Journal of Neurochemistry, 2003, **85**, 1180–1189 doi:10.1046/j.1471-4159.2003.01767.x

An oestrogen membrane receptor participates in estradiol actions for the prevention of amyloid-β peptide$_{1-40}$-induced toxicity in septal-derived cholinergic SN56 cells

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Abstract

Although oestrogen [17β-estradiol (E2)]-related neuroprotection has been demonstrated in different models, the involvement of non-classical oestrogen receptors (ERs) remains unexplored. Using the SN56 cholinergic cell line, we present evidence indicating that an ER associated with the plasma membrane participates in oestrogen-dependent inhibition of cell death induced by amyloid-β peptide (Aβ) toxicity. Similarly to E2 alone, a 15-min exposure to estradiol-horseradish peroxidase (E-HRP) significantly reduced Aβ-induced cell death. This effect was decreased by the ER antagonist ICI 182,780 as well as by MC-20 antibody directed to a region neighbouring the ligand-binding domain of ERα. Using confocal microscopy on unpermeabilized SN56 cells exposed to MC-20 antibody, we identified a protein at the plasma membrane level. Western blot analysis of purified SN56 cell membrane fractions using MC-20 antibody revealed the presence of one band with the same electrophoretic mobility as intracellular ERα. Using conjugated forms of the steroid, E-HRP and E2 conjugated to bovine serum albumin-FITC, we demonstrated by confocal microscopy that SN56 cells contain surface binding sites for E2. Binding of both conjugates was blocked by pre-incubation with E2 and decreased by either ICI 182,780 or MC-20 antibody in a concentration-dependent manner. Thus, a membrane-related ER that shares some structural homologies with ERα may participate in oestrogen-mediated neuroprotection.

Keywords: β-amyloid, neuroprotection, oestrogen, oestrogen receptor, SN56 cell line.

J. Neurochem. (2003) **85,** 1180–1189.

Oestrogen has traditionally been thought to act by binding nuclear oestrogen receptors (ERs), which act as transcription factors and regulate gene expression (Truss and Beato 1993; MacGregor and Jordan 1998). However, emerging data in cells from different origins, including neurones, have implicated alternative mechanisms of oestrogen [17β-estradiol (E2)] action often originating in the cell membrane (Falkenstein *et al*. 2000; Nadal *et al*. 2001). These effects can occur within minutes and trigger multiple signal transduction pathways (Nadal *et al*. 2001). Some data in neural tissue have evidenced the binding of E2 to membrane-related ERs (mER; Norfleet *et al*. 1999, 2000; Milner *et al*. 2001), suggesting a modulation of a variety of intracellular actions by alternative pathways. Among the most studied are rapid effects of oestrogen against different types of injury through activation of mitogen-activated protein kinase cascades (Watters *et al*. 1997; Singer *et al*. 1999; Fitzpatrick *et al*. 2002) that contribute to neuroprotection by mechanisms still poorly understood.

In vitro paradigms that imitate some aspects of Alzheimer's disease (AD) pathology have demonstrated that oestrogen has the ability to prevent neuronal death from

Received December 8, 2002; revised manuscript received January 31, 2003; accepted February 9, 2003.

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Abbreviations used: Aβ, amyloid-β peptide; AD, Alzheimer's disease; DAB, diaminobenzidine tetrahydrochloride; E2, 17β-estradiol; E-BSA-FITC, estradiol-bovine serum albumin-fluorescein isothiocyanate; E-HRP, estradiol-horseradish peroxidase; ER, oestrogen receptor; LBD, ligand binding domain; mER, membrane-related ER; NGS, normal goat serum; PBS, phosphate-buffered saline.

amyloid-β peptide (Aβ) toxicity (Goodman *et al*. 1996; Green *et al*. 1996; Mook-Jung *et al*. 1997) which, otherwise, accumulates provoking neuronal mortality. Oestrogen has been shown to reduce the generation of Aβ in neuroblastoma cells and primary neurones (Xu *et al*. 1998; Greenfield *et al*. 2002) as well as in animal models (Petanceska *et al*. 2000; Zhen *et al*. 2002), suggesting that lowering the levels of Aβ may be a mechanism by which oestrogen may prevent the onset of AD. However, the importance of ER in these oestrogenic actions is still largely unknown. Recent data from different groups, including our own, have demonstrated the involvement of classical ERs in neuroprotection against serum deprivation (Gollapudi and Oblinger 1999) and Aβ toxicity (Kim *et al*. 2001; Marin *et al*. 2001b; Fitzpatrick *et al*. 2002). On the other hand, it remains unexplored in neurones whether these oestrogen preventive actions from injury could be partially regulated by rapid intracellular pathways through a plasma membrane receptor, as has been shown in other target cells from different origins (Watson and Gametchu 1999; Falkenstein *et al*. 2000; Coleman and Smith 2001). So far, a single report by Fitzpatrick *et al*. (2002) in hippocampal-derived cells (HT22) transfected with either ERα or ERβ has documented the involvement of ER in rapid preventive actions of oestrogen against Aβ-induced toxicity. This is in agreement with a non-classical pathway for oestrogen neuroprotective effects although both the existence of a plasma mER counterpart and its possible participation in neuroprotection have not been documented until now.

We have previously reported that a 24-h treatment with oestrogen prior to amyloid-β 1–40 ($A\beta_{1-40}$) produced 85% prevention of SN56 cell death with the participation of ERα, which was up-regulated under these conditions (Marin *et al*. 2001b). Here, we present evidence of cell death prevention against amyloid-β injury after short exposure to E2 and its membrane-impermeant form [estradiol-horseradish peroxidase (E-HRP)] that is diminished by either the anti-oestrogen ICI 182,780 or MC-20 antiserum directed to classical ERα. Our results have also revealed the presence of a putative plasma mER that may be recognized by E2 at the surface of SN56 cells. Overall, these data suggest that SN56 cells express a membrane form of ER that may participate in oestrogen neuroprotective actions against Aβ-induced injury.

