Role of ERβ palmitoylation in the inhibition of human colon cancer cell proliferation

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Abstract

The cellular functions regulated by 17β-estradiol (E2) start after the hormone binds to its receptors (i.e., ERα and ERβ). These act as ligand-dependent transcription factor transactivating target genes. In addition, E2 induces non-genomic actions, whose activation is triggered by a fraction of the ERs localized at the plasma membrane. Palmitoylation allows ERα to localize at the plasma membrane, to associate with caveolin-1, and, upon E2 stimulation, to activate rapid signals relevant for cell proliferation. The existence of a mechanism, which allows ERβ localization at the plasma membrane and its putative role in anti-proliferative E2 effects is completely unknown. Here, the susceptibility of ERβ to undergo palmitoylation and the role played by this process has been analyzed in DLD-1 containing endogenous ERβ or in HeLa cells transiently transfected with ERβ or ERα expression vectors. As for ERα, palmitoylation is necessary for ERβ localization at the plasma membrane and its association with caveolin-1 but, in contrast to ERα, the E2 binding increases ERβ association with caveolin-1 and the p38 member of MAPK family. Moreover, the palmitoyl acyl transferase (PAT) inhibitor blocks the ability of ERβ–E2 complex to activate p38 impairing the receptor-dependent activation of downstream pro-apoptotic cascade (i.e., caspase-3 activation and poly(ADP-ribose)polymerase (PARP) cleavage). Consequently, palmitoylation must be considered to be a molecular device for ERβ, which allows these receptors to interact with the plasma membrane and to regulate E2-induced non-genomic functions relevant to the anti-proliferative effect of this hormone.

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Introduction

Knowledge of the pleiotropic functions regulated by 17β-estradiol (E2) in different tissues and organs has evolved rapidly during the past decade. It is now well established that the E2 effects in living cells are mediated by a complex signaling network rather than a single uniform mechanism. In fact, in addition to its role as gene transcription regulator (Acevedo & Kraus 2004), sustained by the two estrogen receptors (ERα and ERβ), E2 induces rapid, membrane starting, non-genomic actions (Levin 2005, Marino *et al.* 2005, Song *et al.* 2005, Leclercq *et al.* 2006).

Among other physiological roles, there is growing evidence that the rapid actions activated when E2 binds to the α isoform of ER are important for cell proliferation. The E2 treatment of mammary-derived MCF-7 cells triggers ERα association with Src, and the regulatory subunit of phosphatidyl inositol 3-kinase, (PI3K) leading to DNA synthesis (Castoria *et al.* 2001). Moreover, in HepG2 cells, multiple and parallel membrane starting pathways are rapidly activated by the ERα–E2 complex (Marino *et al.* 1998, 2002, 2003). The blocking of phospholipase C/protein kinase C, mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK), and PI3K/AKT pathways completely prevents the E2-induced DNA synthesis (Marino *et al.* 2002, 2003). All these results point to the belief that membrane localized ERα is the primary endogenous mediator of rapid E2 actions important for cell proliferation and survival.

However, recent reports have demonstrated that E2 could even decrease cell growth by significantly increasing apoptosis in several cell types (see Song & Santen 2003 for review). In particular, epidemiologic, clinical, and experimental evidence show that E2 confers protection against prostate and colon cell proliferation (Horvath *et al.* 2001, Konstantinopoulos *et al.* 2003, Bardin *et al.* 2004, Koehler *et al.* 2005).

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ERβ seems to participate in these E2-induced blockages of cell proliferation. A progressive decline of ERβ expression has been reported in multistage mammary carcinogenesis (Roger *et al.* 2001) and prostate cancer (Horvath *et al.* 2001); moreover, ERβ is the predominant ER expressed in colonic tissues and its expression is selectively lost in human malignant colon tissue (Konstantinopoulos *et al.* 2003, Wada-Hiraike *et al.* 2006). It has been proposed that ERβ could act as a dominant regulator of E2 signaling (Koehler *et al.* 2005), and when co-expressed with ERα, it would cause a concentration-dependent reduction in ERα-mediated transcriptional activation (Pettersson *et al.* 1997). The ERβ-directed repression of ERα-mediated effects includes cell proliferation (Imamov *et al.* 2005, Koehler *et al.* 2005). Data from gene expression in cell cultures and knockout mice, clearly indicate that E2-activated ERβ must have some function as a tumor suppressor by modulating the proliferative effects of ERα (Couse & Korach 1999, Cheng *et al.* 2004, Paruthiyil *et al.* 2004, Strom *et al.* 2004).

These studies support a functional antagonism between ERα and ERβ with respect to the E2-induced cell proliferation, but failed either to ascertain the putative role of ERβ in E2-induced apoptosis or to identify the signal transduction pathways involved. However, the ability of the ERβ–E2 complex to activate rapid non-genomic mechanisms has been reported (Castoria *et al.* 2001, Kousteni *et al.* 2001, Geraldes *et al.* 2003, Mori-Abe *et al.* 2003). We recently demonstrated that E2-induced rapid signal transduction pathways in ERβ-transfected HeLa cells appear to play a major role in mediating anti-proliferative properties of this steroid hormone. The action of E2 in these cells results from binding to ERβ which, in turn, acutely promotes the rapid and persistent phosphorylation of the p38 member of MAPK family, thus triggering downstream activation of a pro-apoptotic cascade (Acconcia *et al.* 2005*a*). The rapidity by which these cellular cascades are activated raises the need for a receptor localized at the plasma membrane. Although a subpopulation of ERβ localized within caveolar rafts, responsible for rapid endothelial nitric oxide synthase stimulation by E2 has been reported in the plasma membrane of endothelial cells (Chambliss *et al.* 2002), the mechanism allowing ERβ localization at the plasma membrane and its putative involvement in the anti-proliferative effect mediated by ERβ–E2 complex is completely unknown. We previously demonstrated that the Cys447 residue present in the ERα ligand binding domain (LBD) is palmitoylable and this lipid modification is necessary for the induction of the non-genomic ERK/MAPK signal transduction pathway, which is relevant to E2-induced cell proliferation (Acconcia *et al.* 2005*b*). Although, the homology between ERα and ERβ LBDs is only 59% (Ascenzi *et al.* 2006), the amino acid sequence encompassing the palmitoylated Cys447 and Cys132 residues of ERα and caveolin-1, respectively, is highly homologous to that surrounding the Cys399 residue of ERβ (Acconcia *et al.* 2003). Based on these findings, we postulated that ERβ serves as a palmitoyl acyl transferase (PAT) substrate and that the receptor palmitoylation is important for the localization at the membrane and for the activation of the E2-dependent pro-apoptotic cascade in colon cancer cells. Our results indicate that ERβ palmitoylation is a major determinant for ERβ–membrane and ERβ–protein interaction (i.e., caveolin-1 and p38/MAPK) important for the E2-induced protective effect against colon cancer.

