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Estrogenic effect of the MEK1 inhibitor PD98059 on endogenous estrogen receptor alpha and beta☆

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ABSTRACT

Estrogens are key regulators in mammary development and breast cancer and their effects are mediated by estrogen receptors alpha (ERα) and beta (ERβ). These two receptors are ligand activated transcription factors that bind to regulatory regions in the DNA known as estrogen responsive elements (EREs). ERα and ERβ activation is subject to modulation by phosphorylation and p42/p44 MAP kinases are the best characterized ER modifying kinases. Using a reporter gene (3X-ERE-TATA-luciferase) to measure activation of endogenous ERs, we found that MEK1 inhibitor PD98059, used in concentrations insufficient to inhibit MEK1 activation of p42/p44 MAP kinases, exerted estrogenic effects on the reporter gene and on the ERE-regulated RIP 140 protein. Such estrogenic effects were observed in mammary epithelial HC11 cells and occur on unliganded ERα and ligand activated ERβ. Additionally, concentrations of PD98059 able to inhibit p42/p44 phosphorylation were not estrogenic. Further, inhibition of p42 MAP kinase expression with siRNAs also resulted in loss of PD98059 estrogenic effect. In summary, PD98059 in concentrations below the inhibitory for MEK1, exerts estrogenic effects in HC11 mammary epithelial cells.

1. Introduction

Estrogens are key players in mammary gland development and breast cancer [1], being their effects mediated by two estrogen receptors (ER): ERα and ERβ [2]. Signaling through ERα results in cell proliferation of breast epithelium and cancer cells, while in many cell systems, ERβ was shown to counteract ERα-induced proliferation and to increase apoptosis [3,4].

ERα and ERβ bind the endogenous hormone 17β-estradiol (E2) with the same affinity, but show differential binding to other natural and synthetic compounds [5–7]. Initial E2 displacement experiments showed that ERβ displays higher affinity (20–30-fold) for flavonoids than ERα [5]. As such, ERβ has 20 times higher affinity for the flavone apigenin and the isoflavone genistein. Furthermore, both compounds were able to induce transactivation of 3xERE-Luciferase (ERE-Luc) reporter genes by overexpressed ERα or ERβ [5].

Flavonoids are found widely distributed in plants and are regularly ingested by humans in the form of fruits, vegetables and their derivatives. Beneficial effects of flavonoids are currently under study due to their possible effects against atherosclerosis, osteoporosis and certain cancers [8]. As such, the high daily intake of isoflavones in the form of soy and soy-derived products is thought to contribute to the lower incidence of breast cancer in Asian populations due to isoflavones' higher affinity for ERβ [9].

The flavone PD98059 (also named PD098059) is a widely used mitogen activated protein kinase 1 (MEK 1) inhibitor. PD98059 binds to inactive MEK 1 and prevents its activation by c-Raf, thereby hindering phosphorylation of p42/p44 MAP kinase [10]. In cell based assays, the EC50 for PD98059 ranges between 10 and 50 μM and this compound is commonly regarded in the literature as a MEK 1 specific inhibitor. However, we and others have found that PD98059 exerts estrogenic effects on reporter assays carried out on at least two types of progenitor cell lines: mesenchymal KS483 [11] and epithelial HC11 [12]. In addition, it was shown that some batches of PD98059 are capable of displacing (3H)-E2 from recombinant ERα [13].

Abbreviations: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon nuclear translocator; ER, estrogen receptors; E2, 17β-estradiol; ERE, estrogen responsive element; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; MEK1, mitogen-activated protein kinase kinase 1; PPT, 4,4′,4″-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol; DPN, 2,3-bis(4-hydroxy-phenyl)-propionitrile; DMSO, dimethyl sulfoxyde; p42/p44 MAP kinase, mitogen-activated protein kinase 1 and 3; RIP 140, nuclear receptor-interacting protein 1; SFM, serum free medium; Luc, luciferase; shRNA, short hairpin loop inhibitory RNA; siRNA, short inhibitory RNA.

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PD98059 is a flavone, as such, either it or metabolites derived from it could potentially bind to and activate ER. Therefore, the aim of this work was to further characterize if PD98059 activates endogenous ERα, ERβ or both ER and if such estrogenic effects can also be observed in more complex cellular responses.