Materials and methods

Materials

The 17β- and 17α-estradiol, E-HRP, estradiol-bovine serum albumin-fluorescein isothiocyanate (E-BSA-FITC), trypan blue stain, dextran-FITC and the different compounds for the Na^+-K^+-ATPase and lactate dehydrogenase assays were obtained from Biosigma (Madrid, Spain). ICI 182,780 was a gift from Astra-Zeneca (Madrid, Spain). Colloidal silica was obtained from Nalco Chemical Co. (1060; Chicago, IL, USA). The primary antibody used in most of the experiments shown in this work was MC-20 polyclonal anti-ERα antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) which recognizes amino acids 580–599 in the vicinity of the ligand-binding domain of ERα protein and does not recognize ERβ. Alternatively, the H151 mouse monoclonal antibody directed to the hinge region of ERα (Stressgen Biotechnologies, Victoria, Canada) was also used. The secondary goat biotinylated anti-rabbit antibody was from Vector Laboratories (Burlingame, CA, USA). The cyanine-2 dye-conjugated streptavidin was from Jackson Laboratories (Baltimore, PA, USA). The Hybond-P transfer membranes, horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and the ECL chemiluminescence kit were from Amersham International (Little Chalfont, Buckinghamshire, UK). The imaging densitometer and laser scanning confocal imaging system (MRC 1024) were obtained from Bio-Rad Laboratories (Hemel Hempstead Hertfordshire, UK).

Cell culture and treatments

SN56 cells, a cholinergic cell line from mouse septum origin (kindly provided by Dr Bruce Wainer, Wesley Woods Health Center, Atlanta, GA, USA), were maintained at 37°C as previously described (Marin *et al*. 2001a).

SN56 viability was assessed by the trypan blue dye exclusion method (Black and Berenbaum 1964) followed by cell counting. This method has been shown to be one of the most adequate procedures to measure cell death following Aβ-induced toxicity (Green *et al*. 2000). After a brief treatment with 0.1% trypsin, cells exposed to the different treatments (see below) were resuspended in OptiMEM and 90 μL of each cell suspension were incubated with 10 μL of 0.4% trypan blue stain for 5 min at room temperature. Viable cells (trypan blue-excluding) were counted on a Neubauer haemocytometer. Data are expressed as the mean percentage of total viable cells relative to the untreated control cultures.

17β-Estradiol and estradiol-horseradish peroxidase short-term treatments and competition assays with ICI 182,780 or anti-ERα antibody

SN56 cultures grown in OptiMEM for 24 h were exposed to either E2 or E-HRP at 10 nM for 15 min. After exposure to the steroid, cells were washed in OptiMEM and 5 μM $A\beta_{1-40}$ diluted in 0.05% acetic acid was added for 24 h. As a control of $A\beta_{1-40}$ toxicity, some cultures were exposed to the peptide alone. For competitive assays with ICI 182,780 (10 μM), cells were pre-incubated with the anti-oestrogen for 2 h. Cells were then concomitantly exposed for 15 min to 10 μM ICI 182,780 and 10 nM of either E2 or E-HRP. Cultures were washed in OptiMEM and toxicity was induced with 5 μM $A\beta_{1-40}$ for 24 h. For competitive assays with anti-ERα MC-20, cultures were previously exposed to the antibody (1 : 50) for 2 h. MC-20 was then washed out in OptiMEM and cells were incubated for 15 min with either E2 or E-HRP (10 nM). Finally, cultures were treated with 5 μM $A\beta_{1-40}$ for 24 h and proceeded to survival quantification.

Fluorescence immunocytochemistry SN56 cultures exposed for 24 h to vehicle (0.001% ethanol) or 10 nM E2 in the presence or absence of 5 μM $A\beta_{1-40}$ were fixed under unpermeabilized conditions in phosphate-buffered saline (PBS), pH 7.4, containing 2% paraformaldehyde, 1% glutaraldehyde and 120 mM sucrose (P/G) for 30 min at room temperature. The non-permeabilizing fixative conditions employed here (P/G in the absence of detergent)

have been previously reported to preserve plasma membrane integrity and to prevent anti-ER antibodies crossing the plasma membrane (Norfleet *et al*. 1999, 2000; Clarke *et al*. 2000; Powell *et al*. 2001). To visualize intracellular ERα, cells were fixed in P/G in the presence of NP-40 (0.5%) for 1 min, in order to permeabilize the plasma membrane. Unpermeabilized and permeabilized cells were washed in PBS and incubated with 50 mM ammonium chloride for 1 h at room temperature to minimize generation of aldehyde groups. After washing again in PBS, cell plates were incubated at room temperature with 1 : 200 normal goat serum (NGS) to reduce non-specific binding. MC-20 antibody (4 ng/μL) was incubated in PBS with 1 : 200 NGS for 2 h at room temperature. The secondary biotinylated anti-rabbit antibody was previously adsorbed in SN56 cells fixed in the absence of the primary antibody and then incubated at 1 : 200 NGS in PBS for 1 h at room temperature. Staining was revealed by incubation with cyanine-2 dye-conjugated streptavidin (1 : 500) for 30 min at room temperature. After washing in PBS, cells were mounted in PBS/glycerol (1 : 1). No fluorescence was detected in the absence of primary antibody. Immunosignals were processed using a laser scanning confocal imaging system (MRC 1024; Bio-Rad Laboratories, Hercules, CA, USA).