Materials and methods

Reagents

E2, gentamicin, penicillin, GenElute plasmid maxiprep kit, Dulbecco Modified Eagle Medium (DMEM), RPMI-1640 medium (without phenol red), charcoal-stripped fetal calf serum, and the PAT inhibitor 2-bromohexadecanoic acid (2-bromo-palmitate; 2-Br) ($IC_{50} \simeq 4.0$ μM; Varner *et al.* 2003), were purchased from Sigma-Aldrich (St Louis, MO, USA). The p38/MAPK inhibitor, SB 203 580 (SB), was obtained from Calbiochem (San Diego, CA, USA). The ER inhibitor ICI 182 780 (ICI) was obtained from Tocris (Ballwin, MO, USA). 9,10-[^{3}H]-palmitic acid (specific activity 57.00 Ci/mmol) was purchased from Dupont-NEN (Boston, MA, USA). Lipofectamine reagent was obtained from Gibco-BRL Life-technology. The luciferase kit was obtained from Promega. Bradford Protein Assay was obtained from Bio-Rad Laboratories. The monoclonal anti-phospho-ERK, anti-AKT, anti-Src, anti-caspase-3, anti-poly(ADP-ribose)polymerase (PARP), and anti-β-actin as well as the polyclonal anti-ERK, anti-caveolin-1, anti-ERα MC20 (C-terminus), and anti-ERβ L20 (C-terminus) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal anti-ERβ 14C8 (N-terminus) antibody was purchased from Genetex (San Antonio, TX, USA). The policlonal anti-phospho-AKT, anti-phospho-p38, and anti-p38 antibodies were obtained by New England Biolabs (Beverly, MA, USA). The modulator of non-genomic activity of estrogen receptor (MNAR, also named Pro-, Glu-, and Leu-rich protein-1; PELP1) polyclonal antibody was purchased from Benthyl Laboratories

(Montgomery, TX, USA). CDP-Star, chemiluminescence reagent for Western blot was obtained from NEN. All the other products were from Sigma-Aldrich. Analytical or reagent grade products, without further purification, were used.

Cell culture and count

The ERs-devoid human cervix epithelioid carcinoma cell line (HeLa; Marino *et al.* 2002) and the ERβ containing human colon adenocarcinoma cells (DLD-1; Marino *et al.* 2006) were used as experimental models. Cells were routinely grown in air containing 5% CO_2 in modified, phenol red-free, DMEM (HeLa cells) or RPMI-1640 (DLD-1 cells) media, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml). Cells were passaged every 2 days and media changed every 2 days.

DLD-1 cells were grown to approximate 70% confluence in six-wells plates then stimulated. Thirty hours after treatment (10 nM E2, 1 μM ICI 182 780, 10 nM E2–BSA), cells were harvested with trypsin and centrifuged. Cells were stained with the Trypan blue solution and counted in a hemocytometer (improved Neubauer chamber) in quadruplicate.

Plasmids

The reporter plasmid containing the promoter of complement component 3 gene, retaining a natural estrogen responsive element (ERE), linked to the gene of luciferase (pC3), the reporter plasmid containing the promoter of cyclin D1 pXP2-D_1-2966-luciferase (pD1), the expression vector pCR3.1-β-galactosidase, wild type human ERα pSG5-HE0, human ERβ (pCNX2-ERβ) have been described elsewhere (Herbert *et al.* 1994, Marino *et al.* 2002, Acconcia *et al.* 2004, 2005*a*). A luciferase dose-response curve showed that the maximum effect was obtained when 1.0 μg of plasmids was transfected together with 1.0 μg of pCR3.1-β-galactosidase to normalize for transfection efficiency (approximate 55–65%). Plasmids were purified for transfection using the GenElute plasmid maxiprep kit according to the manufacturer's instructions.

Transfection and luciferase assay

HeLa and DLD-1 cells were grown to ~70% confluence and then transfected using lipofectamine reagent according to the manufacturer's instructions. Six hours after transfection, the medium was changed and 24 h after the cells were stimulated with 10.0 nM E2 for 6 h. The cell lysis procedure as well as the subsequent measurement of luciferase gene expression was performed using the luciferase kit according to the manufacturer's instructions with an EC & G Berthold luminometer (Bad Wildbad, Germany). When indicated, the PAT inhibitor 2-Br (final concentration 10.0 μM) was added 15 min before E2 administration.

Cell labelling with [^{3}H]-palmitate and immunoprecipitation

Twenty-four hours after transfection with either plasmid containing ERα or ERβ, HeLa cells and untransfected DLD-1 cells were incubated with 0.5 mCi/ml [^{3}H]-palmitate at 37 °C for different times ranging between 0 and 240 min. DLD-1 and HeLa cells were stimulated with different concentrations of E2 (0.1, 1.0, 10.0, and 100.0 nM) for different times (10, 60, and 240 min) in the presence of [^{3}H]-palmitate. Cells were then washed in ice-cold PBS, harvested with trypsin (1%, v/v), and lysed in 50 μl lysis buffer (10.0 mM Tris, pH 7.5, 1.0 mM EDTA, 0.5 mM EGTA, 10.0 mM NaCl, 1% (v/v) Triton X-100, and 1% (w/v) sodium cholate) containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml leupeptin, and 5.0 μg/ml aprotinin). The cell lysates were then clarified by centrifugation and immunoprecipitated as described previously (Acconcia *et al.* 2005*b*). Briefly, equal amounts of soluble cell extracts were incubated with different anti-ERβ antibodies. The optimal signal was obtained using 1.0 μg anti-ERβ L10 (C-terminus) together with 1.0 μg of anti-ERβ 14C8 (N-terminus). The lysates and antibodies were incubated for 90 min at 4 °C, and then 20 μl of protein-A agarose were added for 30 min at 4 °C. After centrifugation (50 000 ***g*** for 15 min), the supernatant and the immunoprecipitated proteins were separated in 7–10% SDS-PAGE. In some experiments, the radioactivity present in immunoprecipitated proteins and in the supernatant was monitored by counting with a Camberra Packard Liquid β-counter (Milan, Italy).

True-blot immunoprecipitation

The cell lysates, prepared as described above, were clarified by centrifugation and immunoprecipitated with TrueBlot (eBioscience, San Diego, CA, USA), which preferentially detects the native disulfide form of mouse IgG or rabbit IgG reducing interference by approximate 55 kDa heavy and approximate 23 kDa light chains of the immunoprecipitating antibody. Briefly, after stimulation, equal amounts of soluble cell extracts were incubated with either 2.0 μg of

anti-caveolin-1 or anti-p38 or anti-Src or anti-MNAR or anti-14C8 ERβ antibody. The lysates and antibodies were incubated at 4 °C for 1 h, then 20 µl of Anti Mouse IgG Beads (eBioscience) were added and samples incubated for 1 h on a rocking platform at 4 °C. Samples were centrifuged at 50 000 ***g*** for 10 min, the supernatant was removed completely and beads (pelleted) were washed three times with 100 µl of lysis buffer. SDS-reducing sample buffer (20 µl, containing 50 mM DTT) was added and samples were boiled at 100 °C for 5 min. Proteins were resolved using 10% SDS-PAGE at 100 V for 1 h and then electrophoretically transferred to nitrocellulose for 45 min at 100 V at 4 °C. The nitrocellulose was treated with 5% (w/v) non-fat dry milk (Bio-Rad Laboratories) in 150 mM NaCl, 50.0 mM Tris HCl (pH 8.0), 0.1% (w/v) Tween-20, and then probed at 4 °C overnight with anti-ERβ antibody. The antibody reaction was visualized with the chemiluminescence reagent for Western blot.

Immuno-fluorescence studies

DLD-1 cells were grown on coverslips, treated as above specified, and fixed in 2% (w/v) freshly de-polymerized *p*-formaldehyde (Sigma-Aldrich) and 0.2% (v/v) glutaraldehyde (Agar Scientific Ltd, Stanstead, UK) in PBS. Cells were then incubated with 14C8 anti-ERβ mouse monoclonal antibody diluted 1:100 in PBS containing 1% (w/v) BSA and 0.1% (v/v) Triton X-100. After washing, cells were incubated with goat anti-mouse fluorescein isothiocynate (FITC)-conjugated antibody (Vector Laboratories, Burlingame, CA, USA; diluted 1:200 in PBS containing 1% (w/v) BSA and 0.1% (v/v) Triton X-100). Finally, slides were mounted with Vectashield (Vector Laboratories) and localization of the receptor was examined with a Zeiss LSM510 confocal microscope. Pictures were electrically captured and composed by Photoshop 7.0 software.

