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The ER α membrane pool modulates the proliferation of pituitary tumours

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Abstract

The molecular mechanisms underlying the ER α nuclear/cytoplasmic pool that modulates pituitary cell proliferation have been widely described, but it is still not clear how ER α is targeted to the plasma membrane. The aim of this study was to analyse ER α palmitoylation and the plasma membrane ER α (mER α) pool, and their participation in E2-triggered membrane-initiated signalling in normal and pituitary tumour cell growth. Cell cultures were prepared from anterior pituitaries of female Wistar rats and tumour GH3 cells, and treated with 10 nM of oestradiol (E2). The basal expression of ER α was higher in tumour GH3 than in normal pituitary cells. Full-length palmitoylated ER α was observed in normal and pituitary tumour cells, demonstrating that E2 stimulation increased both, ER α in plasma membrane and ER α and caveolin-1 interaction after short-term treatment. In addition, the *Dhhc7* and *Dhhc21* palmitoylases were negatively regulated after sustained stimulation of E2 for 3 h. Although the uptake of BrdU into the nucleus in normal pituitary cells was not modified by E2, a significant increase in the GH3 tumoural cell, as well as ERK1/2 activation, with this effect being mimicked by PPT, a selective antagonist of ER α . These proliferative effects were blocked by ICI 182780 and the global inhibitor of palmitoylation. These findings indicate that ER α palmitoylation modulated the mER α pool and consequently the ERK1/2 pathway, thereby contributing to pituitary tumour cell proliferation. These results suggest that the plasma membrane ER α pool might be related to the proliferative behaviour of prolactinoma and may be a marker of pituitary tumour growth.

Key Words

- ▶ pituitary tumour
- ▶ palmitoylation
- ▶ oestrogen receptor alpha
- ▶ proliferation

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Introduction

Oestrogens act as important regulators of cell proliferation, cell survival and differentiation in a variety of organ systems and tissues and have been implicated in the aetiology of a variety of malignant cancers and benign tumours, such as pituitary adenomas (Spady *et al.* 1999). Most of the effects of oestrogen are mediated through its two receptors: oestrogen receptor alpha (ER α) and beta

(ER β) (Mitchner *et al.* 1998). ER α expression has been detected in both normal and tumour cells secreting PRL and gonadotropin (Friend *et al.* 1994), and at higher levels in macroadenomas than in microadenomas, and in non-invasive tumours than in invasive ones (Meitzen *et al.* 2013). It has been demonstrated that an oestrogen receptor antagonist inhibited pituitary tumour growth in

a prolactinoma experimental model (Heaney *et al.* 2002), thereby making ER α a potential target for the treatment of high ER α -expressing pituitary adenomas (Gao *et al.* 2017).

In addition to the classic nuclear genomic action, oestrogens have been found to induce rapid effects within minutes of administration, which are mediated through a subpopulation of oestrogen receptors associated with the plasma membrane, a process usually termed 'membrane-initiated steroid signalling' (MISS), 'nongenomic' or 'extranuclear' effects (Watson *et al.* 2005, Ueda & Karas 2013). Related to this, we previously demonstrated that 17 β -oestradiol (E2) and FGF2 exerted a cooperative effect on lactotroph proliferation, principally by signalling initiated at the plasma membrane and mediated by the MEK/ERK1/2 pathway (Sosa *et al.* 2013).

The molecular mechanisms underlying the ER α nuclear/cytoplasmic pool modulating adenohypophyseal cell activity have been widely described. Although it is still not clear how ER α is targeted to the plasma membrane in normal and pituitary tumour cells, it has been reported that one of the requirements for ER to be located at the plasma membrane is the presence of a hydrophobic segment as part of the receptor structure (Marino *et al.* 2006, Morrill *et al.* 2015). A post-translational modification of ER α has been previously described, which includes the addition of a palmitate molecule (S-acylation, commonly called palmitoylation) in cysteine residues of the ligand-binding region of the gonadal steroid receptors (Acconcia *et al.* 2005, Pedram *et al.* 2007), by the palmitoyl-acyltransferases (PATs) DHHC7 and DHHC21 (Pedram *et al.* 2012). Adding lipid residues increases hydrophobicity, promoting steroid receptor translocation to the caveolae regions of the plasma membrane (Razandi *et al.* 2002, Peffer *et al.* 2014), with the different isoforms of caveolin (caveolin-1 and caveolin-2) being involved in this mechanism (Le Lay & Kurzchalia 2005, Totta *et al.* 2015). ER α localisation in caveolae regions has been described in ovarian, prostate and breast tumour cells, suggesting an interaction between caveolin-1 and steroid receptors, which contributes to mER α localisation as well as to the activation of extranuclear E2 signalling (Pedram *et al.* 2002, Acconcia *et al.* 2003, Park *et al.* 2009). However, the functional role of palmitoylation in normal and pituitary tumour cell proliferation and signalling has not yet been explored.