2. Materials and methods

2.1. Hormones and inhibitors

E2 and ICI 182 780 were from Sigma. 4,4′,4″-(4-Propyl-(1H)-pyrazole-1,3,5-triyl) trisphenol (PPT) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) were from Tocris. PD98059 was purchased from Sigma and from Calbiochem. Note that initial experiments were carried out with PD98059 from Sigma which was then replaced by the compound from Calbiochem. Therefore, except for Fig. 1A, all the experiments presented used PD98059 from either, two batches from Calbiochem. Stock solutions where prepared in absolute ethanol (estrogens and antiestrogens) or DMSO (PD98059) and used to a maximum concentration of 1/1000 (solvent/growth medium).

2.2. Cell culture

Generation of HC11 cells stably expressing a 3xERE-TATA-Luciferase reporter gene (H-ERE cells) has been described previously [12]. HC11 and H-ERE cells were routinely grown in complete medium (phenol red-free RPMI 1640, 10% FBS, 50 μg/ml gentamicin, 5 μg/ml insulin and 10 ng/ml EGF). Twenty-four hours before experimental treatment, medium was changed to serum-free medium (SFM: phenol red-free RPMI 1640, 50 μg/ml gentamicin, 5 μg/ml insulin, 2 mg/ml fetuin (Sigma), and 35 μg/ml human transferrin (Roche)) and experiments were carried out in SFM. In mammary epithelial cells, E2 bound ER may induce expression of amphiregulin [14] leading to activation of Raf-MEK regulated signaling and possible activation of ER by p42/p44 [15]. Therefore, in order to prevent possible MEK 1 activation through this paracrine mechanism, cells were always pre-incubated with PD98059 for 30 min, thereafter hormones were added to the same growth medium already containing PD98059. Untreated groups received the same volume of solvents.

2.3. Transient transfections and luciferase assay

HC11 cells at 70% confluence were transfected with a luciferase reporter construct (3x-ERE-TATA-Luc [16]) and/or pSuper plasmid (Oligoengine) containing shRNAs to block either ERα or ERβ expression (shERα, shERβ and the control mutated sequence (shNS) [4]. In selected experiments cells were transfected with 60 ng siRNA to block p42 MAP kinase or the fluorescent non specific control (Santa Cruz). Transfections were carried out in SFM using Fugene 6 (Roche). After 24 h, cells were treated with 10 nM E2, PPT, DPN and/or 100 nM ICI 182 780. Luciferase activity was measured with luciferin kit (Biotherma). Pilot experiments were carried out in transiently transfected HC11 cells in which transfection efficiency was controlled using pRL-TK Renilla luciferase and dual luciferase kit (Promega). No differences were observed between normalization to Renilla or to protein concentration. Therefore we chose to continue with the later as cell number is affected by estrogenic compounds and ERα/ERβ ratios [4]. Experiments were repeated at least three times and carried out in triplicates. Representative experiments are shown.