RNA isolation and quantitative RT-PCR analysis (qRT-PCR)

The sequences for gene-specific forward and reverse primers were designed using the OligoPerfect Designer software program (Invitrogen). The following primers were used: for human ERα (ESR1/NR3A1, GeneBank Accession No. AY425004), 5′-TCCTAGCAGGGAGATGAGGA-3′ (forward) and 5′-CCTTTATGGCCAGCAATCAT-3′ (reverse), for human ERβ (ESR2/NR3A2, GeneBank Accession No. AY785359), 5′-GGCGCGATCTTG-GCTCAC-3′ (forward) and 5′-TGGCTGGACGT-GGTGGCA-3′ (reverse), and for β-actin (GeneBank Accession No. X00351), 5′-AGAAGGATTCC-TATGTGGGCG-3′ (forward) and 5′-CATGTCGTC-CCAGTTGGTGAC-3′ (reverse).

Total RNA was extracted from DLD-1 cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. To determine hER-β gene expression levels (as well to confirm the absence of hER-α), cDNA synthesis and qPCR were performed using a one-step qRT-PCR kit ('SuperScript III Platinum – SYBR Green One-Step' kit; Invitrogen) according to the manufacturer's instructions. cDNA synthesis and qPCR was carried out in a ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as follows: first strand cDNA synthesis was performed at 50 °C for 5 min, followed by an automatic hot-start Taq DNA Polimerase activation step at 95 °C for 5 min, and then by 25 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 40 °C for 1 min. Gene expression was verified by electrophoresis on 2% agarose gel. Each sample was tested in duplicate and the experiment repeated four times.

Electrophoresis and immunoblotting

After treatment, cells were lysed and solubilized in 0.125 M Tris, pH 6.8, containing 10% (w/v) SDS, 1.0 mM phenylmethylsulfonyl fluoride, and 5.0 µg/ml leupeptin; then the cell lysates were boiled for 2 min. In some experiments cells were homogenized using ten strokes of the pestle of Potter homogenizer until about 90% of the cells were broken. Homogenates were centrifuged at 1000 ***g*** for 10 min to pellet the nuclear fraction. Soluble and particulate fractions were obtained by centrifuging the supernatants at 100 000 ***g*** for 30 min. Proteins were solubilized as above described. Total proteins were quantified using the Bradford Protein Assay. Solubilized proteins (20 µg) were resolved by 10% SDS-PAGE at 100 V for 1 h at 24 °C and then electrophoretically transferred to nitrocellulose for 45 min at 100 V and 4 °C. The nitrocellulose was treated with 3% (w/v) BSA in 138.0 mM NaCl, 25.0 mM Tris, pH 8.0, at 24 °C for 1 h and then probed overnight at 4 °C with either anti-phospho-ERK or anti-phospho-AKT or anti-phospho-p38 or anti-ERβ antibodies. The nitrocellulose was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical Company, Rockford, IL, USA) for 10 min at room temperature and then probed with either anti-ERK or anti-AKT or anti-p38 or anti-caspase-3 or anti-PARP and anti-β-actin antibodies. Antibody reaction was visualized with chemiluminescence Western blotting detection reagent

(Amersham Biosciences). The PAT inhibitor 2-Br (10.0 μM) or the p38 inhibitor SB 203 580 (5 μM) was added 30 min before E2 administration.

Statistical analysis

A statistical analysis was performed by utilising Student's *t*-test with the INSTAT software system for Windows. Some data were analyzed by one-way ANOVA and *post hoc* Bonferroni's test (INSTAT software system for Windows). In all cases probability (*P*) values below 0.05 were considered significant.

Results

E2 reduces DLD-1 cell growth

The main difficulty in studying ERs action mechanism in cancer cells derives from the expression of a great number of ERs splice variants (Herynk & Fuqua 2004). To bypass this problem we chose human colon adenocarcinoma (DLD-1) cells as an experimental model, which contain only one ERβ isoform corresponding to 54 kDa protein (Fig. 1A and B). As already reported (Marino *et al.* 2006), E2 stimulation decreases DLD-1 cell growth. This effect requires ERβ, since it is completely prevented by ICI 182 780 (Fig. 1, panel C). Stimulation of DLD-1 cells with the E2 cell membrane impermeable E2-BSA, a well-known agent able to discriminate between non-genomic vs genomic effects of ER(s) (Marino *et al.* 2003), affected DLD-1 cell growth, as did E2 (Fig. 1, panel C). This indicates the pivotal role of plasma membrane-starting signals in E2-induced anti-proliferative effects.

ERβ is a palmitoylable protein

We first verified the occurrence of ERβ palmitoylation in DLD-1 cells. Cells were incubated with [^{3}H]-palmitate at 37 °C for different periods (from 0 to 240 min) and the amount of radioactivity in both the immunoprecipitate and the supernatant was determined (Fig. 2A). ERβ and radioactivity were not detected in the supernatant fractions (Fig. 2A and data not shown). [^{3}H]-palmitate incorporation in immunoprecipitated ERβ was complete within 120 min and remained unchanged over 240 min (Fig. 2B). As a positive control, the radioactivity present in the palmitoylated caveolin-1 (Resh 1999), immunoprecipitated from DLD-1 cells, was measured (Cav-1) (Fig. 2C). A significant decrease in ERβ and caveolin-1 palmitoylation occurred in DLD-1 cells pre-treated with the PAT inhibitor, 2-bromohexadecanoic acid (2-Br) (Fig. 2C). These findings indicate that ERβ, like caveolin-1, undergo PAT-dependent palmitoylation.

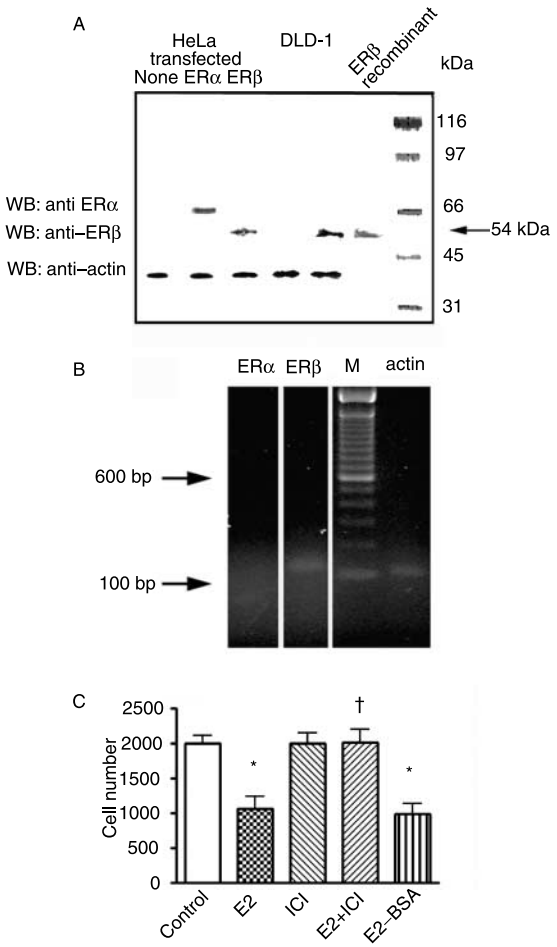
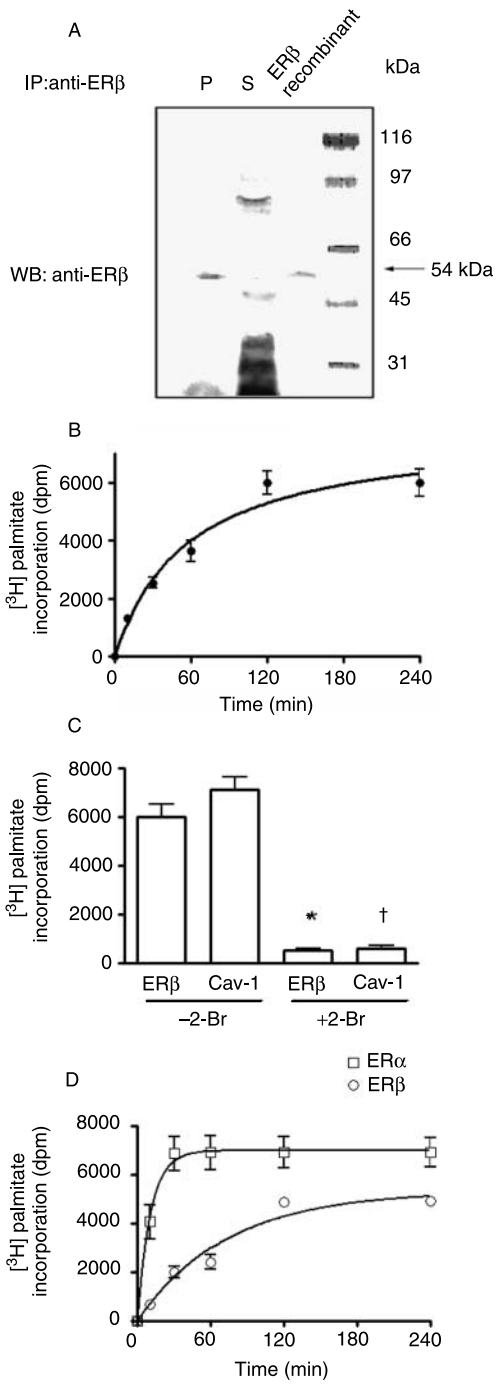