The results reported in the literature are related only to total ER α expression; thus, it is interesting to evaluate the mER α expression in pituitary tumours. In the present study, we speculated that the mER α pool modulates cell proliferation in pituitary tumours. Thus, we tested

the hypothesis that the increase of mER α mediated by palmitoylation triggers ERK1/2 phosphorylation and consequently pituitary tumour cell growth.

Materials and methods

Cell cultures

A pool of 3-month-old female Wistar rats, ($n=12$), bred and housed at the Animal Research Facility of National University of Cordoba, was assigned to each culture taken at random cycle stages. The protocol for the dissociation of pituitary cells has been described previously (De Paul *et al.* 2011). The normal pituitary primary culture includes different type of cells, with the lactotroph (54.1%) and somatotroph (21.8%) being the two principal cell populations (data not shown). After 3-day culture, the cells were maintained in DMEM without phenol red and serum for 24 h before applying the treatments. The experiments were approved by the Institutional Animal Care Committee of the School of Medicine, University National of Cordoba.

The rat GH3 lactosomatotroph cell line is derived from rat prolactin-secreting pituitary tumours that synthesise both prolactin and growth hormone and has been used as a prolactinoma model (Boockfor *et al.* 1985, Chao *et al.* 2014). The cells were cultured in Ham's F-12 medium, supplemented with 2.5% foetal bovine serum and 15% horse serum (Gibco). The cell cultures with a confluence of 80% were maintained in DMEM without phenol red and serum for 24 h and then submitted to different experimental protocols

GH3 and primary adenohypophysis cells were stimulated for 30 min with E2 (10 nM), a selective ER α agonist: 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-tryl) Triphenol (10 nM, PPT) or epidermal growth factor (EGF) (10 ng/mL). For some experiments, the cells were pre-incubated with the global inhibitor of palmitoylation, 2-bromohexadecanoic acid (2BP; 10 μ M) or ER pure antagonist: ICI 182780, for 30 min.

Determination of palmitoylated proteins by acyl-biotin exchange (ABE) assay

The palmitoylated proteins were determined by the acyl-biotin exchange (ABE) assay according to Wan *et al.* (2007) with modifications. The cells were extracted in cold lysis buffer (1.25% Igepal CA-630, 1 mM EDTA and protease and phosphatase inhibitors) and the proteins were

concentrated by precipitation in chloroform-methanol and suspended in the SB buffer (50mM Tris-HCl, pH7.4, 5mM EDTA, 4% SDS) with N-methylmaleimide (NEM-10mM). Next, 1mM NEM was added to the LB buffer (50mM Tris-HCl, pH7.4, 5mM EDTA, 150mM NaCl), which was incubated overnight at 4°C. The samples were divided into two equal portions H and Tris, with the H samples being diluted in HB buffer (1M hydroxylamine, 150mM NaCl, 0.2% Triton and 1mM HPDP-Biotin) and the Tris samples being diluted with the same buffer without hydroxylamine. Purification of the biotinylated proteins was completed by diluting with LB buffer containing streptavidin-agarose beads. Finally, the samples were re-suspended in 35 μ L LB containing 0.1% SDS, 0.2% TritonX-100 and 1% β -mercaptoethanol and heated to 95°C. The proteins were analysed by western blot using primary antibody anti-ER α (1/200; Santa Cruz Biotechnology).

The GH3 cell line was transfected with the plasmid encoding SX8-Cherry as a control of palmitoylated proteins. The expression plasmid (1 μ g) and the transfection reagent PEI (2 μ L, Sigma-Aldrich) were added for 2h and then the GH3 cells were maintained in Ham's F-12 an additional 24h.

Analysis of cell-surface proteins by biotinylation

The cell cultures were washed with PBS buffer and the cell-surface labelled proteins were purified using a cell-surface protein isolation kit (Pierce). The proteins from the supernatant and pellet fractions were analysed by western blot using the specific primary antibodies (Santa Cruz Biotechnology): anti-phosphorylated ER α (1/200), anti- β -actin (1/1000), anti-FGFR (1/200) and anti-EGFR (1/400).

Immunoprecipitation

The protein extract was subjected to immunoprecipitation using anti-ER α (5 μ g/mL). The immune complexes were adsorbed and precipitated using protein G-Sepharose beads (Sigma-Aldrich), washed and denatured by boiling for 5 min in sample buffer. The samples were analysed by western blot using anti-ER α (1/200) and anti-caveolin-1 (1/1000; Cell Signaling Technology).