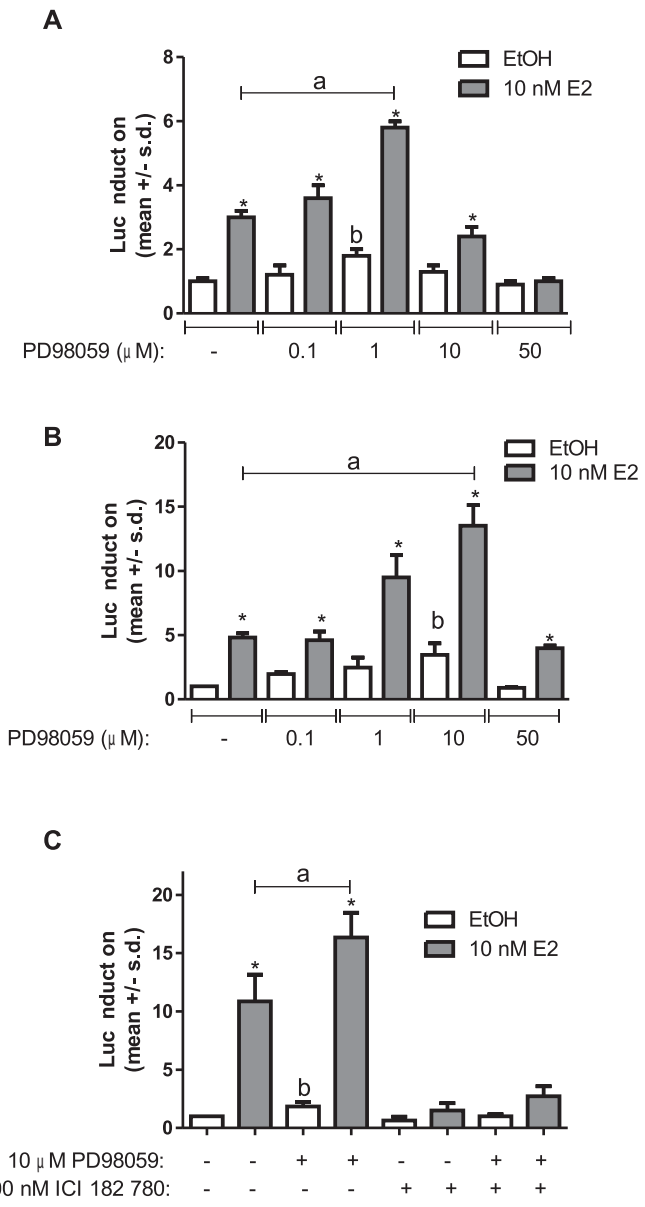

Fig. 1. PD98059 estrogenic effects on ERE-regulated reporter gene. H-ERE cells were treated with the indicated concentrations of PD98059 in the absence or presence of 10 nM E2 (A and B) and 100 nM ICI 182 780 (C). Luciferase activity was measured following 24 h treatment. (A) PD98059 from Sigma; (B and C) PD98059 from Calbiochem. Significant differences were analyzed with one-way ANOVA and Tukey's multiple post-test. $^{*}p < 0.05$ E2 vs control (EtOH); $^{a}p < 0.05$ E2 + PD98059 vs E2; $^{b}p < 0.05$ PD98059 vs control.

2.4. Whole-cell extracts

Cell pellets were resuspended in lysis buffer (1% NP40, 50 nM Tris–HCl (pH 7.5), 140 nM NaCl, 2 nM EDTA, protease inhibitor cocktail (Roche), 1 nM phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktail 1 and 2 (Sigma)), incubated for 20 min on ice, and centrifuged at 20,000 × *g* for 20 min at 4C. Protein concentration was quantified with Bradford reagent (Bio-Rad).

2.5. Western blot

Cell extracts were resolved on SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham). The luminescent signal was detected with Enhanced Chemiluminescence (Amersham) or West Pico (Pierce) kits. Experiments were repeated at least three times.

Primary antibodies were rabbit anti-phospho p42/p44 and rabbit anti p42/p44 from Cell Signaling and rabbit anti-RIP 140 from Santa Cruz Biotechnology.

2.6. Cell counting

Ten thousand cells were seeded in 24-well plates. After 24 h, cells were rinsed in PBS, and medium changed for 10 nM E2, PPT or DPN in SFM. Half volume of medium was changed every 48 h. Following 4 days, cells were detached with trypsin-EDTA and counted in a Neubauer chamber. Cell number index was calculated compared to the untreated control arbitrarily set to 1. The results are presented as mean ± SD of two independent experiments carried out in quadruplicate. Statistical significance between treated and untreated groups was evaluated with one-way ANOVA and Dunnet's or Tukey's post test.

3. Results

Previously it was reported that 0.1–1 μM PD98059 activated transcription of a 3xERE-TATA-luciferase reporter (ERE-luc) in HC11 cells that express endogenous ERα and ERβ [12]. To establish if this observation was due to a particular PD98059 preparation, three different commercial batches of PD98059 were tested in concentrations of 0.1–50 μM (Fig. 1A; Sigma and Fig. 1B; one of two Calbiochem batches). In the absence of ligand, all PD98059 preparations stimulated transcription of ERE-luc. Stimulation followed a bell-shaped dose response curve where the curve corresponding to PD98059 from Calbiochem showed a ten fold displacement to the right as compared to that from Sigma. Additionally, stimulation of ERE-luc with 17β-estradiol (E2) was further significantly increased by pre-incubation with 1 or 10 μM PD98059 as compared with E2 alone (Fig. 1A and B, respectively). Note that after substraction of basal luciferase units from each PD98059 concentration group, the additive effect with E2 remained significant. To rule out the possibility that PD98059 effects were related to clonal selection of stably transfected H-ERE cells, HC11 wild type cells were transiently transfected with the same ERE-luc construct, treated in the same manner as above and a similar effect was observed (not shown).