Figure 1 Level of ERs in DLD-1 cells. (A) Western blot analysis of ERα and ERβ levels were performed in DLD-1 and HeLa cells. HeLa cells were untransfected (none) or transfected with ERα or ERβ expression vectors. Fifty four kilodaltons of human ERβ recombinant protein (5 ng, Invitrogen) was used as control. (B) Identification of ERβ mRNA by RT-PCR using specific sense/antisense primers. Amplification products were separated by electrophoresis on 2% agarose and identified by ethidium bromide staining. ERβ = 108 bp; ERα = 248 bp. (C) DLD-1 cells grown for 30 h in the presence of E2 (10 nM) and/or ICI 182 780 (ICI, 1 μM) or E2–BSA (10 nM) and then counted. The data are the mean values ± S.D. of five independent experiments carried out in duplicate. $P < 0.001$, calculated with Student's *t*-test, compared with respective unstimulated values (control) (*) or with E2-stimulated values (†). For details see text.

The time course for [^{3}H]-palmitate incorporation in ERβ was different to that reported for ERα (Acconcia *et al.* 2004). Since, the PATs are a heterogeneous group of enzymes, which differ depending on the cell type (Smotrys & Linder 2004), we compared the kinetics of [^{3}H]-palmitate incorporation in the same cell line. The ERs-devoid human cervix epithelioid carcinoma cell

(HeLa) were transiently transfected with either ERα-or ERβ-encoding vectors and then incubated with [^{3}H]-palmitate for 4 h at 37 °C. After ERα or ERβ immunoprecipitation, the radioactivity present in the supernatant was determined. ERα palmitoylation was very rapid being complete within 10 min and remaining constant over 240 min (Fig. 2D). Kinetics of [^{3}H]-palmitate incorporation in ERβ was slow in both transfected HeLa and DLD-1 cells (Fig. 2, compare panels B and D). This suggests that ERβ is a worse substrate than ERα for PAT.



ERβ palmitoylation is negatively modulated by E2

To assess the ability of E2 to modulate ERβ palmitoylation, DLD-1 cells were incubated with [^{3}H]-palmitate for 4 h in the presence of different E2 concentrations. Physiological E2 concentration (1–10 nM) decreased the amount of [^{3}H]-palmitate incorporated in ERβ by more than half; a higher E2 concentration (i.e., 100 nM) was more efficient to decrease palmitate incorporation in ERβ, whereas hormone lower concentration was ineffective (i.e., 0.1 nM; Fig. 3A). The time course of 10 nM E2 stimulation in DLD-1 cells showed that 60 min of E2 stimulation reduced the [^{3}H]-palmitate incorporation in ERβ by 38% (Fig. 3B) with an increase in the protein level (Fig. 3C). The increase in ERβ levels were also detected 120 and 240 min after E2 stimulation (Fig. 3C), which is in good accordance with data reported in the literature (Chiang *et al.* 2000, Matthews & Gustafsson 2003 and literature therein). The kinetics of E2-induced de-palmitoylation is very similar for both ERs, as demonstrated in transfected HeLa cells (Fig. 3D).

ERβ palmitoylation is necessary for receptor–protein association

Besides, lipid modification, the association with membrane proteins could allow extrinsic proteins to localize at the plasma membrane. After E2 binding, ERα is able to associate to adaptor and/or signaling proteins, which in turn are responsible for signaling cascade activation important for cell proliferation (Greger *et al.* 2006). This prompted us to evaluate the ability of ERβ to interact with some of these signaling proteins, both in the absence and in the presence of 10 nM E2 for 15 min. No association between ERβ and Src or MNAR was observed before

Figure 2 ERβ palmitoylation. (A) Western blot analysis of immunoprecipitated ERβ in the pellet (P) or the supernatant (S). Fifty four kilodaltons of human ERβ recombinant protein (5 ng, Invitrogen) was used as control. (B) Time course of [^{3}H]-palmitate incorporation in immunoprecipitated ERβ in DLD-1 cells. Data are the means of six independent experiments ± S.D. (C) [^{3}H]-palmitate incorporation (120 min) in immunoprecipitated ERβ or caveolin-1 (Cav-1) in the presence or absence of PAT inhibitor 2-Bromo-palmitate (2-Br, 10 μM). (D) Time course of [^{3}H]-palmitate incorporation in HeLa cells transfected with ERα or ERβ expression vectors. ERs were immunoprecipitated and radioactivity counted. Data are the means of four independent experiments ± S.D. of duplicate analyses. For details see text.

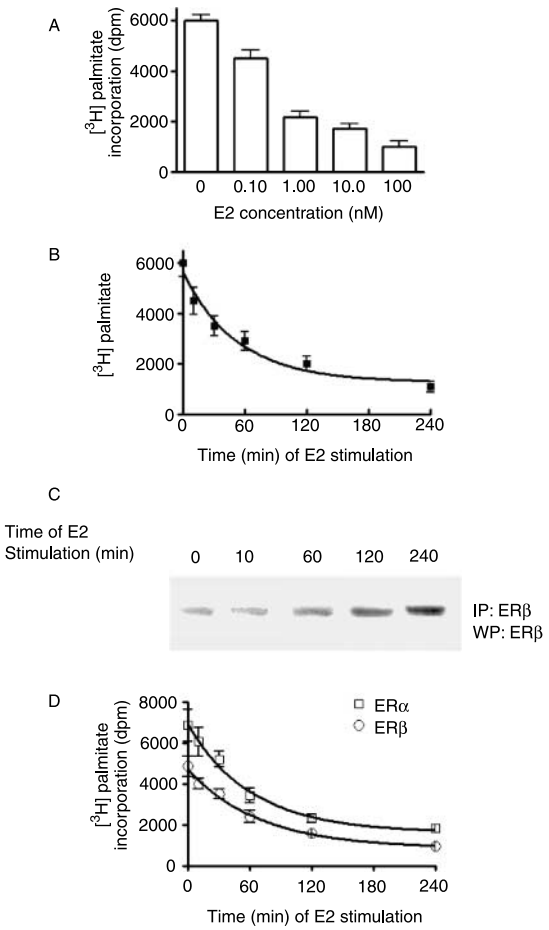


Figure 3 E2 effect on ERβ palmitoylation. (A) Dose-response curve of E2. [^{3}H]-palmitate was incorporated in DLD-1 cells for 120 min, then different E2 concentrations were administrated for further 120 min and ERβ was immunoprecipitated. Data are the means of five independent experiments ±S.D. (B) Time course of E2 stimulation. [^{3}H]-palmitate was incorporated in DLD-1 cells for 120 min, then cells were stimulated with 10 nM E2 at different time and ERβ was immunoprecipitated. Data are the means of five independent experiments ±S.D. (C) Typical Western blot of immunoprecipitated ERβ from DLD-1 cells stimulated with 10 nM E2 at different time. (D) HeLa cells were transfected with human ERα or human ERβ expression vectors. [^{3}H]-palmitate was incorporated in HeLa cells for 120 min, then cells were stimulated with 10 nM E2 at different time and ERβ or ERα were immunoprecipitated. Data are the means of four independent experiments ±S.D. For details see the text.