Preparation of cell lysates for western blotting analysis

The samples were lysed in cold lysis buffer and the total homogenate (50 μ g) was separated using 12%

polyacrylamide gel. The proteins on nitrocellulose membrane were blocked with 5% non-fat dried milk and 0.1% Tween20 at RT and incubated overnight with primary antibodies 1/700 anti-diphosphorylated ERK1/2 (Sigma-Aldrich) and 1/1000 anti-total ERK1 (Santa Cruz Biotechnology, Inc). The blots were incubated with peroxidase-conjugated anti-rabbit (1/5000) or anti-mouse (1/2500 Jackson ImmunoResearch Labs Inc) secondary antibodies and then revealed with ECL detection reagents (Immun-Star HRP-Substrate Kits, Bio-Rad). Finally, the emitted light was captured by the C-DiGit Chemiluminescence Scanner (LI-COR Biosciences), and signals were quantified with ImageJ software.

Gene expression analysis by qPCR

qPCR analysis of cDNA was performed on an ABI Prism 7500 detection system (Applied Biosystem) using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) and the upper and lower gene-specific primer sequences used were DHHC-7 (NM_133394.1) 5'-GAGGATGGACC ACCACTGTC-3' and 5'-CATGATAGCCAGCTCATGC-3'; DHHC-21 (XM_006238345.1) 5'-GAGGATGGACCACCA CTGTC-3' and 5'-TCATGATAGCCAGCTCATGC-3'; DHHC-11 (NM_001039342.2) 5'-AACAACTTGACTTGGCC TACG-3' and 5'-GGCGAAAGAGTAGACAGCA-3' and β -actin (NM_031144) 5'-CCCACACTGTGCCCATCTA-3' and 5'-CGGAACCGCTCATTGCC-3'.

Immunogold electron microscopy

The subcellular localisation of the ER α and caveolin-1 in normal and GH3 cells was examined by ultrastructural immunocytochemical techniques applying previously standardised protocols (Petiti *et al.* 2015). Thin sections in the grids were incubated with anti-ER α (1/200) followed by anti-caveolin-1 (1/500) antibodies overnight at 4°C. Then, the sections were incubated with anti-rabbit or anti-mouse secondary antibodies conjugated to 15nm and 5nm colloidal gold particles (1/30, Electron Microscopy Science) and examined in a Zeiss LEO 906-E transmission EM (TEM) (Zeiss).

Immunofluorescence

For mER α staining, non-permeabilised live cells were incubated with ER α /Alexa Fluor 594 for 15 min at 4°C prior to fixation. The ER α /Alexa Fluor 594 complex was prepared by mixing an adequate dilution of ER α primary antibody and Alexa Fluor 594 secondary antibody for 30 min at 4°C before incubation with the cells.

Images were obtained using a Confocal Laser Scanning Microscope FluoView FV 300 (Olympus) and processed using FV10-ASW 1.6 Viewer software.

Correlative light and electron microscopy

Correlative light and electron microscopy (CLEM) was carried out on ultrathin cryosections by applying the Tokuyasu technique as described by Oorschot (Oorschot *et al.* 2014). The cryosections were transferred on formvar-coated 100 μ m mesh nickel grids and incubated with anti-ER α antibody 1/50 in 1% BSA-PBS, followed by incubation with anti-rabbit Alexa-Fluor594 (1/300, Invitrogen) and DAPI (Sigma-Aldrich) for 1 h at 37°C.

For fluorescence light microscopy (FLM), grids layered with a 200 nm coat of 2% methylcellulose were mounted with 50% glycerol. For TEM observation, grids were unmounted, washed in milli-Q water and incubated in 0.4% uranyl acetate/1.8% methylcellulose. Fluorescence images were obtained using a Confocal Laser Scanning Microscope FluoView FV 1200 (Olympus) and, EM images using a Zeiss LEO 906-E TEM. The analysis was carried out with ImageJ software.

Immunocytochemical detection of bromo-deoxyuridine uptake

Cells at the DNA-synthesising stage were identified by immunocytochemical detection of BrdU. After 30 min of E2 stimulation, BrdU (100 nM) was added for an additional 24 h. The cells attached to the coverslips were fixed in 4% formaldehyde in PBS for 2 h at room temperature and BrdU incorporation detection was performed as described by Ferraris (Ferraris *et al.* 2014). A total of 1000 cells were examined using a systematic process on each glass slide to establish the proportion of positive BrdU in the total cells.

Data analysis

A statistical analysis was carried out on three replicates measured from three independent cell cultures, with ANOVA-Tukey using InfoStat software (Grupo InfoStat, Facultad de Ciencias Agropecuarias, UNC). The results are given as the means $\pm$ s.e.m., and the significance levels were set at $P < 0.05$.