Next, it was investigated if induction of the ERE-luc reporter gene by PD98059 occurred through activation of ER. For this purpose, H-ERE cells were pre-incubated with PD98059, after 30 min, E2 and the ER antagonist ICI 182 780 were added and ERE-luc transactivation quantified as a measure of luciferase activity (Fig. 1C). PD98059 alone and PD98059 + E2 stimulated ERE-luc expression while addition of ICI 182 780 prevented ERE-luc induction. These results indicate that the estrogenic effects of PD98059 observed as increased transactivation of ERE-luc reporter gene are mediated by ER.

ER activity is modulated by phosphorylation and binding to transcriptional co-regulators. In this context, p44/p42 MAP kinases play a pivotal role [15]. Therefore, it was expected that MEK1 inhibition by PD98059 impaired ER transactivation of ERE-luc. Since, in our experimental conditions, this was not observed, p44/42 phosphorylation status was evaluated by immunoblot of whole cell extracts from cells treated with estrogenic (10 μM) and non estrogenic (50 μM) PD98059 concentrations (Fig. 2A). As expected, a non estrogenic concentration of 50 μM PD98059, prevented p44/p42 phosphorylation, while the most estrogenic concentration of 10 μM had no effect. To further establish if loss of PD98059 estrogenic effect at higher concentrations was due to inhibition of p44/p42, siRNAs to block p42 expression where transiently transfected into H-ERE cells, 24 h later cells were pre-incubated with 10 μM PD98059 (estrogenic concentration) and ERE-luc transacti-

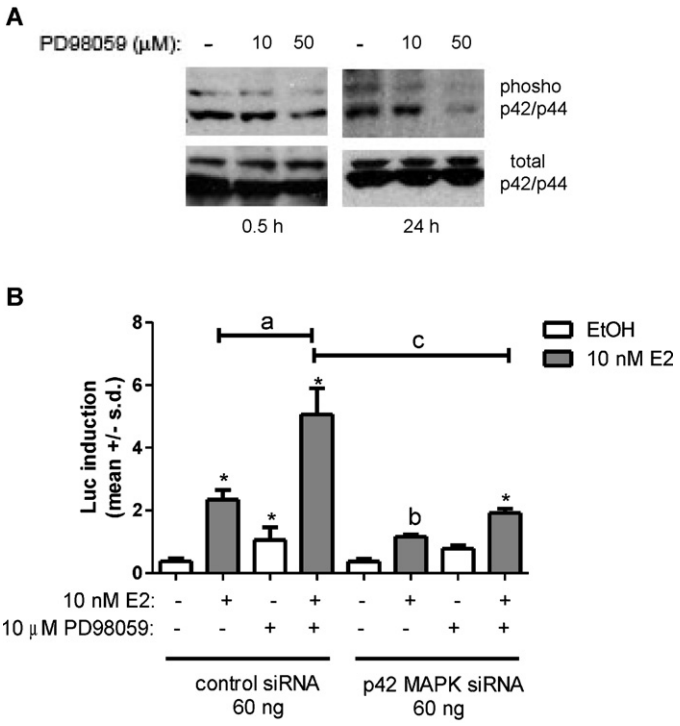

Fig. 2. Estrogenic PD98059 concentrations do not affect p42/p44 MAP kinase phosphorylation. (A) HC11 cells were treated with PD98059 for a period of 24 h and p42/p44 phosphorylation was analyzed by immunoblot. (B) H-ERE cells were transiently transfected with siRNAs to block p42 MAP kinase expression or a control non specific sequence. Following 24 h, cells were treated and luciferase activity analyzed as described in Section 2. Differences between treatments were analyzed with one-way ANOVA and Tukey's multiple post test. *$p < 0.05$ E2 vs control (EtOH); [a]$p < 0.05$ E2 + PD98059 vs E2; [b]$p < 0.05$ p42 siRNA + E2 vs control siRNA + E2; [c]$p < 0.05$ p42 siRNA + E2 + PD98059 vs control siRNA + E2 + PD98059.

