complexity of our understanding of estrogen action (Toran-Allerand 2004). Among them, ERα has been reported to influence the development and the physiology of neurons. For instance, the development of the brain is associated with the perinatal expression of ERα in

Figure 4 (A) Neuroprotective effects of estradiol against buthionine sulfoximine (BSO). PC12 ERα (density adjusted to 8–10 × 10³ cells/cm² in 6-well plates (1.5 ml/well) in phenol red-free DMEM containing 10% charcoal-stripped FCS and 5% charcoal-stripped HS, antibiotics and 1 g/l glucose) were treated with 300 μM BSO (i) after 24 h of pre-incubation (preincubation-24 h) with 17βE2 (0.1, 1, 10 nM) or 17αE2 (1, 100 nM) or (ii) simultaneously (no preincubation) with 17βE2 (0.1, 1, 10 nM). Cell death was measured 24 h after the treatment with BSO and expressed as a percentage of the total cell population (adherent, viable and dead cells in the supernatant; means ± S.E.M. of individual determinations for 3–8 distinct experiments). Columns with different superscripts differ significantly (*P* < 0.05). (B) Cell death of PC12 control or PC12 ERαCF (truncated ERα) (for density and medium, see panel A) pretreated or not with 10 nM 17βE2 for 24 h. At this time, 300 μM BSO were added for 24 h. Cell death was expressed as a percentage of the total cell population (means ± S.E.M.; *n* = 4–6). Columns with different superscripts differ significantly (*P* < 0.05).

different brain structures such as the preoptic area or the hypothalamus (Gerlach *et al.* 1983). In the adult brain, estradiol protects against brain injury through blood flow-independent mechanisms (Dubal *et al.* 2001) and injury up-regulates the expression of ER α in regions that are protected by estradiol (Dubal *et al.* 1999). In mouse, the ER α knock-out, but not the ER β knock-out, abolishes the protective effects of the steroid (Dubal *et al.* 2001). Despite the huge amount of experimental evidence for a critical role of ER α in the brain, the precise ER α -mediated mechanisms remain to be defined.

Several data have recently emphasized the importance of the A/B domain in the functions of ER α . An insertional disruption of the ER α gene in the first exon coding for the A/B domain (Couse *et al.* 1995, Pendaries *et al.* 2002) induces physiological deficiencies in the corresponding ER $-/-$ mice (ER α -Neo KO mice), such as abolition of reproductive function. Although totally abolishing the production of the full length ER α , the insertional disruption does not suppress the expression of a naturally occurring N-terminal A/B truncated form of the receptor, referred to as ER α 46 (Flouriot *et al.* 2000). It is produced by an alternative splicing event which skips the first coding exon of the ER α gene, targeted by the disruptive insertion. As this form is devoid of the transactivation function AF-1 present in the A/B domain of ER α , the phenotype of the ER α -Neo KO mice could be viewed as being an ER α AF-1 knock-out. Conversely, this isoform is thought to be responsible for residual estrogen responsiveness such as the persistence of some degree of uterine hypertrophy or the preservation of endothelial NO production in ER α -Neo KO mice (Figtree *et al.* 2002, Pendaries *et al.* 2002). Therefore, the ER α A/B domain appears to be dispensable to mediate some ER α functions. Up to now, such an isoform of ER α has unfortunately not been detected in the brain of ER α wild-type or ER α $-/-$ mice, making elusive the relative importance of the A/B domain in ER α -mediated effects in the brain.

The goal of this study was to determine the possible relevance of the A/B domain of ER α in some estradiol-mediated effects in the brain, such as neuronal differentiation and neuroprotection. To solve this problem, we have developed a cellular model in which ER α can mediate both neurotropic and neuroprotective effects, focusing on the role of estrogens on neurite outgrowth and resistance to some oxidative stress of PC12 cells expressing the full-length ER α . Estradiol markedly potentiates the NGF-induced neurite outgrowth of PC12 ER α cells. Such a marked enhancement of NGF-stimulated neurite outgrowth of ER α -transfected PC12 cells by estradiol had previously been associated with a modulation of some cytoskeletal mRNAs, such as peripherin and α -tubulin (Gollapudi &

Figure 5 (A) Cell death of PC12 ER α , preincubated for 24 h with 17 β E2 (1 nM) in the presence or not of ICI 182,780 (ICI; 100 nM) and then treated with BSO. Cell death was measured 24 h after BSO and expressed as a percentage of the total cell population (means $\pm$ S.E.M. of individual determinations for 3–8 distinct experiments). Columns with different superscripts differ significantly ($P < 0.05$). (B) Content of glutathione (GSH) in the PC12 ER α clone. Cells were preincubated in the absence (control) or in the presence of 1 nM 17 β E2 for 24 h. At this time, 300 μ M BSO or solvent were added for 6 h. Results are expressed as μ g GSH/mg protein (means $\pm$ S.E.M.; $n = 4$ –6). Columns with different superscripts differ significantly ($P < 0.05$).