or after E2 stimulation of DLD-1 cells (Fig. 4A and B, respectively). This result was followed by the inability of ERβ to induce ERK or AKT phosphorylation (Fig. 4C and D, respectively). On the contrary, the association of ERβ with caveolin-1 is present even in the absence of E2 (Fig. 5A and B). This association increase 60 min after E2 stimulation (Fig. 5A and B). Notably, E2-induced ERβ-caveolin-1 association was completely prevented by pre-treatment with the PAT inhibitor, 2-Br (Fig. 5C). No variation in the level of caveolin-1 was present after E2 stimulation (Fig. 5B).

As a whole, these results suggest that ERβ palmitoylation is necessary for receptor localization at the plasma membrane. Immunofluorescence and cell fractionation results are consistent with these results (Fig. 6). Under basal conditions, ERβ is expressed in both membrane and cytoplasm (Fig. 6A, left panel and Fig. 6B), while upon E2 stimulation, the receptor is mainly present at the cell periphery corresponding to the plasma membrane (see the arrows in Fig. 6A, central panel and Fig. 6B). Pre-treatment with the PAT inhibitor abrogates the ability of E2 to re-localizate ERβ to the plasma membrane compartment (Fig. 6A, right panel and Fig. 6B).

ERβ palmitoylation is necessary for E2-induced pro-apoptotic effects

In DLD-1 cells, the rapid, persistent E2-induced activation of the p38/MAPK pathway is sufficient and necessary for E2-induced apoptosis (i.e., caspase-3 activation and PARP cleavage; Marino *et al.* 2006). We next determined whether ERβ palmitoylation could have an impact on these rapid non-genomic ERβ activities. E2 induced the rapid and persistent phosphorylation of p38 in DLD-1 cells (Fig. 7A). We investigated whether a physical association existed between p38 and ERβ by immunoprecipitation. Under basal conditions a complex formed by the unphosphorylated form of p38 and ERβ has been detected (Fig. 7B). After 10 min of E2 treatment, ERβ–p38 association as well as p38 phosphorylation significantly increased (Fig. 7B). However, when the cells were pre-treated with the PAT inhibitor, 2-Br, the E2-induced activation of this signaling kinase was completely blocked even though the basal p38 levels were unaffected (Fig. 7C).

We have recently demonstrated that ER-dependent caspase-3 activation and PARP cleavage are some of the downstream events triggered by E2-induced p38 activation in ERβ-transfected HeLa cells (Acconcia *et al.* 2005*a*). Accordingly, in DLD-1 cells, E2 induced the cleavage of the caspase-3 proform (32-kDa band), resulting in the production of the active subunit of the protease (17-kDa band; Fig. 8A). To confirm that the appearance of the 17-kDa band was associated with an increase in caspase-3 activity, we analysed one of the known substrates of caspase-3, namely PARP. This 116-kDa, DNA repair enzyme, is cleaved by active caspase-3, so produces the inactive 85-kDa fragment. The E2 treatment of DLD-1 cells resulted in the conversion of PARP into the inactive

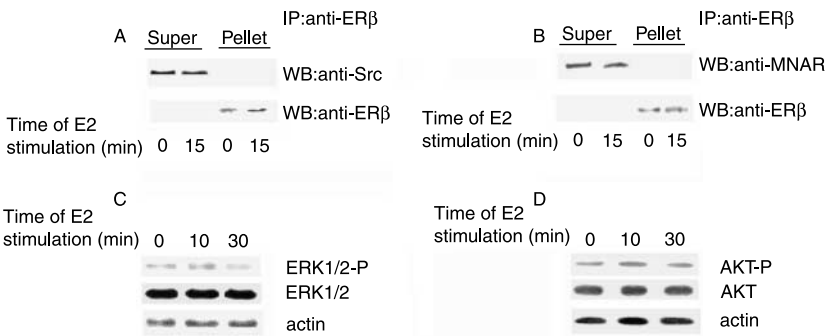


Figure 4 ERβ association to Src and MNAR and activation of ERK/MAPK and PI3K/AKT pathways in DLD-1 cells. Cells were grown in the absence (0) or stimulated for 15 min with 10 nM E2, ERβ was immunoprecipitated with anti-ERβ antibody followed by Western blot with anti-Src (A) or with anti-MNAR (B) and anti-ERβ antibodies. Western blot analysis of ERK (C) and AKT (D) phosphorylation in DLD-1 cells were performed on unstimulated (0) and stimulated cells for 10–30 min with E2 (10 nM). Data represent a typical Western blot of three different experiments. For details see text.

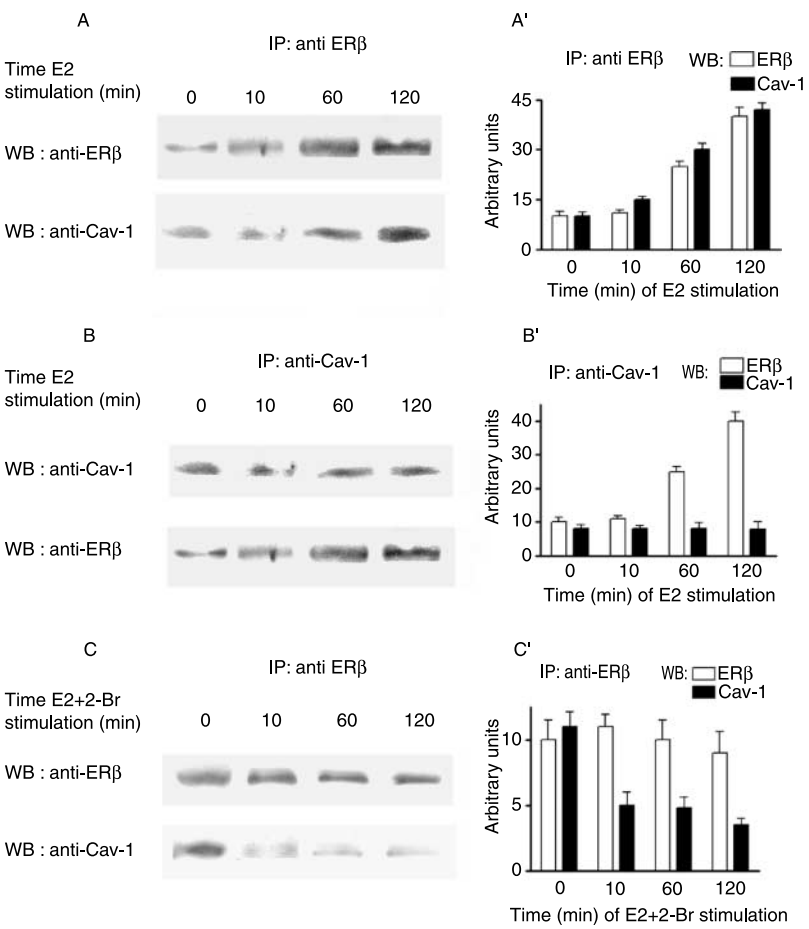


Figure 5 Role of ERβ palmitoylation in ERβ association to caveolin-1. DLD-1 cells were stimulated with 10 nM E2 for different times then were subjected to ERβ immunoprecipitation (A and A′) or caveolin-1 immunoprecipitation (B and B′) followed by Western blot with anti-caveolin-1 or with anti-ERβ antibodies. (C and C′) DLD-1 cells were pre-treated for 30 min with 10 μM PAT inhibitor 2-Br then stimulated with 10 nM E2 for different times and subjected to ERβ immunoprecipitation or caveolin-1 immunoprecipitation followed by Western blot with anti-caveolin-1 or with anti-ERβ antibodies. (A, B, and C) Typical Western blot; (A′, B′, and C′) densitometric analysis of four different experiments. Data are the mean ± S.D. For details see text.

