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17β-Estradiol induces ERβ up-regulation via p38/MAPK activation in colon cancer cells

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Abstract

Estrogen receptors (ERα and ERβ) mediate opposite functions on cancer growth induced by 17β-estradiol (E2). E2 binding to ERα induces a cancer promoting response, whereas E2 binding to ERβ exerts a protective action against cancer growth. Moreover, E2 can diversely modulate the ERα and ERβ levels intensifying or decreasing their actions in target tissues. Only molecular mechanisms at the root of E2 ability to down-regulate the ERα levels are known. Here, we report the first molecular mechanism underlying E2-induced ERβ up-regulation in DLD-1 colon cancer cells. E2 induces a short term (2 and 4 h after stimulation) translation of ERβ mRNA followed by a late (24 h after stimulation) enhanced transcription. Both processes required the E2-induced persistent and palmitoylation-dependent p38/MAPK activation. Overall, our data suggest a finely tuned control exerted by rapid signals on different cellular molecular events important for the protective effects of E2 against colon cancer growth.

Keywords: 17β-Estradiol; Estrogen receptor β; Non-genomic signals; p38/MAPK; Colon cancer cells

Besides their classical role in sexual development and function, estrogen receptors (ERα and ERβ) mediate 17β-estradiol (E2) functions in many non-reproductive tissues [1]. Knockout mice analysis showed that ERα and ERβ have markedly different tissue distribution [2] and mediate diverse E2 effects [3,4]. Evidences accumulated suggesting ERα as mediator of the E2-induced cancer promoting response in several target cells [5–7], whereas the loss of ERβ (the predominant ER subtype expressed in human colon) is associated with advanced stages of colon cancer and tumor cell de-differentiation, thus suggesting its protective role in colon cancerogenesis [8–13].

In cancer progression, the analysis of the various roles played by ER isoforms, is further complicated by the fact that E2 can modulate ERα and ERβ expression intensifying or decreasing their actions in target tissues. E2 administration to ovariectomized rats reduces the uterine levels of ERα by approximately 60% [14] and E2 exposure down-regulates the steady-state level of ERα in MCF-7 cells, as reflected by the protein half-life decrease [15,16]. The potential E2-dependent regulation of ERβ levels, which can play a fundamental role in the control of cell proliferation, is less known although a time-dependent E2-induced increase in ERβ mRNA has been observed in the human cell lines of breast cancer T47D [4,17], prostate cancer DU145 [18], and colon cancer DLD-1 [13].

E2 exerts its powerful effects on cell physiology mainly by regulating gene transcription, even if post-transcriptional regulation of gene expression (including mRNA translation) represents a mechanism to control and modify the flow of genetic information into the proteome [19]. This latter system is commonly used by E2 to auto-regulate the expression of the mRNAs that encode its own receptor proteins [20], but the molecular mechanisms involved are unknown.

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The discovery of membrane-associated ER pools capable of eliciting both genomic and non-genomic responses [1,21–24] have led to a deep re-evaluation of the mechanisms underlying E2 action. Recent investigations have revealed that ERs are highly mobile proteins continuously shuttling between the plasma membrane and other cellular compartments [22,25,26]. Ligand- and/or protein-induced ER conformational changes regulate such movements leading to specific responses. Receptor anchorage at specific sites as well changes in receptor levels could be determined by these shuttling mechanisms [25]. Indeed, we previously demonstrated that in human DLD-1 colon cancer cells, ERβ palmitoylation was necessary for its localization at the plasma membrane and to associate with caveolin-1 and the p38 member of MAPK family [13]. Moreover, the palmitoyl acyl transferase (PAT) inhibitor 2-bromohexadecanoic acid (2-Br) blocked 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 PARP cleavage) [13].

In order to verify the hypothesis that E2 might triggers such rapid, non-genomic action by the up-regulation of ERβ levels, we used the DLD-1 model system, devoid of ERα [13], and demonstrated that E2 rapidly increases ERβ levels via the palmitoylation-dependent persistent p38/MAPK activation. In turn, p38/MAPK yields both rapid translational and slow transcriptional mechanisms able to maintain high level of ERβ important for the E2 anti-proliferative effects.