Oblinger 2001). A physiological range (nanomolar concentrations) of 17 β E2 protected PC12 cells expressing ER α against the toxicity of BSO, an irreversible specific inhibitor of γ -glutamylcysteine synthase (Griffith & Meister 1979a) which causes deprivation of glutathione stores (Griffith & Meister 1979b), but did not protect control cells. Altogether, our data verify that some neuroprotective effects of estradiol require ER α and could not be related to antioxidant properties of the steroid at high concentrations (micromolar range) with a hydroxyl group in the C3 position (Sugioka *et al.* 1987, Behl *et al.* 1997). Moreover, because neuroprotective effects of estradiol require a period of pretreatment and are reversed by ICI 182,780, we suggest that an ER α -mediated alteration of gene expression is required to afford neuroprotection, as previously reported in other models such as organotypic cortical explant cultures (Wilson *et al.* 2000). It has been suggested that a possible synergistic interaction between glutathione and E2 is involved in the neuroprotective potency of the steroid in the HT22 cell line exposed to the neurotoxic beta-amyloid peptide (Green *et al.* 1998). Such an hypothesis cannot be held here, because protection was observed in the HT22 cell line lacking functional ER and because neuroprotective effects of estrogens in the present report require ER α . Moreover, because 17 β E2 does not restore the levels of glutathione following treatment with BSO, neuroprotection may involve mechanisms downstream of the synthesis of reactive oxygen species, such as activation of antiapoptotic pathways. Indeed, BSO is one of the major factors controlling the redox status of cells and suppresses glutathione peroxidase (Griffith & Meister 1979b). It leads to a programmed cell death (Serghini *et al.* 1994, Higuchi & Matsukawa 1999, Leon *et al.* 2003), apoptosis being associated with high molecular-weight DNA fragmentation (Higuchi & Matsukawa 1999). Some antiapoptotic effects of estradiol in nigral dopaminergic neurons treated with BSO have already been reported to be mediated by transcription through an AP-1 site downstream from JNK and caspase-3 activation (Sawada *et al.* 2000). Such a mechanism cannot be considered here because neuroprotective effects of estrogens in dopaminergic neurons were observed after both 17 α E2 and 17 β E2 and appeared to be mediated by ER β . Moreover, the neuroprotection by estradiol could not be attributable to a general steroid structure because testosterone or progesterone did not provide protection against BSO (data not shown).

The role of the A/B domain in the differentiating and neuroprotective effects described above was studied using PC12 cells expressing a truncated ER α (ER α CF). We have demonstrated that the A/B domain of ER α is essential for mediating both the protective effects of estrogens against oxidative stress and the potentiation of NGF-induced differentiation.

Estradiol influences, in complex ways, the expression of numerous genes in multiple regions of the brain that are theoretically relevant to the effects of estradiol observed in PC12 cells expressing ER α . For instance, estradiol modifies the expression of genes involved in the dendritic and axonal elongation such as GAP-43 or Tau (Ferreira & Caceres 1991, Shughrue & Dorsa 1993), in the balance of apoptosis and of survival such as Bcl2 or bcl-XL (Pike 1999, Zhao *et al.* 2004) or in the driving of the growth factor effects, like TrkA or p75^{NGFR} (Sohrabji *et al.* 1994, Toran-Allerand 1996). These ligand-activated mechanisms may involve a direct control of target gene transcription from both the ERE and various alternate response elements such as AP-1 motifs. Putative ERE found in target genes such as TrkA, BDNF, Bcl-2, Bcl-XL and p75^{NGFR} support this idea (Sohrabji *et al.* 1994, 1995, Toran-Allerand 1996). As the A/B domain harbors the activation function AF-1, ER α -mediated genomic effects seem to be relevant in the differentiating and neuroprotective effects of estradiol in PC12 ER α cells. Accordingly, we have recently reported that the transcriptional activity of ER α is essentially mediated by AF-1 in differentiated cells (Mérot *et al.* 2004). We thus demonstrated that P19 cells which transiently expressed ER α CF (but not P19 cells expressing ER α) became transcriptionally inactive following a retinoic acid-induced differentiation into a neural phenotype. The same strategy has therefore been conducted here in PC12 cells to assess the relevance of AF-1 in ER α transcriptional activity. An enhancement of the role of AF-1 in ER α activity following differentiation is suggested in PC12 cells, as shown by the measurement of the agonistic activity of 4-OHT. As a result, it appeared that ER α CF was much less efficient than ER α in the activation of transcription in differentiated cells. Nevertheless, unlike P19 cells, we observed a residual transcriptional activity of ER α CF. Because the percentage of differentiated cells after 48 h of NGF exposure does not go beyond 60%, we suppose that a mixing of transfected differentiated and undifferentiated PC12 cells in culture may, in part, mask the strict AF-1 cell context of differentiated cells. A cross-talk between ER α and NGF signaling could explain the increase in AF-1 activity of differentiated PC12 cells. Indeed, like epidermal growth factor or insulin-like growth factor-I, NGF activates extracellular signal-regulated protein kinase (ERK), resulting in the direct phosphorylation of ER α on serine 118 (a phosphorylation site of the B domain), promoting an AF-1-dependent transcriptional activity (Ho & Liao 2002). However, the agonist activity of 4-OHT does not seem to be linked to the phosphorylation status of serine 118 (data not shown), suggesting that the enhancement of AF-1 in ER α transcriptional activity is likely to be a combination of several mechanisms involving specific cofactor expression and post-translational modifications.