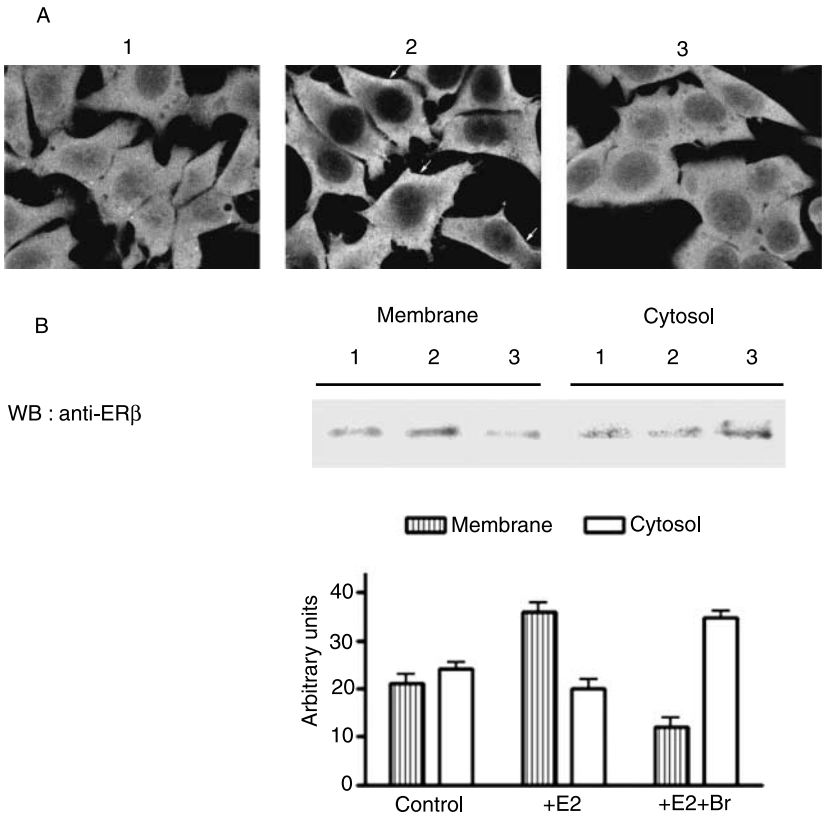


Figure 6 (A) Immuno-fluorescence analysis of unstimulated (left panel), 10 nM E2 stimulated for 120 min (central panel), and pre-treated for 30 min with 10 μM PAT inhibitor 2-Br then stimulated with 10 nM E2 for 120 min (right panel) DLD-1 cells. White arrows indicate the membrane localization of ERβ. (B) Cell fractionation assay of unstimulated (1, control), 10 nM E2 stimulated for 120 min (2, +E2), and pre-treated for 30 min with 10 μM PAT inhibitor 2-Br then stimulated with 10 nM E2 for 120 min (3, +E2+Br) DLD-1 cells. Typical Western blot (upper panel) and densitometric analysis (bottom panel) of four different experiments. Data are the mean±S.D. For details see text.

85-kDa fragment (Fig. 8B). In contrast, neither caspase-3 nor PARP was affected by E2 after the pre-treatment of DLD-1 cells with the PAT inhibitor 2-Br or with the p38 inhibitor SB 203 580 (Fig. 8). Notably, no changes in the pro-apoptotic cascade were detected after treatment with both inhibitors, when used alone. Thus, palmitoylated ERβ must mediate the E2-induced p38 and caspase-3 activation as well as PARP cleavage. These findings demonstrate the critical role played by palmitoylation in ERβ-mediated anti proliferative E2-induced effects.

ERβ palmitoylation is not necessarily essential for E2-induced transcriptional activity

We previously demonstrated that ERα palmitoylation is required for E2-induced gene transcription (Acconcia *et al.* 2005*b*). Since, we wanted to compare the ERα and ERβ transcriptional activity in the same cellular context, the ERs devoid HeLa cells were used as experimental model. HeLa cells were co-transfected with the ERE containing pC3 promoter and ERβ or ERα expression vectors. As expected, in HeLa cells transfected with ERα, the E2 treatment induced a three- and a two-fold increase of pC3 and pD1 promoter activities, respectively (Fig. 9). On the other hand, in ERβ-transfected HeLa cells E2 induced a twofold increase only on the pC3 promoter activity (Fig. 9). Notably, the pre-treatment of ERα or ERβ-transfected HeLa cells with the PAT inhibitor 2-Br reduced the E2-inducible pC3 promoter activity by 30% without affecting the basal pC3 promoter activity (Fig. 9). As expected, the ERα palmitoylation has a powerful effect on the E2-induced cyclin D1 promoter activity, which is totally impaired by 2-Br treatment, whereas ERβ was unable to mediate cyclin D1 promoter activity, both in the presence or absence of 2-Br.