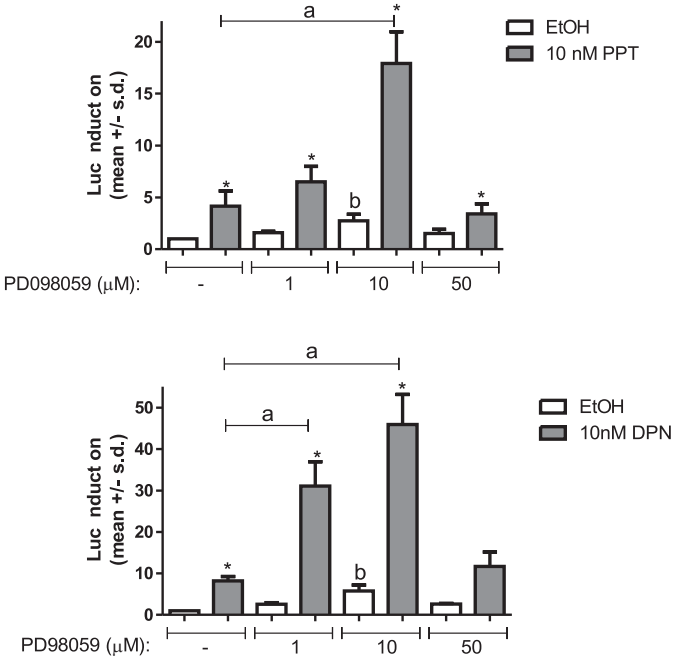

Fig. 3. PD98059 stimulates ERα and ERβ mediated transactivation. H-ERE cells were incubated with PD98059 in the indicated concentrations with or without 10 nM ERα and ERβ selective agonists PPT and DPN, respectively. Following 24 h incubation, luciferase activity was measured. Significant differences were analyzed with one-way ANOVA and Tukeyís multiple post-test. *$p < 0.05$ PPT or DPN vs control (EtOH); [a]$p < 0.05$ PPT or DPN + PD98059 vs PPT or DPN; [b]$p < 0.05$ PD98059 vs control.