Results

ER α expression in normal and pituitary tumour cells

First, we analysed the expression of total ER α in normal and pituitary tumour cells. The expression of ER α was higher

in tumour GH3 than in normal pituitary cells, and this did not change with the 30 min E2 treatment (Fig. 1A). Next, to determine whether ER α was palmitoylated in normal adenohypophysis and in GH3 pituitary tumour cells, the ABE assay was carried out followed by western blot. ER α full-length expression was observed as bands at around 66 kDa, and this protein was detected as palmitoylated in line H of ABE, in normal adenohypophysis cells (Fig. 1B, top) and in the GH3 cell line (Fig. 1B, bottom). Furthermore, an additional ER α -immunoreactive band around 50 kDa was detected in the palmitoylated proteins line in GH3 cells, possibly corresponding to the splicing variant of ER α . The SX8-Cher transfection in GH3 tumour cells showed a 70 kDa band in line H of ABE, confirming the presence of this palmitoylated protein (Fig. 1B, bottom).

E2 regulates Dhhc7 and Dhhc21 palmitoylase expression

Considering that the PATs are key to regulating the subcellular localisation of different ER α pools, *Dhhc7* and *Dhhc21* were evaluated by qPCR. As shown in Fig. 1C and D, the *Dhhc7* and *Dhhc21* mRNA basal levels were higher in tumour than in normal cells. Then, we evaluated whether E2 was able to regulate the mRNA expression levels of these enzymes, with a significant decrease in *Dhhc7* (Fig. 1E) and *Dhhc21* (Fig. 1F) mRNA levels being observed in normal and GH3 cells stimulated with E2 for 3 h compared to control. However, this reduction was transient, as *Dhhc7* and *Dhhc21* mRNA levels returned to baseline values after 6 or 9 h of E2 treatment. The expression of *Dhhc11* mRNA levels, used as a negative control, did not vary after E2 treatment either cell type (Fig. 1G).

Membrane ER α expression is regulated by palmitoylation

To explore whether palmitoylation could promote changes in mER α expression, pituitary cells were pre-treated with 2BP, the global inhibitor of palmitoylation, and then stimulated with E2 for 30 min. In unstimulated and nonpermeabilised pituitary cells, endogenous ER α -specific immunostaining was observed at the plasma membrane in some normal pituitary and pituitary tumour cells. However, E2 treatment for 30 min increased ER α expression at the plasma membrane, which was more frequently observed in tumour cells and was reverted when the cells were pre-treated with 2BP

Figure 1

(A) Basal expression of total ER α in normal and pituitary tumour cells and after E2 treatment for 30 min. (B) Protein extracts from pituitary cells were tested for palmitoylated ER α following the ABE assay. Biotinylated proteins were purified, separated by SDS-PAGE and stained with anti-ER α antibody. ER α palmitoylation is prominent in protein extracts that were treated with hydroxylamine (H). In addition, in GH3-transfected SX8-Cher tumour cells, palmitoylated SX8 was used as a control of palmitoylated protein. The input in (A) and (B) was 10% of total protein extract and Tris: pull-down without H. Relative mRNA expression levels of *Dhhc7* (C) and *Dhhc21* (D) in normal pituitary and GH3 tumour cells. *Dhhc7* (E), *Dhhc21* (F) and *Dhhc11* (G) mRNA expression levels after treatment with E2 (10 nM) at 3, 6 and 9 h. A significant decrease in the *Dhhc7* and *Dhhc21* mRNA levels was observed in normal and GH3 cells stimulated with E2 for 3 h compared to control. Expression levels were calculated by quantitative real-time PCR analysis. The β -actin gene was used as the internal reference gene and the $\Delta\Delta$ CT method was used for relative quantification and expressed as fold over control: * $P < 0.05$ *Dhhc7* vs C and $\Delta P < 0.05$ *Dhhc21* vs C.

(Fig. 2A, B and C). We visualised the expression of ER α by CLEM (Fig. 2B and D), which enabled simultaneous observation of a given subcellular structure. In normal (Fig. 2B) and tumour GH3 (Fig. 2D) cells, the ER α was localised at the plasma membrane when the cells were treated with E2 for 30 min.

The changes in mER α expression in pituitary cells were analysed by cell-surface biotinylation. As shown in Fig. 2, western blot analysis revealed the presence of ER α in the pellet fraction (containing the cell-surface biotinylated proteins) and in the supernatant fraction (with the intracellular unbiotinylated proteins).

In the pellet fraction, under baseline conditions, lower ER α protein expression was observed in both normal (E) and tumour (F) cells, whereas E2 treatment for 30 min significantly increased mER α expression, which was completely reversed by the 2BP pre-treatment.