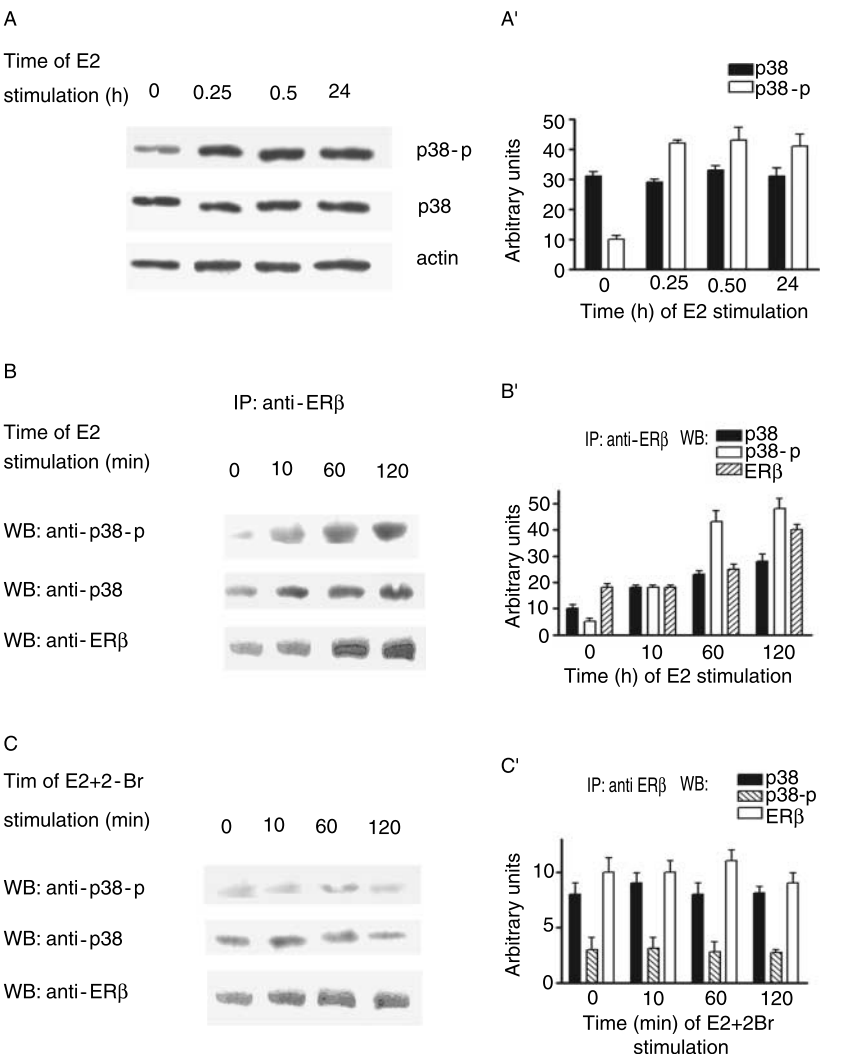


Figure 7 Role of ERβ palmitoylation on p38/MAPK activation. (A and A′) Time course analysis of p38/MAPK phosphorylation was performed on untreated (0) and E2-treated (10 nM) DLD-1 cells at the indicated times. The amount of protein levels were normalized by comparison with actin expression. (B and B′) DLD-1 cells were stimulated with 10 nM E2 for different times then were subjected to ERβ or p38 immunoprecipitation followed by Western blot with anti-p38 or anti-p38 phosphorylated (p38-P) or anti-ERβ antibodies. (C and C′) DLD-1 cells were pre-treated for 30 min with 10 μM PAT inhibitor 2-Br then stimulated with 10 nM E2 for different times and subjected to ERβ or p38 immunoprecipitation followed by Western blot with anti-p38 or anti-p38 phosphorylated (p38-P) or anti-ERβ antibodies. (A, B, and C) Typical Western blot; (A′, B′, and C′) densitometric analysis of four different experiments. Data are the mean±S.D. For details see text.

Discussion

Recently, various studies have shown decreased expression of ERβ mRNA and protein (or an increased ERα/ERβ mRNA ratio) in cancer vs normal tissues in many tumors, including breast, ovary, colon, and prostate (Foley *et al.* 2000, Campbell-Thompson *et al.* 2001, Roger *et al.* 2001, Fixemer *et al.* 2003, Bardin *et al.* 2004). Moreover, ERβ gene is localized on chromosome 14q (see Ascenzi *et al.* 2006 and references therein), the loss of which has been detected in breast, ovarian, prostate, and colon cancers (Young *et al.* 1993, Bandera *et al.* 1997, Loveday *et al.* 2000, Kasahara *et al.* 2002). These overall findings suggest a potential tumor-suppressive function for ERβ (Iwao *et al.* 2000) that opposes the effects mediated by ERα–E2 complex, which drives cells to proliferation. Thus, the same hormone elicits different effects on cell proliferation by binding to different receptors.

Then, which are the molecular mechanisms underlying the above opposite effects? Like most other members of the nuclear receptor family, ERs have a modular architecture of four interacting domains: the N-terminal A/B domain, the C or DNA binding domain

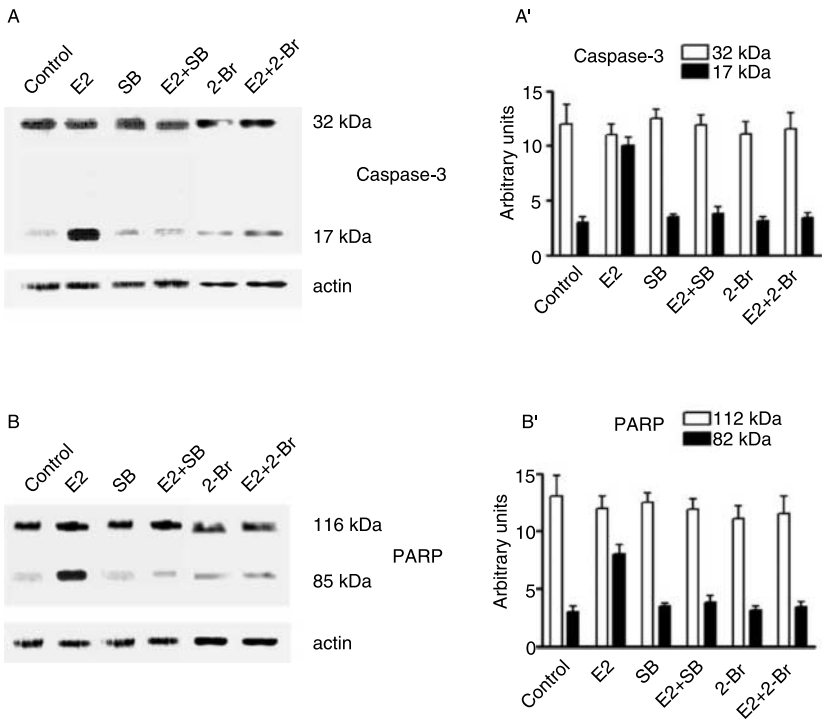


Figure 8 Role of ERβ palmitoylation on p38-dependent pro-apoptotic cascade activation. (A and A′) Western blot analysis of caspase-3 and PARP (panel b) activation were performed on unstimulated or 24 h E2-treated (10 nM) DLD1 cells. When indicated 5 μM p38/MAPK cascade inhibitor SB 203 580 (SB) or 10 μM PAT inhibitor 2-Br was added 30 min before E2 administration. The amount of protein levels were normalized by comparison with actin expression. (A and B) Typical Western blot; (A′ and B′) densitometric analysis of five different experiments. Data are the mean ± S.D. For details see text.

(DBD), the D or hinge domain and the C-terminal E/F or LBD (Marino *et al.* 2005, Ascenzi *et al.* 2006). The homology in the DBD is 97% (Ascenzi *et al.* 2006), which suggests that ERα and ERβ are capable of regulating gene transcription through a classical mechanism involving the consensus ERE element, but ERβ seems to be a weaker transactivator (Cowley & Parker 1999).

To date, only a limited number of genes have been shown to be regulated by this classical mode of action. E2 also transcriptionally regulates target genes via ERs though a non-ERE mode of action. These effects are mediated through promoter elements that bind various transcription factors (e.g., AP-1-binding sites and Sp1-binding sites; Webb *et al.* 1998, Porter *et al.* 1997). At these sites, ERα and ERβ could have opposite transcriptional effects in some circumstances. In fact, ERβ is not able to potentiate an AP-1-containing reporter in the presence of E2 in a tissue-specific manner (Paech *et al.* 1997). In addition, E2 interaction with ERβ does not result in the formation of a transcriptionally active complex at a promoter containing Sp1 elements (Saville *et al.* 2000). More significant differences are reported between ERβ and ERα actions with respect to their ability to activate rapid E2-induced signals. The activation of ERK/MAPK, PI3K/AKT, and PKC, rapidly generated after E2 binding to ERα in different cell lines, are all defined as necessary and sufficient for E2-induced G1 to S phase progression, to increase survival pathways (e.g., Bcl-2), and to regulate the transcription of AP-1- and Sp1-dependent genes important for cell cycle modulation (e.g., cyclin D1). Interestingly, the transcriptional activity of the E2–ERα complex could be inhibited by pre-treating cells with the ERK inhibitors PD98059 and U0126 (Levin 2005, Marino *et al.* 2005).