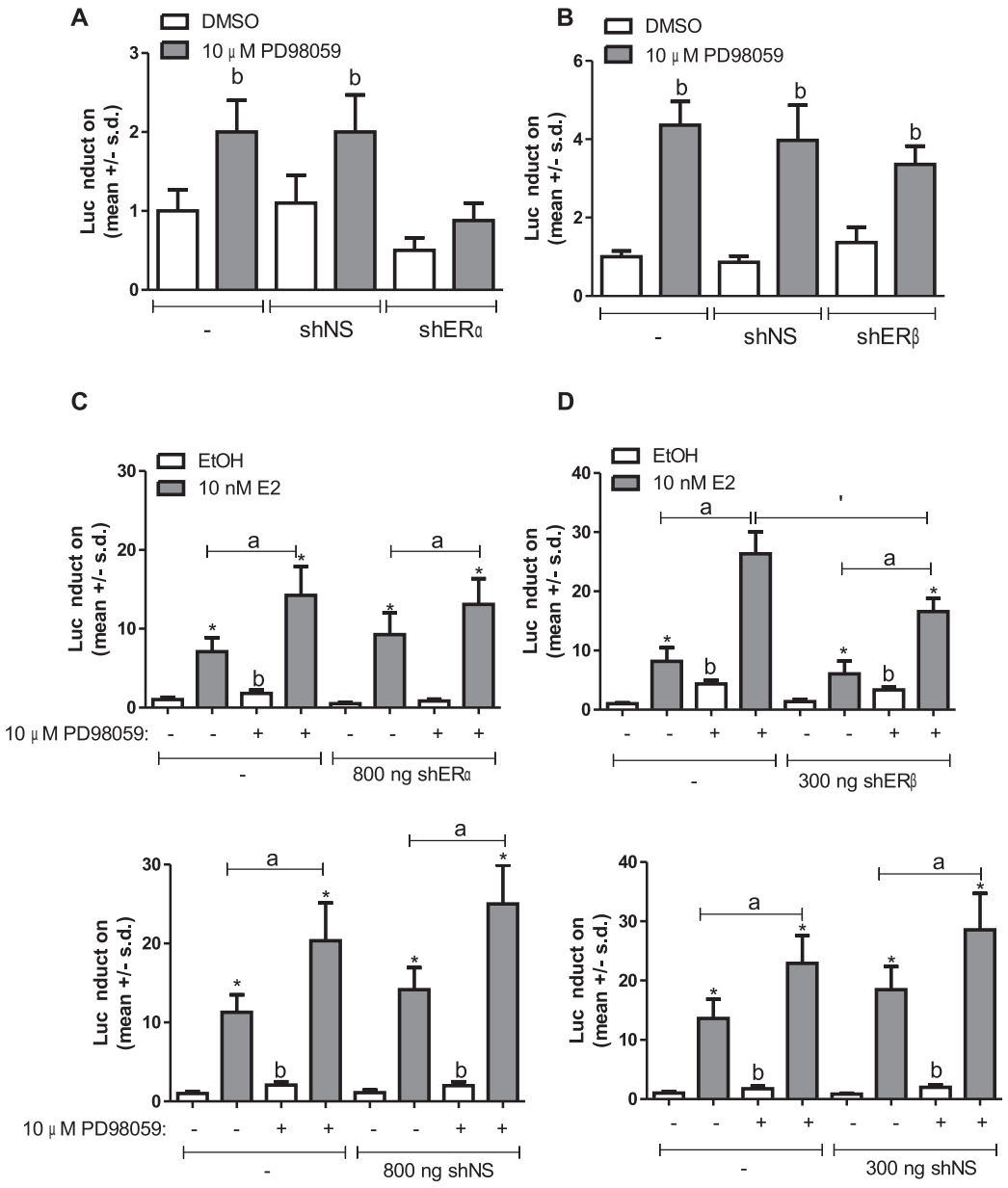

Fig. 4. Estrogenic concentrations of PD98059 affect unliganded ERα and ligand activated ERβ. H-ERE cells were transiently transfected with shRNAs to inhibit ERα expression (shERα; A and C), ERβ (shERβ; B and D) or same concentration of a mutated sequence (shNS). Cells were treated with 10 μM PD98059 with or without 10 nM E2 and luciferase activity was measured as described in Section 2. Significant differences were analyzed with one-way ANOVA and Tukey's multiple post-test. $^{*}p < 0.05$ E2 vs control (EtOH); $^{a}p < 0.05$ E2 + PD98059 vs E2; $^{b}p < 0.05$ PD98059 vs control (DMSO) and $^{c}p < 0.05$ shERβ + E2 + PD98059 vs E2 + PD98059.

vation was measured. As expected, knock down of p42 inhibited ER transcriptional activity induced by E2 alone, PD98059 alone or PD98059 + E2 (Fig. 2B). In summary, this results show that the estrogenic effect of PD98059 is – at high concentrations-overriden by its inhibition of the MEK1/p42/p44 pathway.

Once established that PD98059 can exert estrogenic effects in the absence of MEK1 inhibition and taking into account that flavones display a higher affinity for ERβ, we evaluated if such effects were observed on ERα, ERβ or both ER. For this purpose, H-ERE cells were treated with 10 nM PPT or DPN (ERα and ERβ selective agonists, respectively). PD98059 exerted a synergic effect on ERE-luc activation induced by ligand bound ERα (PPT) and ERβ (DPN), being the effect on ligand bound ERβ, more potent and already significant with 1 μM PD98059 (Fig. 3). To further analyze the effect of PD98059 on ERα and ERβ, shRNAs were used to alternatively block the expression of one or the other receptor (shERα, shERβ and shNS) while incubating with the endogenous hormone E2. The effective shRNA concentrations to achieve specific down regulation of each receptor were evaluated in pilot experiments (Supplementary Figure 1). Treatment with shERα, abolished stimulation of ERE-luc by PD98059 (Fig. 4A) while no significant inhibition was observed with shERβ (Fig. 4B). On the contrary, ERE-luc activation by E2 alone or E2 + PD98059 was not affected by knock down of ERα (Fig. 4C), but was reduced to nearly 40% in cells with knock down of ERβ (Fig. 4D). In summary, these results suggest that PD98059 can activate ERβ as well as unliganded ERα; still, E2 + PD98059 additive effects on ERE-luc transactivation partially rely on ERβ.