Isolation of SN56 cell plasma membrane

Plasma membrane of both untreated and E2-treated SN56 cells was isolated using the cationic colloidal silica technique, a method that has been previously demonstrated to allow a high purification of plasma membrane fractions (Chaney and Jacobson 1983; Schmidt *et al*. 1985). The biochemical purity of plasma membrane preparation was assessed by determination of Na^+-K^+-ATPase activity (Díaz *et al*. 1998) and lactate dehydrogenase activity (Bergmeyer 1974) in both the plasma membrane and cytosolic fractions.

Western blot analysis

Both purified membrane and cytosolic fraction extracts from vehicle- and 10 nM oestrogen-treated cultures were mixed in sample buffer (625 mM Tris-HCl, 1% sodium dodecyl sulphate, 10% glycerol, 5% β-mercaptoethanol and 0.001% bromophenol blue, pH 6.8) and boiled at 95°C for 5 min. Whole extracts from untreated SN56 cells were used as control. Samples were electrophoresed on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to Hybond-P transfer membranes. The blots were then incubated with MC-20 anti-ERα antibody raised in rabbit (see Materials) and diluted 1 : 100. As a control of plasma membrane extraction and protein transfer, Hybond-P membranes were re-blotted with a rabbit antibody directed to the Na^+-K^+-ATPase β_1-subunit (1 : 5000) and a mouse anti-HSP90 antibody (1 : 1000; Stressgen Biotechnologies). Antibody labelling was revealed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1 : 20 000) and visualized with the ECL chemiluminescence kit.

Estradiol-peroxidase binding assay

SN56 cultures grown in OptiMEM for 24 h on glass coverslips were fixed in P/G for 1 min at room temperature. Cells were washed in PBS and incubated with 100 nM E-HRP overnight at 4°C. For competition assays, cultures were previously exposed for 2 h at room temperature to a 300-fold excess of E2, 17α-estradiol or ICI 182,780 diluted in 1% BSA and 3% dimethylsulphoxide in PBS and then coincubated with E-HRP overnight at 4°C. The peroxidase reaction was developed by the addition of 0.5 mg/mL 3,3′-diaminobenzidine tetrahydrochloride (DAB) in the presence of 0.3 mg/mL hydrogen peroxide for 30 min, as indicated in the FAST DAB enhancer kit (Sigma, St Louis, MO, USA). Light absorbed at 488 nm by the DAB product of peroxidase reaction was monitored in transmission photographs obtained by confocal microscopy and used as an index of the amount of E-HRP bound to the plasma membrane. A comparative numerical assessment was made by quantifying the percentage of absorbed light in individual cells exposed to the different compounds with respect to E-HRP alone, as previously described (Nadal *et al*. 2000). As a control, the absorbed light obtained by unspecific HRP staining in the presence of vehicle (1% BSA and 3% dimethylsulphoxide in PBS) was also quantified. A lower percentage of absorbed light represents a higher competition for an E-HRP binding site. Identical confocal settings and background parameters were used in all experiments. As a control of plasma membrane integrity during E-HRP treatment, both unpermeabilized and detergent-treated permeabilized fixed cells were alternatively incubated for 1 min at room temperature with 2.5 mg/mL dextran-conjugated FITC. This molecule has the same molecular weight as E-HRP (40 000 Da) and is thus considered as a good control of permeability in membrane binding assays (Nadal *et al*. 1998; Norfleet *et al*. 1999). Staining obtained with this compound was immediately visualized for labelling by confocal microscopy.

Estradiol-bovine serum albumin-fluorescein isothiocyanate binding assay

SN56 cultures grown in OptiMEM for 24 h on glass coverslips were washed in PBS. For competition assays, prior to the addition of E-BSA-FITC, cultures were exposed to a 10-fold excess of E2, ICI 182,780 (at 10, 50 or 100 μM) or MC-20 antibody (1–8 ng/μL) at 37°C for 2 h. Cells were finally washed three times in PBS. As a control of unspecific binding, other cells were incubated with 100 μM of BSA for 30 min and then exposed to 10 μM 17β-estradiol-6-(*o*-carboxymethyl) oxime-BSA-FITC for 1 h at 37°C. The fluorescent light visualized at the cell surface was monitored by taking emission images of the laser scanning confocal microscope and the intensity of the fluorescent area was quantified by using lasersharp software (Bio-Rad). Local background was subtracted. Identical experimental conditions were used for all treatments. No labelling was detected when coverslips were coincubated with MC-20 antibody and an excess (100 μM) of BSA in the absence of the hormone.

Statistical analyses

Data are expressed as mean ± SEM and were analysed by one-way ANOVA followed by Tukey's *post-hoc* test to compare between groups. Statistical significance is indicated in the figures from $p < 0.05$.

Results

Short-term exposure to either 17β-estradiol or estradiol-horseradish peroxidase reduces SN56 cell death during amyloid-β peptide injury, an effect that is attenuated by the anti-oestrogen ICI 182,780

Exposure of SN56 cells to 5 μM $A\beta_{1-40}$ for 24 h induced 65% cell death as compared with vehicle-treated cultures

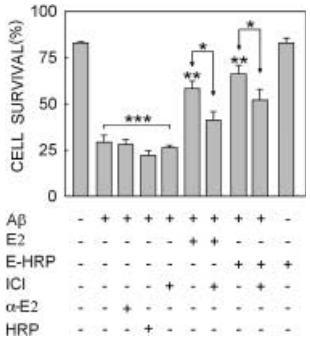

Fig. 1 Neuroprotective effects of short exposures to 17β-estradiol (E2) or the membrane-impermeant estradiol-horseradish peroxidase (E-HRP) against amyloid-β peptide $(A\beta)_{1-40}$-induced toxicity. SN56 cultures were incubated with either E2, E-HRP or the stereoisomer 17α-estradiol (α-E2) (all at 10 nM) for 15 min prior to being exposed to $A\beta_{1-40}$ (5 μM). As a control of cell injury, some cultures were incubated with the amyloid alone. For ICI 182,780 (ICI) treatments, cells were pre-incubated for 2 h with an excess (10 μM) of the anti-oestrogen and then cells were coincubated with both the anti-oestrogen and the hormone for 15 min. Cell viability by trypan blue exclusion was measured 24 h later (see Materials and methods). Vehicle-treated cells were used as an experimental control (first bar on the left). $^*p < 0.05$; $^{**}p < 0.001$ versus Aβ; $^{***}p < 0.0001$ versus vehicle-treated cells; five assays per group.