Besides direct regulation of gene transcription, evidence has clearly emerged that ER α can regulate gene expression by signaling pathways initiated outside the nucleus (Abraham *et al.* 2004, Edwards 2005). These so-called nongenomic effects include activation of cell signaling molecules such as protein kinases. In several models, the neurotropic and neuroprotective effects of estrogen have been linked to a rapid stimulation of the mitogen activated protein kinase (MAPK) pathway and/or the phosphoinositol 3-kinase (PI3K) pathway (Singer *et al.* 1999, Kuroki *et al.* 2001, Zhang *et al.* 2001, Fitzpatrick *et al.* 2002, Mize *et al.* 2003, Dominguez *et al.* 2004). Such rapid nongenomic effects could potentially promote differentiating and protective effects of estrogens in PC12 ER α cells. Numerous studies focusing on the molecular mechanism underlying estradiol-dependent activation of Ras/MEK/ERK and/or PI3K/Akt pathways have identified c-src as a critical upstream effector in cortical explants, osteoclasts and breast cancer cell lines (Migliaccio *et al.* 1998, Kousteni *et al.* 2001, Nethrapalli *et al.* 2001). Functional interactions between c-src and the E domain of ER α are able to promote estradiol-dependent c-src activation. Thus, the E domain of ER α was sufficient to mediate estradiol protection against apoptosis in osteoclasts, osteocytes, embryonic fibroblasts and HeLa cells via activation of the src/Ras/MEK/ERK pathway (Kousteni *et al.* 2001). Such a mechanism, involving the E domain of ER α , might confer an estradiol-induced neuroprotection in PC12 ER α CF. This is not true in the present study. Importantly, no ER α -dependent activation of ERK1/2 was detected in ER α and ER α CF PC12 cells (data not shown). Nevertheless, several intracellular signaling pathways can be activated by E2/ER α and the role of extranuclear ER α -initiated mechanisms cannot be ruled out in the present study. Furthermore, both nuclear and extranuclear ER α signaling may converge to afford the neuroprotective effects of estradiol, as suggested by the study of Mize *et al.* (2003). Indeed, activation of the MAPK cascade by estradiol has been reported to be involved in the protection of different HT22 ER α clones from oxidative stress (Singer *et al.* 1999, Mize *et al.* 2003). This estradiol-induced neuroprotection was observed in an HT22 ER α clone, but also in an HT22 clone expressing an ER α modified in its ERE-binding domain (HE27) (Mize *et al.* 2003). Nevertheless, the neuroprotection in the HE27 clone was moderate when compared with the neuroprotection afforded by estradiol in the ER α clone, despite a similar ER α -dependent activation of ERK. This suggests that both activation of the MAPK cascade and ERE-dependent transcriptional regulation promote ER α -mediated neuroprotection by estradiol in this model. Such an interplay between nongenomic and genomic pathways has been evidenced in the neuroblastoma cell line SK-N-BE2C transiently transfected with ER α and

treated with estradiol, in which early membrane estrogenic events potentiated the delayed transcriptional response of ER α (Vasudevan *et al.* 2001).

In conclusion, we provide evidence for a critical role of the A/B domain in both differentiating and protective effects of estradiol in PC12 cells. Nevertheless, multifactorial and inter-connected ER α -mediated effects may promote tropic and/or protective effects in the brain, depending on the cell context and the duration of estradiol impregnation. Further experiments in PC12 cells will focus on this point and on the characterization of some signaling pathways involved in the differentiating and neuroprotective effects of estradiol.

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