E2 induces ER α and caveolin-1 association

As interaction between ER α and caveolin-1 has been described in different tissues (Wang *et al.* 2011, Peffer *et al.* 2014), we evaluated whether E2 could promote any interaction in pituitary cells by using a

Figure 2

Cell-surface immunostaining of ER α in non-permeabilised normal (A) and tumour GH3 (B) cell cultures treated with E2 (10 nM) for 30 min with or without pre-treatment of 2BP. Microphotograph represent the merging of light transmitted and immunofluorescence field. CLEM of normal pituitary (C) and GH3 tumour (D) rat cell cultures were treated with E2. (1) Immunofluorescence labelling for ER α , (2) TEM images for the whole section and (3) CLEM overlay of TEM on the corresponding FLM image. Arrows indicate ER α at plasma membrane. Bar: 10 μ m. Biotinylation of cell-surface biotinylated proteins in normal pituitary and tumour GH3 rat cells. Surface membrane proteins were biotinylated in normal control (E) and tumour GH3 (F) pituitary cells or in cells treated with E2 (10 nM) for 30 min with or without 2BP pre-incubation. Whole-cell lysates were subjected to avidin pull-down using streptavidin-agarose beads, and the recovered cell-surface biotinylated proteins (pellet) and intracellular unbiotinylated proteins (supernatant) were analysed by western blot and stained with anti-ER α antibody. In the pellet fraction, ER α expression showed an increase after E2 treatment but a decrease after pre-treatment with 2BP. The ER α expression did not change after treatment in the supernatant. The expression of the FGF and EGF receptors and β -actin confirmed equivalent total protein loading. Images correspond to a representative experiment from a total of three with similar results. Values are expressed as mean $\pm$ S.E.M. * P < 0.05 E2 vs C and $^{\wedge}$ P < 0.05 E2-2BP vs E2. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-18-0418>.

co-immunoprecipitation assay. As shown in Fig. 3, in normal (A) and GH3 (C) unstimulated cells, a basal interaction was observed between both proteins, which was significantly increased by E2 treatment. Interestingly, 2BP treatment was able to reverse the E2-induced ER α /caveolin-1 interaction, revealing similar expression levels as the controls.

In addition, we analysed the fine localisation of ER α with caveolin-1 by means of TEM immunogold labelling in normal (B) and tumour GH3 (C) cells. As shown in Fig. 4B and C, the immunoreactivity for ER α (15 nm gold particle) was distributed in the cytoplasm and occasionally in the plasma membrane in normal and tumour control cells, whereas ER α localisation was frequently observed the

plasma membrane, with caveolin-1 (5 nm gold particles) being close to each other in E2-treated cells.

The involvement of palmitoylation and mER α in cell proliferation

To analyse the contribution of mER α to cell proliferation, we determined the BrdU uptake into the nucleus of normal and pituitary tumour cells incubated with a palmitoylation inhibitor. The percentage of control normal BrdU-positive cells was 2.6%, with no changes observed after the different treatments (Fig. 4A and B). However, in non-stimulated GH3 cells, the proliferation was around 30%, showing a significant increase after E2 stimulation that was mimicked for PPT respect to control. The E2 effect was blocked partially by ICI 182780 and the global inhibitor of palmitoylation, 2BP (Fig. 4C and D).

Considering previous results from our laboratory concerning the involvement of the MEK/ERK1/2 and PI3K/AKT pathways in pituitary tumour cell proliferation (Petiti *et al.* 2010, 2015), we determined the phosphorylation of ERK1/2 and AKT in pituitary tumour cells. Figure 4E shows the significant increase in phosphorylated ERK1/2 after E2 and PPT treatments observed for 30 min, which was blocked when the cells were pre-incubated with ICI 182780 or 2BP, suggesting that ER α palmitoylation may be required to activate these kinases. The expression of phosphorylated AKT increased after E2 or PPT treatments for 30 min, while pre-incubation with ICI 182780 or 2BP did not revert this activation, suggesting that AKT may

contribute to the pituitary tumour proliferation induced by E2 in a mER α -independent manner.

Additionally, we tested if the effect of the inhibitor of palmitoylation could affect the cell response to different growth stimulatory factors. With this aim, we analysed the proliferation and activation of ERK1/2 in GH3 cells stimulated with the EGF for 30 min, in the presence or absence of the pre-incubation with 2BP. As shown in Fig. 4, the EGF treatment significantly increased the uptake of BrdU and ERK1/2 phosphorylation (Fig. 4F and G), effects that were not reverted when the cells were pre-treated with 2BP, suggesting that the cell response to palmitoylation inhibitor is ER specific.

The above results indicate that the plasma membrane ER α localisation mediated by ER α palmitoylation triggers ERK1/2 phosphorylation and consequently pituitary tumour cell growth.

Discussion

This study found that the subpopulation of ER α localised in the plasma membrane induced pituitary tumour proliferation by the mER α /caveolin1/ERK1/2 pathway. The E2 stimuli significantly increased mER α expression, ER α interaction with caveolin-1, ERK1/2 phosphorylation and finally led to pituitary tumour proliferation, which were partially reversed by the PAT inhibitor.