(Fig. 1). Pre-incubation with 10 nM E2 for 15 min reduced cell death to 30%, suggesting that oestrogen-mediated neuroprotection may be promoted at the plasma membrane level. To explore this possibility, SN56 cells were incubated with E-HRP for 15 min prior to $A\beta_{1-40}$ exposure showing that cell mortality was reduced in a similar range as with E2 (25%). Neuroprotective effects of both forms of oestrogen were partially prevented (in about 35%) by 2 h treatment with the ER antagonist ICI 182,780 (10 μM). Neither ICI 182,780 nor HRP alone altered Aβ-induced cell death. No significant neuroprotective effect was observed in the presence of 10 nM of the relatively inactive stereoisomer 17α-estradiol under the same experimental conditions. Furthermore, in unstressed conditions, E-HRP did not modify cell viability as compared with vehicle-treated cells.

Immunoreactivity for ERα at the plasma membrane domain of SN56 cells

Since oestrogen seems to initiate neuroprotective actions at the plasma membrane level, we explored the potential existence of an ER-like agent at the surface of SN56 cells either grown in standard conditions or during toxicity. SN56 cultures were treated with E2 in the presence of $A\beta_{1-40}$ peptide and fixed under either non-permeabilizing or detergent-permeabilizing conditions. As a control, some cultures were treated with vehicles (0.001% ethanol for E2 and 0.05% acetic acid). When unpermeabilized cells were incubated with MC-20 antibody directed to the carboxy-terminal region of ERα, some immunosignals associated with the cell surface were observed by confocal microscopy (Figs 2a and b). Some staining was also noticed in neurites (arrowheads), confirming the membrane-related labelling of anti-ERα antibody. In permeabilized cells, a strong staining was observed at the cytoplasmic and nuclear levels (Figs 2d and e). The intensity of immunosignals at the cell surface was not affected by the treatment with E2 and $A\beta_{1-40}$ peptide. We also performed another group of experiments using H151 monoclonal antibody directed to the hinge region of ERα (Figs 2c and f). In contrast to MC-20, no staining was observed in unpermeabilized oestrogen-treated cells incubated with H151 antibody (Fig. 2c), whereas this antibody recognized an intracellular protein in permeabilized conditions (Fig. 2f). The absence of immunostaining for this antibody at the surface of cells has also been previously reported in rat pituitary GH3/B6/F10 cells (Norfleet *et al.* 2000). The distinct pattern of immunostaining obtained with MC-20 and H151 anti-ERα antibodies suggests the presence of a protein related to the plasma membrane of SN56 cells (mER) that is structurally related to ERα, at least concerning the epitope recognized by MC-20.

An ERα-like agent is present in SN56 cell plasma membrane fractions

To confirm the membrane-related immunolocalization of ER observed by confocal microscopy, we performed plasma membrane isolations in SN56 cells either untreated or treated with 10 nM E2 using the cationic colloidal silica technique. Western blot analyses of SN56 cell fractions probed with MC-20 antibody revealed a membrane-related protein migrating at 67 kDa, the level of intracellular ERα, in either whole cell or cytosolic extracts (Fig. 3, ERα). In addition, another band migrating at approximately 80 kDa was identified in both purified cell membrane extracts and whole cell extracts (Fig. 3, *). The same electrophoretic pattern was obtained in both vehicle- and oestrogen-treated cultures (Fig. 3, M-1 and M-2, respectively). As membrane isolation controls, incubation with a specific antibody directed to the β_1-subunit of Na^+-K^+-ATPase revealed, as expected, a 45-kDa band specifically in the membrane fractions, whereas a 90-kDa band was observed only in cytosolic extracts with an anti-HSP90 antibody. Plasma membrane preparations were highly enriched on Na^+-K^+-ATPase (i.e. 1304% recovered Na^+-K^+-ATPase activity over 100% activity found in total extracts and < 0.1% found in the cytosolic fraction) indicating a very high degree of purity in the fraction and were 95% free of lactate dehydrogenase activity, thus ruling out significant cytosolic contamination.

Plasma membrane-related effects of 17β-estradiol in cell survival are reduced by MC-20 antibody

To further support the existence of an mER involved in neuroprotection against $A\beta_{1-40}$-induced injury, we exposed SN56 cultures to MC-20 anti-ERα antibody (4 ng/μL) for