Limited, conflicting data are reported for ERβ-mediated rapid signals. A subpopulation of ERβ transfected in CHO cells is capable of activating IP_3 production, ERK/MAPK and c-Jun kinase phosphorylation (Razandi *et al.* 1999). Geraldes and coworkers reported that E2 reduces ERK/MAPK activity through ERβ stimulation in porcine smooth muscle cells (Geraldes *et al.* 2003). Moreover, contradictory evidence on the ability of ERβ to activate or inactivate Src and p38 kinases have also been reported (Castoria *et al.* 2001, Kousteni *et al.* 2001, Geraldes *et al.* 2003, Mori-Abe *et al.* 2003). We recently reported the ERβ–E2

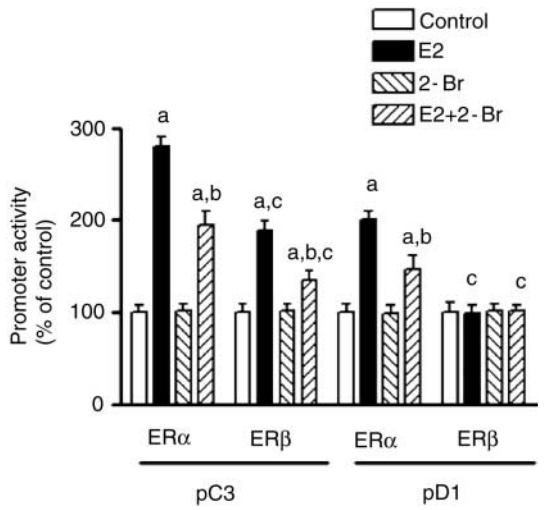


Figure 9 Role of ERs palmitoylation on the E2-induced genomic activity in HeLa cells. Cells were co-transfected with human ERβ or human ERα expression vectors together with pC3-luciferase (pC3) or pD1-luciferase (pD1) constructs and pre-treated 30 min with 10 μM PAT inhibitor 2-Br before E2 administration (10 nM for 6 h). The data are the mean values ±S.D. of five different experiments. $P<0.001$ was calculated with Bonferroni's test: a, significantly different from control value (open bar); b, significantly different from E2 stimulated samples; and c, significantly different from ERα transfected HeLa cells.

complex ability to activate p38/MAPK in the ER-devoid HeLa cells transiently transfected with the ERβ expression vector (Acconcia *et al.* 2005*b*). The discrepancies reported in these studies could be due to the different cellular models utilized, in which ERβ is over-expressed, or both ERα and ERβ are co-expressed, or different ERβ splicing forms could even be present. This further enhances the complexity in the spectrum of potential cellular responses to E2.

Here, using colon cancer cells which contain only one ERβ isoform, we demonstrated that ERβ is localized to the plasma membrane and originate rapid signal transduction cascades important for anti-proliferative effects of E2. In fact, we prove that ERβ undergoes PAT-dependent palmitoylation even if this isoform is a poor substrate for PAT as compared to ERα (Fig. 2). In addition, the localization to the membrane is dependent on ERβ palmitoylation since the PAT inhibitor impairs ERβ from localizing at the membrane and from interacting with caveolin-1. Similar to that reported for ERα is the time- and concentration-dependent negative regulation of ERβ palmitoylation exerted by E2 (Fig. 3).

Palmitoylation function must be considered more than a simple membrane association of otherwise soluble proteins. In fact, the palmitoylation status of several proteins has also been linked to their activation and their movement within membrane subdomains. As an example, de-palmitoylation of eNOS increases in response to treatment of cells with bradykinin (Robinson *et al.* 1995) and de-palmitoylation has a subtle effect on membrane distribution of G-proteins affecting their partitioning within membrane subdomains (Smotrys & Linder 2004). Thus, palmitate addition is a dynamic modification that is continually turning over on cellular proteins. ERβ and ERα do not contain a trans-membrane domain (Zhang *et al.* 2004) or consensus sequences for miristoylation or prenylation (Acconcia *et al.* 2003), thus their ability to associate with scaffolding or/and signaling proteins at the plasma membrane seems principally due to palmitoylation (Acconcia *et al.* 2005*b*, Levin 2005). In the resting state, ERβ is localized mainly in the cytosol and nucleus of DLD-1 cells and only a little amount of receptor is tethered with caveolin-1 (Fig. 5). After E2 stimulation, ERβ undergoes de-palmitoylation, which increases receptor–caveolin-1 association (Fig. 5) and, thus, its presence at the plasma membrane (Fig. 6). E2 stimulation of ERα-containing cells decreases receptor palmitoylation with a kinetics similar to that reported for ERβ (Fig. 3). The E2-dependent de-palmitoylation decreases ERα–caveolin-1 association allowing ERα association with adaptors and/or signaling proteins (e.g., MNAR, Src, tyrosine kinase receptors), which in turn give rise to rapid signaling cascades (e.g., MAPK and PI3K; Levin 2005, Marino *et al.* 2005, Song *et al.* 2005, Leclercq *et al.* 2006). This does not occur in the presence of ERβ. It has been reported that intact A/B domain and tyrosine 537 in E domain of ERα are both required for receptor interaction with Src in the MNAR–ERα–Src complex and the *in vitro* association between ERβ and MNAR has been reported (Barletta *et al.* 2004, Greger *et al.* 2006). Although ERβ possess a tyrosine residue at 488, which could be subjected to phosphorylation, the ERα and ERβ A/B domain differ in both length and amino acid sequence, exhibiting a low amino acid identity (Ascenzi *et al.* 2006). Moreover, present data indicate that no association between ERβ and MNAR and Src was present in DLD-1 cells before and after E2 stimulation (Fig. 4). On the other hand, E2 increased ERβ level (Chiang *et al.* 2000, Matthews & Gustafsson 2003 and present data) and its association with caveolin-1 (Fig. 5). As a whole, these data raise the intriguing possibility that the short A/B domain of ERβ could facilitate the E2-induced association between ERβ and caveolin-1, impairing its association with MNAR and Src. As a consequence, ERK and AKT

activation does not occur. Experiments in our laboratory are in progress to better define this pathway.

ERK/MAPK as well as PI3K/AKT cascades co-operate in ERα–E2 induced cell proliferation and cell survival, enhancing the expression of the anti-apoptotic protein (Bcl-2) and promoting the G1/S transition via the enhancement of cyclin D1 expression (Marino *et al.* 2002, 2003). In addition, the E2–ERα complex rapidly increased p38/MAPK phosphorylation but the contemporary increase of Bcl-2 levels, mediated by ERK/MAPK and PI3K/AKT pathways, impairs the prolonged p38 activation and the downstream effects of this kinase (Acconcia *et al.* 2005*b*). On the contrary, the rapid increase of p38 phosphorylation induced by the E2–ERβ complex is not modulated by ERK/MAPK and PI3K/AKT pathways, thus a more prolonged p38 phosphorylation occurs (Acconcia *et al.* 2005*b*; Fig. 6).

The ERβ palmitoylation is important for E2-induced cell functions. In fact, ERβ–p38 association and E2-induced prolonged activation of this kinase is prevented by the PAT inhibitor 2-Br (Fig. 6). Moreover, ERβ palmitoylation is necessary for the p38-dependent activation of downstream pro-apoptotic cascade, which involves the caspase-3 activation and PARP cleavage (Fig. 7). To evaluate the impact of palmitoylation on E2-transcriptional effects, we compared ERβ and ERα in a cellular context, which contained the same co-activators. As expected, ERβ is a weaker transactivator than ERα. The palmitoylation of ERβ scarcely influences ERβ genomic activities (i.e., ERE-containing gene promoter transcription). Similar results were obtained in ERα-containing cells, indicating that the rapid palmitoylation-dependent signal transduction pathways are important for the complete transcriptional activity of ERs. This could be due to the ability of such cytosolic signals to modulate the co-activator recruitment and the chromatin activation status (Smith & O'Malley 2004). ERβ and ERα have opposite effects in mediating the E2-induced transcription of cyclin D1 (i.e., non-ERE-containing gene promoter). As expected, ERβ was unable to induce cyclin D1 promoter transcription, whereas a critical requirement of ERα palmitoylation for cyclin D1 promoter transcription was present.

In conclusion, present findings indicate that palmitoylation localizes ERβ at the plasma membrane, and this even if only in little quantity, directs several E2 effects, which allow the protective effect of this hormone in colon cancer. These data, showing the molecular mechanism, which rapidly follows E2 entry in ERβ-containing cells, further sustain the tumor suppressor function played by this receptor isoform. Although ERα is palmitoylated, the outcome effects in cell physiology are opposite to that reported for ERβ. Thus, the expression of each ER isoform and/or their co-expression in the cells could account for the different E2-dependent modulation of cell proliferation reported.

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