We previously identified the presence of ER α in the plasma membrane in normal pituitary cells

Figure 3

Association between ER α and caveolin 1. Normal pituitary (A) and tumour GH3 (C) rat cell cultures were treated with E2 (10 nM) for 30 min with or without pre-treatment of 2BP. The total cell extracts were used for immunoprecipitation (IP) using anti-ER α and the immunoprecipitates were then probed with anti-cav-1. * P < 0.01 E2 vs C and ^ P < 0.05 E2-2BP vs E2. In total anterior pituitary cell culture lysates, both antibodies recognised the antigens (input). Electron micrographs of normal pituitary (B) and tumour GH3 (D) with double immune-labelling of ER α (15 nm gold particle) and cav-1 (5 nm gold particle). The black arrows indicate the ER α -cav-1 adhered to the plasma membrane and the white arrows indicate the cytosolic ER α . g, granule; bar: 100 nm.

Figure 4

Representative micrographs of immunohistochemical staining for BrdU (red) and quantitative analysis in normal (A and B) and tumour GH3 (C and D) cell cultures. The cells were treated with E2 (10 nM) or PPT (10 nM) for 30 min with or without pre-treatment with 2BP or ICI 182780 (I, 100 nM). BrdU was added for an additional 24 h and the data represent the proportion of positive BrdU cells in the total cells. Significant differences were found in GH3 cell proliferation: * $P < 0.01$ E2 or PPT vs C and ^ $P < 0.05$ I+ E2 vs E2. # $P < 0.05$ E2 or PPT vs E2 or PPT and ~ $P < 0.05$ 2BP-I+E2 vs E2. Bar: 100 μ m. (E) Western blotting of ERK1/2 and Akt in total extract from tumour pituitaries treated with E2 (10 nM) or PPT (10 nM) for 30 min with or without pre-treatment of 2BP or ICI 182780 (I, 100 nM). The amount of protein levels was normalised by comparison with total ERK1/2 and AKT expression. A representative panel of three independent experiments is shown. (F and G) Quantitative analysis of BrdU staining and western blotting of ERK1/2 in total extract from cells treated with EGF (10 ng/mL) for 30 min with or without pre-treatment with 2BP (10 μ M). Significant differences were found in GH3 cell proliferation: * $P < 0.01$ EGF vs C, while that cell proliferation and ERK1/2 phosphorylation did not change with 2BP treatment. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-18-0418>.

(Gutierrez *et al.* 2008), and it has also been demonstrated that E2 stimulates the translocation of endogenous ER α and the activation of the PKC α /ERK1/2 pathway (Gutierrez *et al.* 2012, Watson *et al.* 2012, Zarate *et al.* 2012), without any effect on cell proliferation (Sosa *et al.* 2013). Considering that lactotroph cells represent the main phenotype in adult female rat pituitaries that express ER α and that GH3 cells have been employed as a prolactinoma model, we compare the ER α expression in GH3 vs normal pituitary cells. The analysis of mER α expression by western blot and immunofluorescence,

revealed an increased level of this receptor in tumour cells compared to normal pituitary cells. The involvement of mER α in the rapid pro-apoptotic action of E2 in normal pituitary cells has been previously demonstrated (Zarate *et al.* 2012). In contrast, in pituitary tumour GH3B6/F10 cells, high levels of mER α mediated rapid signalling responses to oestrogens, which culminated in functional changes such as prolactin release, cell proliferation, apoptosis and changes in cell shape (Jeng *et al.* 2009, Jeng & Watson 2011). However, these studies in both normal and pituitary tumour cell did not reveal the

contribution of palmitoylation to ER α translocation to the plasma membrane. In the present investigation, we detected palmitoylated ER α in normal and GH3 pituitary tumour cells for the first time and demonstrated that E2 stimulated ER α expression in the plasma membrane, which was reverted by the palmitoylation inhibitor. Thus, palmitoylation (a reversible posttranscriptional modification) should be considered to be more than just a simple membrane association of soluble proteins. In fact, the palmitoylation status of several proteins has also been linked to their activation and the regulation of the traffic and function of both the nuclear/cytoplasmic and the membrane receptor pool (Fukata & Fukata 2010).

In addition to full-length mER, we detected an ER α -palmitoylated variant at around 50 kDa in the membrane fraction of pituitary tumour cells. In agreement with this, other authors have reported that, as well as full-length 66-kDa ER α , truncated forms of this receptor were present in various organs, produced by alternate ER mRNA splicing or specific post-translational processing, often outside the nucleus. In agreement, a 46-kDa truncated variant has been shown to be preferentially palmitoylated and enriched in the cell membranes of endothelial, osteoblast and breast cancer cells (Denger *et al.* 2001, Marquez & Pietras 2001, Li *et al.* 2003), and palmitoylation inhibitors were able to block ER-46 membrane localisation (Acconcia *et al.* 2005). Moreover, variants of lower molecular weights (~39 kDa and ~22 kDa) were detected in the membrane fraction of anterior pituitary cells (Zarate *et al.* 2012) and breast cancer cells, suggesting that these ER α variants may be considered as a target of palmitoylation and result in their localisation in the plasma membrane (Li *et al.* 2003, Wang *et al.* 2006).