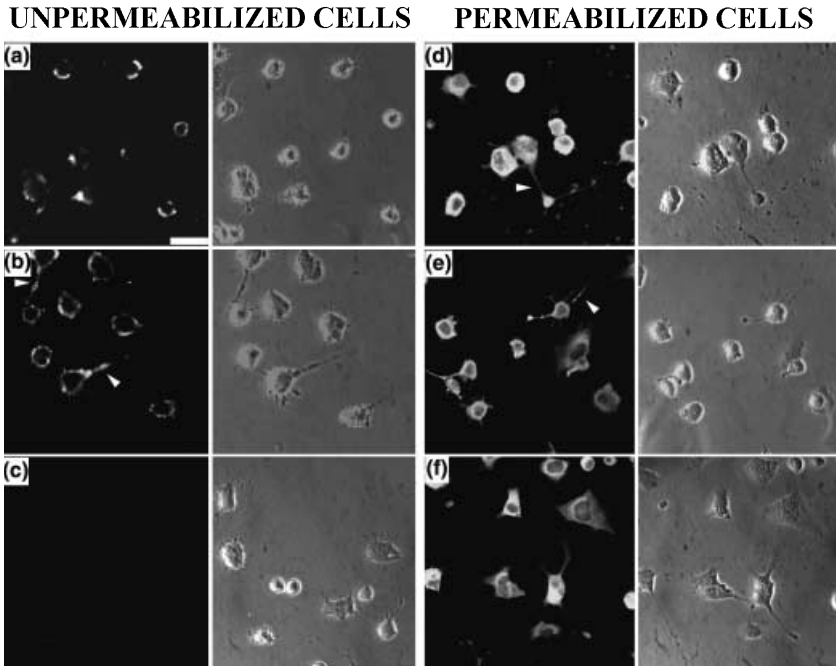

Fig. 2 Distinct immunofluorescent staining of ERα protein during amyloid-β peptide $(Aβ)_{1-40}$ toxicity in the presence of 17β-estradiol. Either non-permeabilized or detergent-permeabilized SN56 cultures were labelled with MC-20 (a, b, d and e) or H151 (c and f) anti-ERα antibodies and biotinylated anti-rabbit secondary antibody linked to Cy2-conjugated streptavidin (see Materials and methods). For MC-20 immunocytochemistry, (a and d) correspond to vehicle-treated cells and (b and e) to 10 nM oestrogen and 5 μM $Aβ_{1-40}$ cotreated cells. (c and f) correspond to oestrogen and Aβ cotreated cells incubated with H151 antibody. Notice the staining at the surface of unpermeabilized cells as compared with the intracellular labelling of permeabilized cells. To visualize cell shapes, equivalent matched phase contrast images are shown on the right of each immunofluorescence image. Notice also the staining in neurites (arrowheads). Four assays per group. Bar, 50 μm.

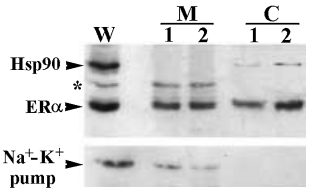

Fig. 3 Analysis of oestrogen receptor-like protein extracted in plasma membrane fractions of either vehicle-treated or estradiol-treated SN56 cells. Plasma membrane fractions extracted by using the cationic silica technique were loaded on sodium dodecyl sulphate–polyacrylamide gel electrophoresis for western blot analysis with MC-20 anti-ERα antibody. As a control, membranes were re-blotted with both a polyclonal antibody directed to the $β_1$-subunit of Na^+-K^+-ATPase and to a monoclonal anti-heat shock protein 90 specific antibody. M, Immunoblotting results obtained with plasma membrane fractions from vehicle-treated (column 1) or 10 nM estradiol-treated (column 2) cells. C, Equivalent cytosolic fractions of these two treatments. Whole SN56 cell extracts (W) were loaded for comparison purpose; additional band at 80 kDa (*). Five assays per group.

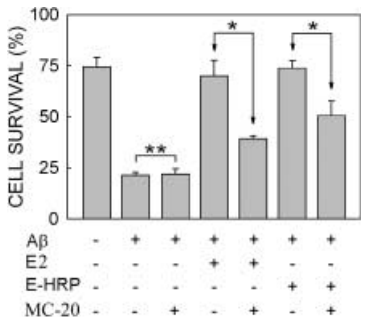

Fig. 4 Specific MC-20 anti-ERα antibody inhibits neuroprotective actions of either 17β-estradiol (E2) or estradiol-horseradish peroxidase (E-HRP). SN56 cultures were pre-incubated with the specific anti-ERα antibody (1 : 50) prior to treatment with either form of steroid for 15 min. Cells were then exposed to 5 μM amyloid-β peptide $(Aβ)_{1-40}$ for 24 h and viability was measured. For comparative purposes, other cultures were concomitantly exposed to the same steroid and Aβ treatments in the absence of the antibody. Both cells exposed to the antibody during injury in the absence of oestrogen as well as vehicle-treated cells were used as a control of viability. *$p < 0.01$; **$p < 0.0001$ versus vehicle-treated cells. Five assays per group.

2 h at 37°C, prior to either E2 or E-HRP exposure. Treatment with this antibody reduced the neuroprotective effect of both E2 and E-HRP in a similar range to ICI 182,780 (35–50%) (Fig. 4), whereas the antibody alone did not affect cell survival. Furthermore, we did not observe any significant modification of oestrogen-related neuroprotection in another group of experiments using H151 antibody (data not shown).

Altogether, these data support the idea that oestrogen is able to prevent $A\beta_{1-40}$-induced SN56 cell death by acting through a potential membrane form of ER that shares some structural similarities with ERα.