Materials and methods

Reagents. E2, gentamicin, penicillin, RPMI1640 (without phenol red), charcoal-stripped fetal calf serum, the PAT inhibitor 2-bromohexadecanoic acid (2-Br), actinomycin, and cycloheximide were purchased from Sigma–Aldrich (St. Louis, MO, USA). The p38/MAPK inhibitor SB-203580 (SB) and the proteasome inhibitor MG-132 were obtained from Calbiochem (San Diego, CA, USA). The ER inhibitor ICI-182780 (ICI) was obtained from Tocris (Ballwin, MO, USA). Bradford Protein Assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). The anti-β-actin and the anti-ERβ L20 (C-terminus) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-ERβ-14C8 (N-terminus) antibody was purchased from Genetex (San Antonio, TX, USA). The anti-phospho-p38 and anti-p38 antibodies were obtained from New England Biolabs (Beverly, MA, USA). The ECL chemiluminescence reagent for Western blot was obtained from Amersham Biosciences (Little Chalfont, UK).

All the other products were from Sigma–Aldrich (St. Louis, MO, USA). Analytical or reagent grade products, without further purification, were used.

Cell culture and counting. Human DLD-1 colon adenocarcinoma cells [11] were routinely grown in air containing 5% CO_2 in modified phenol red-free RPMI1640 medium, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml).

DLD-1 cells were grown to ~70% confluence in 6-well plates and then stimulated. After treatment (10 nM E2, 1 μM ICI-182780, 5 μM SB-203580, 10 μM 2-Br), cells were harvested by trypsinization, centrifuged, stained with Trypan blue solution, and counted in a hemocytometer (improved Neubauer chamber) in quadruplicate.

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, Carlsbad, CA, USA). The following primers were used: for human ERα (ESR1/NR3A1, GenBank Accession No. AY425004), 5′-TCCTAGCAGGGA GATGAGGA-3′ (forward) and 5′-CCTTTATGGCCAGCAATCAT-3′ (reverse), for human ERβ (ESR2/NR3A2, GenBank Accession No. AY785359), 5′-GGCGCGATCTTGGCTCAC-3′ (forward) and 5′-TGGCTGGACGTGGTGGCA-3′ (reverse), and for β-actin (GenBank Accession No. X00351), 5′-AGAAGGATTCCTATGTGGGCG-3′ (forward) and 5′-CATGTCGTCCCAGTTGGTGAC-3′ (reverse).

Total RNA was extracted from DLD-1 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To determine hERβ gene expression levels (as well to confirm the absence of hERα), cDNA synthesis and qPCR were performed using a one-step qRT-PCR kit ("SuperScript™ III Platinum®—SYBR® Green One-Step" kit; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis and qPCR were 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 Polymerase 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 2% agarose gel electrophoresis. Each sample was tested in duplicate and the experiment repeated 4 times.

Electrophoresis and immunoblotting. After treatments, 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] and finally boiled for 2 min. Total proteins were quantified using the Bradford Protein Assay. Solubilized proteins (20 μg) were electrophoretically resolved by 10% SDS–PAGE (100 V, 1 h, 24 °C) and then transferred to nitrocellulose (100 V, 45 min, 4 °C). The nitrocellulose membrane 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-p38 or anti-ERβ antibodies. The nitrocellulose membrane 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-p38 or anti-β-actin antibodies. Antibody reactions were visualized by chemiluminescence with the Western blotting detection reagent.

Results

As already reported, human DLD-1 colon adenocarcinoma cells contain only one ERβ isoform corresponding to a 54 kDa protein [11,13]. The time course of DLD-1 stimulation by E2 (10 nM) showed that ERβ protein levels increased from 1 to 24 h (Fig. 1A). The E2-induced ERβ increase was ER-dependent since it was prevented by a pre-treatment with the pure antiestrogen ICI (Fig. 1A). The dose-dependent effect showed that, after 2 h of stimulation, 1 nM E2 induces ERβ up-regulation with a peak at 10 nM. No further increase was detectable at higher E2 concentration (data not shown). To verify that the E2-induced ERβ up-regulation was independent of a block in protein degradation, DLD-1 was stimulated with 10 nM E2 in the presence or absence of the proteasome inhibitor MG-132 (15 μM). E2 or MG-132 stimulation induced a similar increase of ERβ levels, while the co-treatment was additive (Fig. 1B). This data indicates that the E2 effect is not mediated by a block of proteasomal protein degradation.