Palmitoyl-acyltransferase isoform expression and localisation is tissue specific (Ohno *et al.* 2006), with at least a dozen of the 23 human DHHC genes having been implicated in tumour growth (Yeste-Velasco *et al.* 2015). DHHC7 and DHHC21 are the proteins responsible for the palmitoylation of the sex steroid oestrogen, progesterone and androgen receptors. DHHC-7 and -21 knockdown studies have shown that PATs are required for endogenous ER palmitoylation, membrane trafficking and rapid signal transduction in cancer cells (Pedram *et al.* 2012). In the present study, we observed greater *Dhhc7* and *Dhhc21* mRNA expression in tumour cells than in normal pituitary cells, which may be associated with the proliferative behaviour of GH3 cells. It has been reported that the *Dhhc21* gene is significantly overexpressed in human breast cancer compared with normal breast epithelium. It is possible that alterations in the steroid receptor PAT

abundance or function contribute to increased ER at the plasma membrane in some situations (Pedram *et al.* 2012), thereby making the DHHC-7 and -21 proteins attractive novel targets to selectively inhibit membrane sex steroid receptor localisation and function in pituitary tumours.

Although s-acylation is known to be a major regulator of localisation of cellular protein and pathways, there is still little information about how the dynamics of this process is regulated. It has also been reported that palmitoylation regulation may occur via a regulatory mechanism occurring at the mRNA level of the DHHC enzymes (Chai *et al.* 2013). In this study, E2 treatment for 3 h reduced both *Dhhc7* and *Dhhc21* mRNA expression, whereas at 30 min the plasma membrane ER α pool and the interaction ER α /caveolin were increased. The current knowledge of oestrogen molecular action includes the ability of the E2-ER complex both to induce gene transcription (Smith & O'Malley 2004) and to evoke the membrane-starting activation of specific rapid phosphorylation cascades (ERK/MAPK) (Yang *et al.* 2004). Both these processes are integrated and influence the cellular response to oestrogen, thus highlighting the ER regulation at genomic and nongenomic levels. The fast action/membrane of E2 (30 min) was not in line with that observed after sustained stimulation of E2 for 3 h, which downregulated the mRNA levels of *Dhhc7* and *Dhhc21*, probably as a compensatory mechanism to regulate the ER α pool at the plasma membrane. These results are in agreement those of an investigation that demonstrated that E2 stimulation for 1 to 4 h decreased by 60% the [3H]-palmitate incorporated into ER α in HeLa cells, suggesting that ER α palmitoylation is negatively modulated by E2 (Acconcia *et al.* 2005).

The relationship between ER α and caveolin appears to be important for determining E2 effects on different cell types, with it having been demonstrated that caveolin-1 is an essential for joining ER α to the cell membrane and that this process is facilitated by prior ER palmitoylation (Pedram *et al.* 2007). Our results revealed that the ER α /caveolin-1 interaction increased after E2 stimulus in normal as well as in GH3 pituitary tumour cells. Concurring with our data, an ER α /caveolin-1 interaction was demonstrated in enriched mER α GH3/B6/F10 pituitary tumour cells (Watson *et al.* 2012). In addition, it has been reported that, as E2 is highly concentrated in isolated caveolae, it readily engages ER α bound to caveolin-1, which serves as a scaffold for membrane-localised signalling molecules (Peffer *et al.* 2014). Therefore, caveolin may be a fundamental scaffolding protein whose activation maximises membrane hormone effects and leads to specific biological consequences. This idea is supported

by caveolin knockdown rats as they, showed a reduction in membrane ER α functions, thereby suggesting that trafficking of ER α to the plasma membrane is mediated by caveolin (Christensen & Micevych 2012). It has also been demonstrated that caveolin-1 protein downregulation leads to ER α signalling deregulation in mammary epithelia (Wang *et al.* 2011). We observed that the ER α /caveolin-1 association was palmitoylation dependent, as indicated by the decrease in this association after palmitoylation inhibition. A non-palmitoylable ER α -Cys477Ala mutant was unable to localise at the plasma membrane, interact with caveolin-1 or generate E2-induced rapid membrane-starting signal pathways to regulate cell proliferation (Acconcia *et al.* 2005). Moreover, it has been demonstrated that ER α rapid de-palmitoylation and decoupling the ER α action mechanisms impair the activation of the ERK/MAPK and PI3K/AKT signal transduction pathways (Totta *et al.* 2004, Levin 2005). These reports are in agreement with our results, where it was observed that pre-incubation with 2BP in GH3 tumoural cells decreased the ER α and caveolin-1 interaction, as well as ERK1/2 phosphorylation, suggesting that palmitoylation is necessary for a mediated E2 effect.