Both impermeant conjugates, estradiol-horseradish peroxidase and estradiol-bovine serum albumin-fluorescein isothiocyanate, bind to specific sites of SN56 cell plasma membrane; competition by 17β-estradiol, ICI 182,780 and MC-20 antibody

To further explore a potential membrane-related form of ERα involved in neuroprotection by E2, we studied as a first approach the existence of specific plasma membrane binding sites in SN56 cells using two different compounds, E-HRP and E-BSA-FITC, that are unable to cross the plasma membrane (Pappas *et al*. 1995; Morey *et al*. 1997; Watters *et al*. 1997; Watson and Gametchu 1999; Nadal *et al*. 2000; Russell *et al*. 2000). SN56 cells were exposed to 100 nM E-HRP overnight at 4°C and the HRP reaction was then developed. A strong staining was obtained with the DAB-based primary product of peroxidase reaction, as revealed by transmission laser scanning confocal microscopy (Fig. 5a). The E-HRP binding was competed off by a 300-fold excess of either unlabelled E2 (Fig. 5b) or ICI 182,780 (Fig. 5c). On the other hand, a very small effect on E-HRP staining (15%) was obtained in the presence of 17α-estradiol (Fig. 5d). To confirm the incapability of E-HRP to enter through the plasma membrane, we exposed unpermeabilized P/G-fixed SN56 cells to 2.5 mg/mL dextran-FITC. No intracellular staining was observed with dextran-FITC (Fig. 5e), indicating that E-HRP does not penetrate the SN56 cell membrane under these experimental conditions.

The existence of oestrogen membrane binding sites in SN56 cells was also revealed using FITC-conjugated E-BSA. SN56 cells were incubated with 10 μM E-BSA-FITC observing fluorescent staining at the outer cell membrane (Fig. 6a). Labelling was blocked by a 10-fold excess of E2 (Fig. 6b) but was not affected by 10-fold excess of BSA (Fig. 6c). Interestingly, a complete blockade of immunostaining was obtained with 100 μM ICI 182,780 (Fig. 6d). Quantification of the percentage of emitted light in cells exposed to E-BSA-FITC and ICI 182,780 revealed a dose-dependent reduction in labelling with increasing concentrations of ICI 182,780 (Fig. 6e).

Since the MC-20 antibody recognized an ERα-like agent on the unpermeabilized SN56 cell surface and it was able to interfere in neuroprotective actions of E2 triggered at the plasma membrane, we wondered if this antibody recognition of an mER was related to the site recognized by E2 at the membrane level. Therefore, we also performed double-binding experiments with 10 μM of E-BSA-FITC on SN56 cultures previously exposed to different concentrations (1–8 ng/μL) of MC-20 antibody.

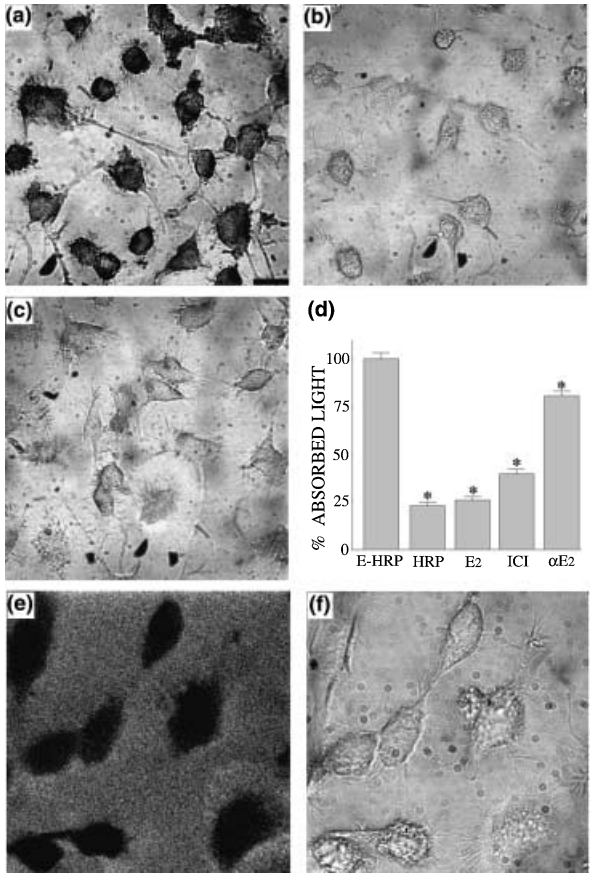

Fig. 5 Estradiol-horseradish peroxidase (E-HRP) binding assay in SN56 cells. Cultures were fixed under non-permeabilizing conditions and incubated with 100 nM E-HRP overnight at 4°C. For double-binding assays, cells were previously exposed to an excess of 17β-estradiol (E2) or ICI 182,780 (ICI). (a) Staining of 100 nM E-HRP in unpermeabilized SN56 cells as observed by confocal microscopy. (b and c) Blockade of E-HRP labelling with 30 μM of E2 (b) and anti-oestrogen ICI (c). (d) Quantification analysis of the competition of E-HRP binding by 30 μM of E2, ICI and 17α-estradiol (αE2) as compared with the maximal absorbed light obtained with E-HRP binding alone. No. of cells per group, 90. $^*p < 0.0001$ versus E-HRP. (e) Exclusion of intracellular staining obtained in the presence of 2.5 mg/mL dextran-fluorescein isothiocyanate. (f) Corresponding brightfield image of (e) obtained by confocal microscopy. Bar, 10 μm.

We visualized by confocal microscopy a significant inhibition of E-BSA-FITC-related fluorescence in the presence of 8 ng/μL MC-20 (Fig. 7b), as compared with control cultures incubated with the steroid alone (Fig. 7a). The inhibitory effect of MC-20 on fluorescent staining was quantified by the percentage of emitted light reduction related to the different doses of the antibody (Fig. 7c). Binding competition results were not reproduced with H151 antibody (data not shown). Thus, it seems that E2 recognizes a membrane-associated ER that shares some structural homologies, at least with the carboxy-terminal