Estrogenic effects of PD98059 have to our knowledge been shown only on ERE-driven reporter genes [11,12] or *in vitro* reconstituted systems [13]. In order to establish if a similar effect could be observed in a more complex, physiologically regulated promoter,

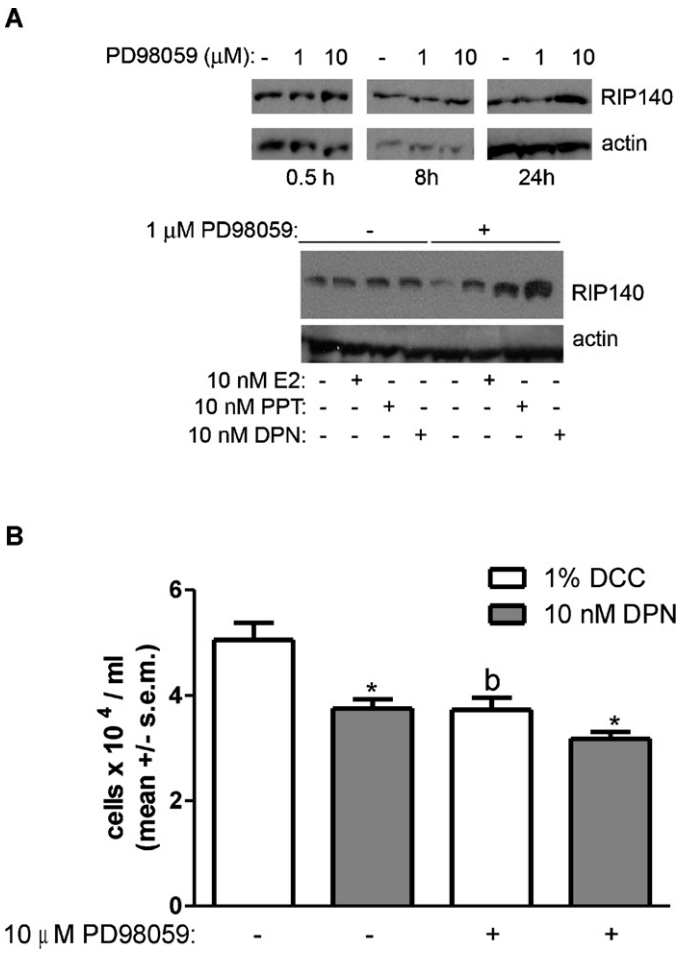

Fig. 5. Estrogenic PD98059 concentrations induce expression of the ERE-regulated protein RIP140 and inhibit cell growth. (A) HC11 cells were treated with PD98059 with or without 10 nM E2, PPT or DPN for 24 h and RIP140 levels were analyzed by immunoblot. (B) Effect of PD98059 on cell number. HC11 cells were treated with P98059 with or without 10 nM DPN for 48 h. Thereafter, cells were counted. Significant differences were analyzed with one-way ANOVA and Dunnet's post-test $^{*}p < 0.05$ DPN treated vs control; $^{b}p < 0.05$ PD98059 alone vs control.

expression of the ERE regulated protein RIP140 was analyzed by immunoblot. No effect was exerted by 1 μM PD98059, while 10 μM PD98059 increased RIP140 levels (Fig. 5A, upper panel). To test if the estrogenic potency of PD98059 was higher on ERβ (as observed in the reporter assays), HC11 cells were incubated with 1 μM PD98059 alone or with 10 nM E2, PPT and DPN. Similarly to the reporter assays, the combined effect of PD98059 with DPN was more potent than that of E2 or PPT (Fig. 5A, lower panel).

In HC11 cells, ERα and ERβ modulate cell number in opposing ways. Activation of ERα induces proliferation resulting in an increase in cell number, while activation of ERβ increases apoptosis, which results in reduced cell number [4]. Therefore, we investigated if and estrogenic concentration of 10 μM PD98059 would have a synergic effect on reduction of cell number by the ERβ agonist DPN. PD98059 alone decreased cell number, and addition of DPN caused a further decrease (Fig. 5B). Therefore, when analyzing complex biological responses such as inhibition of cell culture growth, PD98059 mimics the effects of DPN, an effect which occurs without MEK1 inhibition.