We next evaluated the mechanisms underlying this E2 effect assessing the involvement of E2-induced genomic

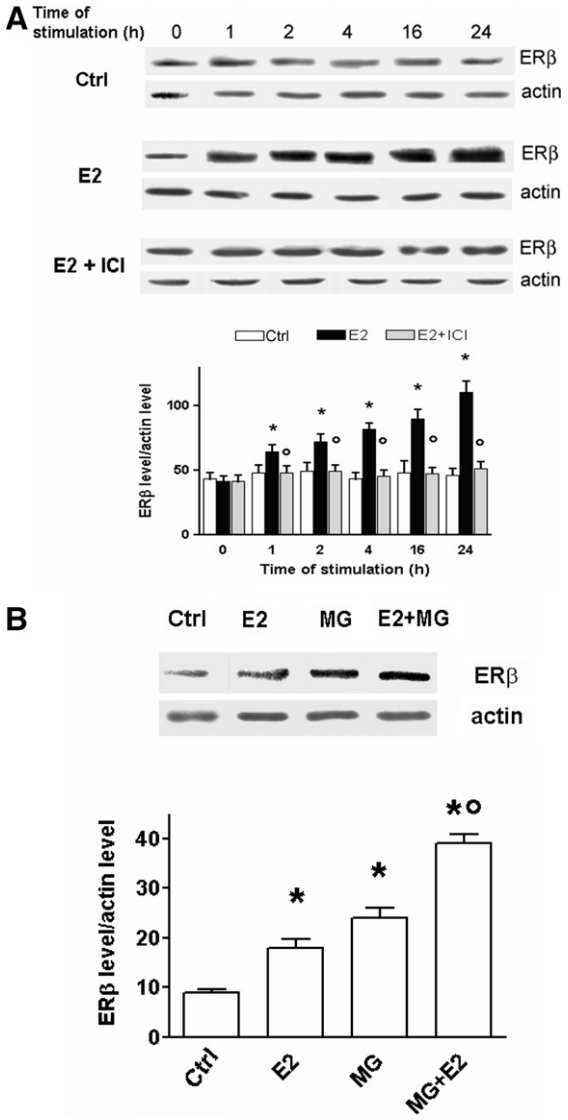

Fig. 1. ERβ levels upon E2 treatment of DLD-1 cells. (A) Western blot analysis of ERβ was performed on cells treated with vehicle (Ctrl), E2 (10 nM) or E2 (10 nM) + ICI (1 μM) in DLD-1 cells at the indicated times. β-Actin expression was used for protein level normalization (upper panel). Densitometric analysis of four different experiments. Data are mean values ± SD. $P < 0.001$, calculated with Student's t test, compared with vehicle stimulated values (Control, Ctrl) (*) or with E2-stimulated values (°) (lower panel). (B) Western blot analysis of ERβ was performed on cells treated for 4 h with vehicle (Ctrl), E2 (10 nM) or E2 (10 nM) + MG-132 (15 μM) in DLD-1 cells. β-Actin expression was used for protein level normalization (upper panel). Densitometric analysis of four different experiments. Data are mean values ± SD. $P < 0.001$, calculated with Student's t test, compared with vehicle stimulated values (Ctrl) (*) or with E2- or MG-132-stimulated values (°) (lower panel). For details see text.

mechanisms. After pre-treating DLD-1 cells with actinomycin, a well-known inhibitor of transcription, the increased ERβ protein level was still observed 2 and 4 h after E2 stimulation, but was completely prevented 24 h after hormone stimulation (Fig. 2A). RNA expression analysis by qRT-PCR confirmed that the E2-induced ERβ gene transcription occurs only 24 h after stimulation. In fact, E2 increased the ERβ mRNA levels only after long incubation time, whereas at shorter stimulation time (i.e., 2 and 4 h) a decrease of ERβ mRNA levels was detected (Fig. 2B). This suggests that the rapid E2-induced ERβ up-regulation could be dependent on the rapid induction of ERβ mRNA translation. Indeed, DLD-1 cells pre-treatment with cycloheximide, a well-known translation inhibitor, completely prevented the rapid E2-induced ERβ protein up-regulation (Fig. 2C).