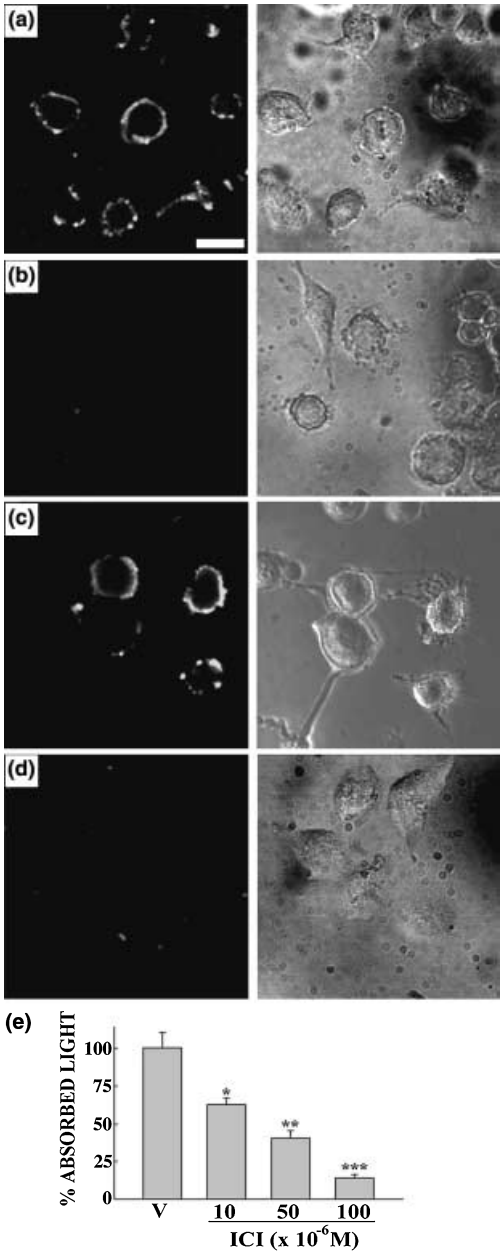

Fig. 6 Estradiol-bovine serum albumin-fluorescein isothiocyanate (E-BSA-FITC) binding of SN56 cells and competition with ICI 182,780. (a) Specific fluorescence staining at the surface of SN56 cells with 10 μM E-BSA-FITC. This labelling was competed off with a 10-fold excess of 17β-estradiol (b) but remained unaffected with a 10-fold excess of BSA (c). (d) Staining was blocked with 100 μM ICI 182,780 (ICI). (e) Quantification of ICI competition at the different doses used. Values are referred to the percentage of absorbed light in vehicle-treated cells used as a control. Corresponding transmission images of the different cultures are shown on the right. Eighty cells per group. *$p < 0.001$ versus vehicle; **$p < 0.001$ versus 10; ***$p < 0.001$ versus 50. Bar, 30 μm.

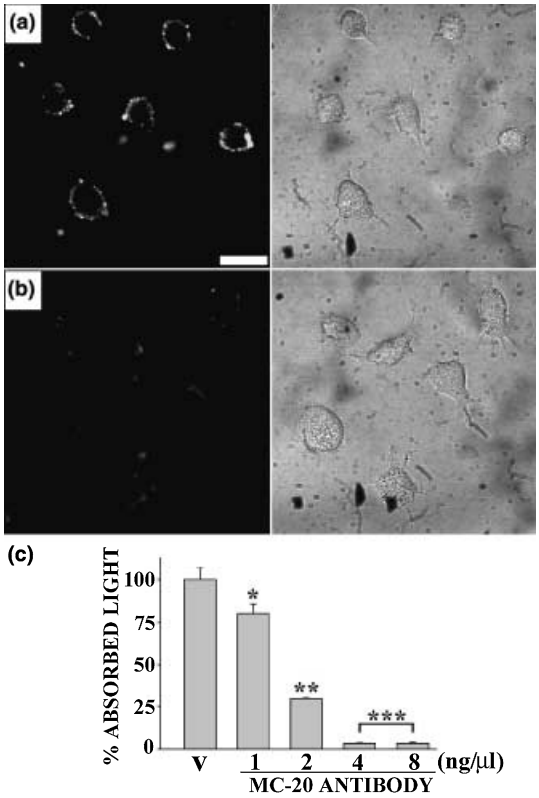

Fig. 7 Blockade of estradiol-bovine serum albumin-fluorescein isothiocyanate (E-BSA-FITC) binding with MC-20 anti-ERα antibody in SN56 cells. (a) Specific fluorescence staining at the surface of SN56 cultures incubated with 10 μM E-BSA-FITC used as a control. This binding was competed off with a previous incubation with increasing concentrations of MC-20 antibody that progressively displaced steroid labelling. Staining was inhibited when cultures were pre-incubated with 8 ng/μL of the antibody (b). (c) Quantification of E-BSA-FITC staining in the presence of different concentrations (1–8 ng/μL) of the antibody. Values are referred to the percentage of absorbed light in vehicle-treated cells used as a control. Corresponding transmission images of the different cultures are shown on the right of each immunofluorescence. Sixty cells per group. *$p < 0.05$ versus vehicle; **$p < 0.001$ versus 1; ***$p < 0.001$ versus 2. Bar, 50 μm.

neighbouring ligand binding domain (LBD) of intracellular ERα.