4. Discussion

Previously, it was shown that in HC11 cells, concentrations of PD98059 between 0.1 and 1 μM stimulated ligand independent and E2-induced ERE-luc transactivation [12]. Simultaneously, Dang and Lowik reported that in osteoprogenitor KS487 cells, PD98059 in concentrations of 1–25 μM, also induced transcriptional activity of ERE-luc [11]. In the present study, we showed that the maximum estrogenic effect of PD98059 is achieved at concentrations 10 times higher (10 μM) than initially reported in the same cells [12], an effect we relate to the different PD98059 sources (initially from Sigma and latter on from Calbiochem). It is interesting to note that in all these studies, despite the differences in the most effective concentration needed to transactivate ERE-luc reporter genes, PD98059 estrogenic effect followed a bell-shaped dose response curve with estrogenic concentrations in the 1–10 μM range and non estrogenic concentrations in higher 50 μM range. Since only non estrogenic concentrations of PD98059 inhibited MEK1 activity and in HC11 cells, ER ligand dependent and independent transcriptional activation of ERE-luc relies at least on p42 MAP kinase, it is probable that the inhibitory effect of PD98059 on p42/p44 MAP kinases overrides the ligand effect of PD98059, therefore impeding ER transcriptional activation. This possibility has been previously proposed by Long et al. and it was anticipated that PD98059 preparations may have contaminants with estrogenic activity as two out of 4 batches tested were capable of displacing (3H)-E2 from recombinant ERα in solution [13]. In this experimental setting, the EC50 for PD98059 was nearly 100 μM, 4 orders of magnitude higher than that of E2. The low affinity of PD98059 for ERα as compared to E2 was similar to that observed for genistein and apigenin in similar assays [5]. However, this experimental setting is far from the actual cellular context where ER affinity for a ligand, and DNA response elements is dictated by interactions with chaperones, transcriptional co-regulators as well as postranscriptional modifications [17]. In line with this, the same PD98059 estrogenic concentration reported by us (10 μM) was found to give a maximum stimulation of EREps2-luc in ER-negative MDA-MD-231 cells transfected with ERα [13]. On the other hand, flavonoids are substrates to cytochrome P450 monooxygenases and phase II conjugation enzymes [8]. Particularly, flavone was shown to increase expression and activity of rat CYP1A2 [18], and to increase activity of UDP-glucuronoslytransferase [19]. Therefore, wether the estrogenic effect observed in PD98059 preparations is related to an ER binding contaminant, to a metabolic product or to the flavone nature of PD98059 which make it a potential ER ligand at μM concentrations (well within the EC50 for ER binding of other flavonoids) deserve further studies. Yet, it is noteworthy that in a similar manner as other flavonoids, PD98059 is more likely to activate ERβ mediated transcription of not only ERE-luc but also more complex ERE-regulated promoters, as well as complex biological responses as shown here for cell growth.

In basal growth conditions, PD98059 exerted low agonistic effects on ERα mediated transcription of ERE-luc. However, in combination with E2, PD98059 displayed an additive effect on both ER, being more potent the effect on ERβ. This is an unusual finding given that compounds with low ER agonistic properties usually inhibit effects caused by E2. However, an alternative explanation relies on the observation that PD98059 may be an aryl hydrocarbon receptor (AHR) ligand with antagonist properties [20]. AHR is a ligand (xenobiotic)-activated transcription factor, which upon ligand binding translocates to the nucleus and heterodimerizes with aryl hydrocarbon nuclear translocator (ARNT). The activated AHR/ARNT heterodimer recruits coregulators and transactivates AHR response elements leading to changes in target gene expression, including cytochrome P450 detoxifying monooxygenases. Crosstalk between AHR and ER has been postulated as one cause of endocrine disruption [21]. More importantly, it has been shown that in HC11 cells, ARNT is also and ER transcriptional co-activator with higher activation potential on ERβ [22]. Therefore, it is possible that the additive effect observed following E2 addition is due to PD98059

antagonism of AHR favouring ARNT binding to ER and subsequent co-activation, rather than PD98059 binding to ER.

Finally, since PD98059, in concentrations which do not inhibit MEK1, may still affect cell physiology as shown on ER regulated proteins and effects on cell number, the use of this compound for the study of signaling events in cells expressing ER should be carefully considered.

5. Conclusion

This work has characterized the dual effect of the flavone PD98059 as putative estrogen preferably activating ERβ, besides the previously known effect as MEK1 inhibitor. It established that PD98059 estrogenic concentrations do not efficiently inhibit downstream targets of MEK1 and thus allow ER transcriptional activity. Estrogenic concentrations of PD98059 can affect transactivation of ERE-regulated reporter genes as well as gene expression indicating that the estrogenic effects of PD98059, even in concentrations which do not inhibit MEK1, may affect cell physiology. Results presented here point to the potential ER agonist nature of PD98059, particularly enhancing ERβ mediated responses. Since ERβ is currently under evaluation as a target for breast cancer treatment, further identification of PD98059, possible contaminating or metabolic products with ER binding/activating activity is of interest.

Conflict of interest

The authors disclose no actual or potential conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2010.12.020.

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