Thus, we verified the putative role played by E2-induced rapid signal transduction mechanisms. In DLD-1 cells, as already reported, E2 induces the rapid (15 min) and persistent (24 h) activation of p38/MAPK (Fig. 3A) which is completely prevented pre-treating cells with the ER inhibitor ICI (Fig. 3A). The addition of palmitate is necessary for ERβ localization at the plasma membrane and its association with caveolin-1 and p38/MAPK. Moreover, the PAT inhibitor 2-Br 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 PARP cleavage) [13]. Such results are confirmed here since pre-treatment of DLD-1 cells with 2-Br completely prevented the E2-induced rapid and persistent p38/MAPK phosphorylation (Fig. 3A), strongly indicating that a membrane-localized ERβ is required for the E2-dependent p38/MAPK activation.

Furthermore, a membrane-localized ERβ as well the rapid E2-induced p38/MAPK activation are required for the rapid E2-induced ERβ up-regulation. Indeed, just 2 h of pre-treatment with either the palmitoylation or the p38/MAPK inhibitor is sufficient to influence the E2-induced ERβ up-regulation because receptor levels were maintained at control values (Fig. 3B). Interestingly, these inhibitors impaired the E2-induced ERβ up-regulation even after 24 h of stimulation (Fig. 3B). This result was further confirmed by the decrease in the E2-induced ERβ mRNA level occurring 24 h after palmitoylation and p38/MAPK inhibitor pre-treatment (Fig. 3C).

Finally, we also evaluated whether high levels of ERβ were necessary for the E2-induced DLD-1 cell growth decrease. As a matter of fact, DLD-1 cells pre-treated with either the ERβ inhibitor (i.e., ICI-182780, data not shown) or the inhibitors of E2-induced ERβ over-expression (i.e., 2-Br and SB-203580, Fig. 4) completely abrogated the E2 effect on cell growth.

Discussion

At present, the rapid activities of ERs are widely accepted and previous disagreement on their existence is quite settled. However, other controversies in this field are still present, related to whether or not all of these rapid effects are of physiological relevance [27]. Nevertheless, the physiological role played by rapid membrane-starting pathways has been clarified at least for some E2 effects. It is assumed that the E2 proliferative effects are exerted via ERα-dependent rapid membrane-starting actions