Discussion

Epidemiological studies of AD in post-menopausal women have suggested that oestrogen replacement therapy may contribute to palliate the symptoms of neurodegeneration (Tang *et al*. 1996; Henderson *et al*. 2000). In agreement with this, accumulating evidence in cellular paradigms that mimic some aspects of AD toxicity supports a neuroprotective role of oestrogen against a variety of toxic agents including Aβ (Green and Simpkins 2000; Garcia-Segura *et al*. 2001). However, the mechanisms and signalling pathways for this

oestrogen-mediated protective action are still not fully understood. Previous work from different groups, including our own, indicates that oestrogen-dependent activation of canonic ER may prevent Aβ-induced neuronal death (Kim *et al*. 2001; Marin *et al*. 2001b; Fitzpatrick *et al*. 2002). We now report that, in addition to classical mechanisms, oestrogen may have unconventional signalling pathways to promote neuroprotection. This is suggested by the fact that a 15-min pre-treatment with either E2 or its impermeant counterpart E-HRP was sufficient to protect SN56 cells from amyloid toxicity. Our results also demonstrate the presence of an ERα-like agent associated with the plasma membrane (mER), as evidenced by the staining with MC-20 antibody on the surface of unpermeabilized SN56 cells and by immunoblotting assays of membrane fractions. In extracts from either whole cells or plasma membranes, another band migrating at a higher M*r* (80 kDa) was observed in addition to mER with an M*r* similar to that of intracellular ERα. This M*r* heterogeneity may be indicative of different post-translational isoforms of ERα, as is the case for glucocorticoid receptors (Powell *et al*. 1999), or alternatively, the result of different susceptibility to proteolysis. The existence of an mER has previously been reported in a variety of cell types (reviewed in Pietras *et al*. 2001), including neurones (Blaustein 1992; Clarke *et al*. 2000; Norfleet *et al*. 2000; Milner *et al*. 2001), and suggests that cell membrane forms of ER may also play a role in oestrogen-dependent responses in target cells.

The putative participation of ERs in oestrogenic neuroprotection during short exposures was also evaluated by pre-treating SN56 cultures with either the ER antagonist ICI 182,780 or MC-20, an antiserum against the carboxy-terminal region of ERα. Both molecules were able to partially prevent the protective effect of oestrogen on $A\beta_{1-40}$-induced cell death. To our knowledge, this is the first demonstration of the involvement of a constitutive ER subpopulation associated with the plasma membrane in oestrogen-dependent neuroprotection against Aβ injury. Previous data in hippocampal-derived HT22 cells transfected with ERα or ERβ have demonstrated a contribution of both ER transcripts in mediating protective effects of estradiol related to rapid signalling pathways (Fitzpatrick *et al*. 2002). However, the putative involvement of constitutive ERβ cannot be quantified in the present paradigm since SN56 cells constitutively express very low amounts of this receptor (Martinez-Morales *et al*. 2001).

A further characterization of an oestrogen-specific binding at the periphery of SN56 cells was performed using E2 either conjugated to BSA and FITC (E-BSA-FITC) or bound to HRP (E-HRP). The reduction in E-BSA-FITC staining observed in double-binding assays with increasing concentrations of ICI 182,780 supports the idea that oestrogen specifically binds to an mER that shares some homologies with classical ERα in functional regions of the molecule. In agreement with this, exposure to MC-20 antibody prior to oestrogen impeded hormone binding to its putative receptor at the cell surface, suggesting that mER and intracellular ER may be similar at least in the epitope recognized by MC-20 antibody. Another piece of evidence in support of this hypothesis was the weak competition for oestrogen membrane sites exerted by 17α-estradiol, a compound that has 100-fold lower affinity for ERα than E2 (Hajek *et al*. 1997). This compound also failed to produce any significant neuroprotection against $A\beta_{1-40}$-related cytotoxicity after 15 min exposure. Previous data in different oestrogen target cells have substantiated the structural relationship between intracellular ER and its membrane-associated counterpart (Migliaccio *et al*. 1996; Norfleet *et al*. 1999; Razandi *et al*. 1999; Watson and Gametchu 1999; Powell *et al*. 2001; Fitzpatrick *et al*. 2002). It has also been suggested that an mER originated from the traditional ER and translocated to the membrane might participate in several oestrogenic actions (Razandi *et al*. 1999; Russell *et al*. 2000; Fitzpatrick *et al*. 2002). Interestingly, a recent work in neocortical explants has proposed the existence of a novel mER that has homology with ERα LBD and is associated with oestrogen-induced activation of the mitogen-activated protein kinase cascade (Toran-Allerand *et al*. 2002). However, whereas oestrogen rapid signalling triggered at the plasma membrane may promote neuroprotection by induction of ER-mediated transcriptional activities (Singer *et al*. 1999), other alternative mechanisms may also be involved depending on both the cell origin and type of injury (reviewed in Green and Simpkins 2000).

In summary, our results show that oestrogen can exert neuroprotective effects against Aβ-induced toxicity in neuronal-derived cholinergic cells by briefly acting at the plasma membrane. This neuroprotective action may be triggered after oestrogen binding to a plasma membrane protein that shares some structural homologies with the LBD of ERα. We have previously found that oestrogen can also prevent $A\beta_{1-40}$-induced cell death through classical intracellular ERs in SN56 cells (Marin *et al*. 2001b). Therefore, it is possible that oestrogen neuroprotective actions mediated through membrane-associated ER could be coordinated with more delayed intracellular responses mediated by activation of nuclear ERs. These interactions might ultimately modulate neuronal responses to oestrogen in the presence of toxic agents causing cellular stress. Further understanding of the discrete actions by which steroids act through both alternative and classical mechanisms to induce neuroprotection may provide alternative strategies for preventing or treating AD-related neurodegenerative disorders.

Acknowledgements

This work was supported by grant SAF2001-3614-C03-01/02. Partial financial support was received from Lilly S.A and Astra-

Zeneca. BG and AM held research fellowships from, respectively, MEC and MCYT (Spain). The authors wish to thank D. Perera and J. Hernández for assistance with membrane extractions, Dr Eduardo Almansa and Rosa Dópido for technical assistance in the enzymatic assays and Dr P. Martin-Vasallo and Dr M. L. García de Paredes (Astra-Zeneca) for their generous gifts of anti-β_1-subunit of Na^+-K^+-ATPase antibody and ICI 182 780, respectively.

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