Several members of the MAP kinase signalling pathway, including Src, Shc and ERKs, are clustered in caveolae-specialised membrane invaginations that are enriched in the caveolin-1 scaffolding protein and compartmentalise signal transduction (Okamoto *et al.* 1998). ER α activation may trigger cell proliferation mediated by ERK (Jeng *et al.* 2009, Watson *et al.* 2010, 2012), with the MEK/ERK1/2 pathway being involved in the pathogenesis of several types of tumours including pituitary adenomas (Vlotides *et al.* 2008, Ebbesen *et al.* 2016). It has been reported that the inhibition of ERK1/2 signalling reduced cell viability in rat tumour cells after exposure to a general antagonist of ER (Gao *et al.* 2017). Here, we observed that the oestrogen antagonist and the palmitoylation inhibitor prevented the activation of ERK1/2 and resulted in a decrease in ER α expression levels in the plasma membrane as well as in cell pituitary proliferation, indicating that rapid E2-induced signals require ER localisation at the plasma membrane. Pedram *et al.* reported that knockdown of DHHC7 or 21 significantly impaired the ability of E2 to stimulate ERK in breast cancer cells (Pedram *et al.* 2012). In addition, the expression of ER α without a palmitoylated site interfered with endogenous ER function and inhibited E2-induced ERK activation, cyclin D1 production, cdk4 activity and G1/S progression, suggesting that the inhibition of mER α expression and its association with the modulation of ERK activity could be put forward as an important therapeutic intervention in breast cancer (Razandi *et al.* 2003).

It is generally accepted that oestrogens act as potent mitogens through ER α , exerting a sustained, dose-dependent trophic stimulus on anterior pituitary proliferation (Nolan & Levy 2009). In our study, in primary pituitary cells with a basal proliferation of 2.6%, E2 treatment was unable to modify this mitotic rate. In contrast, in pituitary tumour cells, which exhibited a high basal proliferative activity, E2 stimulation triggered an increase in BrdU uptake with a significant contribution of mER α . Furthermore, palmitoylation inhibition induced a significant decrease in cell proliferation, which was consistent with previous reports showing that mER α contributes, together with the nuclear ER α pool, to the induction of tumour cell proliferation (Razandi *et al.* 2003). In addition, significantly increased ER α localisation in the plasma membrane has been associated with aggressive breast cancer behaviour or resistance to endocrine therapy (Yang *et al.* 2004, Fan *et al.* 2007).

Moreover, these result indicated that oestrogens were able to trigger a proliferative response in the pituitary tumour cell, associated with high levels of ER α and the activation of ERK1/2 signalling (Watson *et al.* 2008, Jeng *et al.* 2009). In breast cancer cells, E2-ER α -induced cell transition through G1 to the S phase of the cell cycle, which significantly blocked by 2BP or by inhibitors of MEK, suggesting that membrane localisation of palmitoylated ER α leads to a signal transduction that contributes to cell cycle progression (Pedram *et al.* 2007). Additionally, the ERK/MAPK and PI3K/AKT pathways, activated by the E2-ER α complex, cooperatively promote the G1/S transition (Marino *et al.* 2002, Acconcia *et al.* 2005). In our study, we observed that the palmitoylation inhibitor induced a partial reversion in the tumour proliferation by E2 in a pERK1/2-dependent and pAKT-independent manner.

The differences in the proliferation effect observed between normal and pituitary tumour cells under E2 treatment may be explained by the high ER α expression in tumour cells compared to normal cells, as well as by the undetectable subtype ER β expression in GH3 pituitary tumour cells as was previously reported in our laboratory. In addition, we determined the specific role of ER β in the E2 proliferative effect in normal, hyperplastic and pituitary tumour cells, with this hormone being able to increase pituitary cell proliferation only in cells with a high ER α / β ratio, showing that ER β exerts an inhibitory role on the mitogenic activity of pituitary cells (Perez *et al.* 2015). The reason that different cellular phenotypes can respond to the same hormone in a different manner may be due to the diverse expression patterns of ER α and ER β (McDonnell & Norris 2002).

In summary, our results showed that E2 modulated ER α palmitoylation, enhancing the mER α pool and consequently activating the ERK pathway, thereby contributing to pituitary tumour cell proliferation. These findings suggest that mER α could be related to the proliferative behaviour of prolactinoma and be a possible marker of pituitary tumour growth.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The authors would like to make the following declarations about their contributions: L d V S, J P P and A I T conceived and designed the experiments. L d V S, J P P, S C, J P N, F P and P P performed the experiments. L d V S, J P P, S C, J P N and F P analysed the data. L d V S, J P P, A D P, J V-T, S G and A I T prepared the manuscript.

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