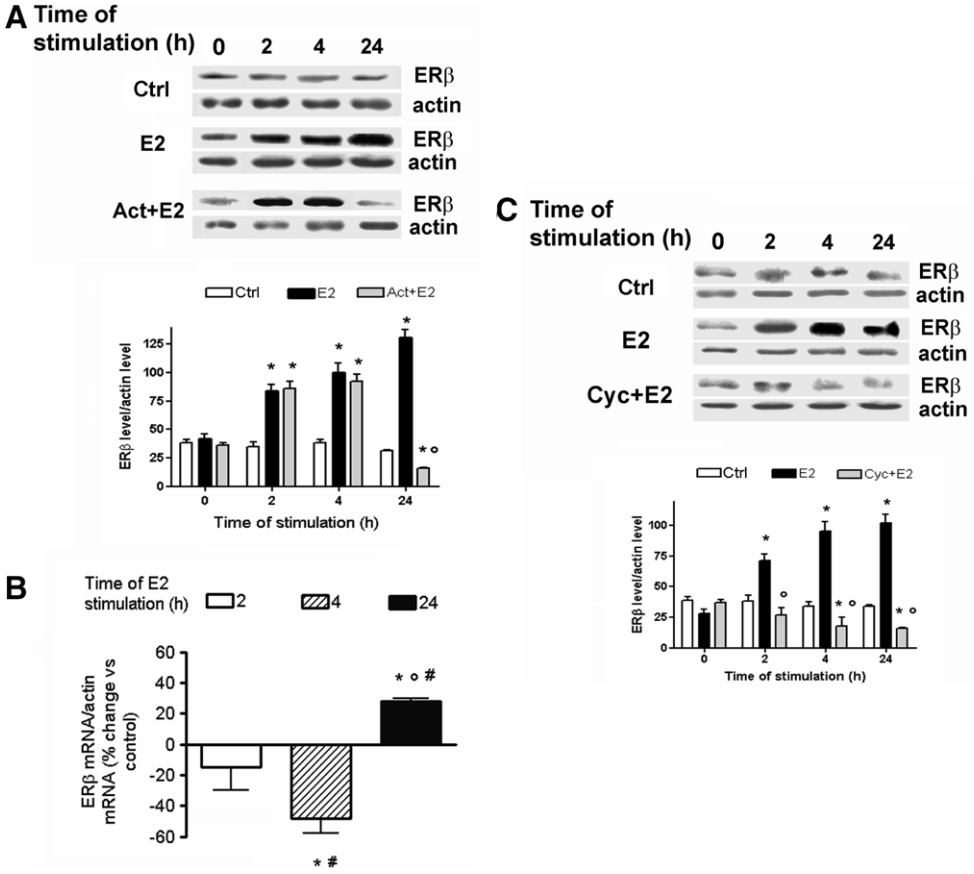

Fig. 2. Mechanisms involved in E2-induced ERβ expression. (A) Western blot analysis of ERβ was performed on cells treated with vehicle (Ctrl), E2 (10 nM) or E2 (10 nM) + actinomycin (Act, 1 μg/ml) in DLD-1 cells at the time indicated. β-Actin expression was used for protein level normalization (upper panel). Densitometric analysis of four different experiments. Data are mean values ± SD. $P < 0.001$, calculated with Student's t test, compared with vehicle stimulated values (Ctrl) (∗) or with 4 h Act + E2-stimulated values (°) (bottom panel). (B) qRT-PCR analysis was performed on total RNA extracted from DLD-1 cells treated with E2 (10 nM) at the time indicated. ERβ mRNA levels are expressed as % change versus vehicle stimulated samples. Data are mean values ± SD of four different experiments. $P < 0.001$, calculated with Student's t test, compared with vehicle stimulated values (∗) or with 2 h E2-stimulated values (#) or with 4 h E2-stimulated values (°). (C) Western blot analysis of ERβ was performed on cells treated with vehicle (Ctrl), E2 (10 nM) or E2 (10 nM) + cycloheximide (Cyc, 10 μg/ml) in DLD-1 cells at the indicated times. β-Actin expression was used for protein level normalization (upper panel). Densitometric analysis of four different experiments. Data are mean values ± SD. $P < 0.001$, calculated with Student's t test, compared with vehicle stimulated values (Ctrl) (∗) or with E2-stimulated values (°) (bottom panel). For details see text.

[5–7,28]. What is the contribution of ERβ-dependent signals to E2-induced cell proliferation?

ERβ appears to act as a dominant regulator in E2 signaling, and when co-expressed with ERα it causes a concentration-dependent reduction of ERα-mediated transcriptional activation [29] and the repression of ERα-mediated effects including cell proliferation [30,31]. Moreover, ERβ has been reported to rapidly induce a persistent membrane-initiated activation of p38/MAPK without any interference on survival/proliferative pathways, thus impairing the activation of cell cycle components (i.e., cyclin D1 expression) [32]. Recently, it has been demonstrated that this rapid signal transduction pathway is important for the E2-induced protective effects against colon cancer cell proliferation [11,13].

Here, we reported for the first time that rapid and prolonged E2-induced p38/MAPK activation is also important for the up-regulation of ERβ levels in DLD-1 colon cancer cells. The E2-induced p38/MAPK activation is crucial to rapidly increase the level of ERβ even in the absence of ERβ gene transcription as demonstrated by actinomycin effect and mRNA levels. In addition, p38/MAPK activation is also required for the initiation of transcription. The mRNAs encoding hormone receptors are commonly regulated by their own hormones to create auto-regulatory feedback loops. Moreover, different hormones, including steroid hormones, regulate concentrations of various gene products primarily by altering mRNA translation and stability [19 and literature cited therein]. Our data demonstrate that E2-induced p38/MAPK is fundamental both for the rapid increase of ERβ mRNA translation and for the slow ERβ gene transcription. The final consequence is an increased level of ERβ which, in the presence of E2, will further increase the hormone protective effect against tumour growth. Indeed, in the presence of the p38/MAPK inhibitor the ERβ levels remain similar to the control and the E2-induced DLD-1 cell number reduction is completely prevented. These results reinforce the interpretation of a

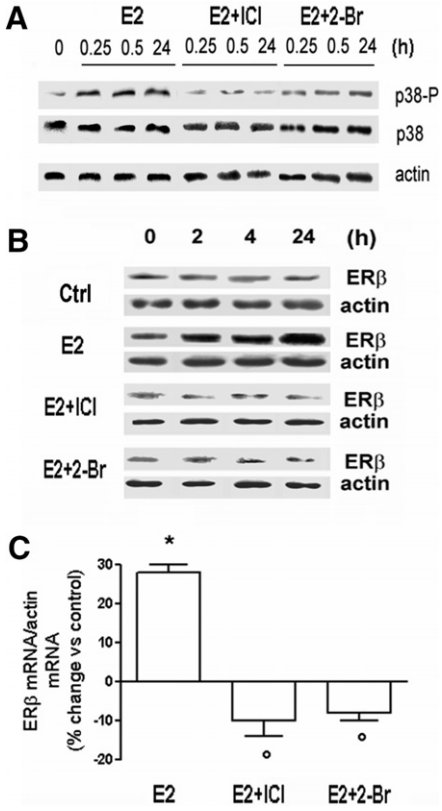

Fig. 3. Involvement of E2-induced p38/MAPK activation in ERβ levels. (A) Analysis of p38/MAPK phosphorylation was performed on DLD-1 cells treated with vehicle (Ctrl) or E2 (10 nM), E2 (10 nM) + ICI-182780 (1 μM) or E2 (10 nM) + 2-Br (10 μM) at the time indicated. β-Actin expression was used for protein level normalization. Representative Western blot of three different experiments. (B) Time course analysis of ERβ level expression was performed on cells treated with vehicle (Ctrl), E2 (10 nM), E2 (10 nM) + 2-Br (10 μM) or E2 (10 nM) + SB 203,580 (5 μM) at the time indicated. β-Actin expression was used for protein level normalization. Representative Western blot of three different experiments. (C) qRT-PCR analysis was performed on total RNA extracted from DLD-1 cells treated with E2 (10 nM), E2 (10 nM) + 2-Br (10 μM) or E2 (10 nM) + SB-203580 (5 μM) for 24 h. ERβ mRNA levels are expressed as % change versus DLD-1 cells treated with vehicle. Data are mean values ± SD of four different experiments. $P < 0.001$, calculated with Student's t test, compared with vehicle stimulated values (*) or with E2-stimulated values (°).

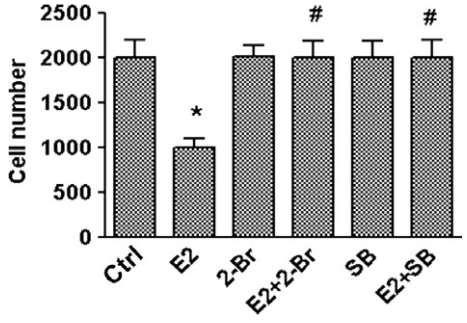

Fig. 4. E2 effect on DLD-1 cell growth. Cells were treated with E2 (10 nM) or 2-Br (10 μM) or SB-203580 (5 μM) for 30 h. Cells were then harvested and counted in a hemocytometer with Trypan Blue solution. Data are mean values ± SD of four different experiments. $P < 0.001$, calculated with Student's t test, compared with vehicle stimulated values (Ctrl) (*) or with E2-stimulated values (#).

role for ERβ levels as a negative regulator of colon tumour growth.

Noteworthy, the increase of translation efficiency in response to the p38/MAPK pathway has already been reported. The AU-rich elements (ARE) present in the tumour necrosis factor-α mRNA can regulate protein expression through translation control [33,34]. When the cell is quiescent, translation of specific ARE-bearing transcripts is repressed. However, upon stimulation of myeloid cells, the p38/MAPK pathway is activated and translation is enhanced while the repressive signals are removed [33]. A similar mechanism also occurs in *Saccharomyces cerevisiae* in response to external stimuli [34], and data here presented strongly suggest that E2 could utilize a similar regulation pathway to rapidly up-regulate ERβ levels.

Finally, since the palmitoylation inhibitor 2-Br completely prevents the activation of p38/MAPK and the increase of ERβ levels, present data further indicate that the rapid E2-induced signals require a membrane-localized receptor.

Overall, our data suggest a finely tuned control exerted by rapid E2-induced signals on different cellular molecular events. In particular, the E2-induced membrane-starting signals occur before the appearance of nuclear effects and the cell context in which the genomic events take place will be different depending on which signaling pathway is activated. Thus, the integration between these molecular events is required to provide plasticity for cell response to sex steroids.

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