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Sosa LV, Gutiérrez S, Petiti JP, Vaca AM, De Paul AL, Torres AI. Cooperative effect of E_2 and FGF2 on lactotroph proliferation triggered by signaling initiated at the plasma membrane. *Am J Physiol Endocrinol Metab* 305: E41–E49, 2013. First published May 7, 2013; doi:10.1152/ajpendo.00027.2013.—In the present work, we investigated the effect of 17β-estradiol (E_2) and basic fibroblast growth factor 2 (FGF2) on the lactotroph cell-proliferative response and the related membrane-initiated signaling pathway. Anterior pituitary mixed-cell cultures of random, cycling 3-mo-old female rats were treated with 10 nM E_2, E_2 membrane-impermeable conjugated BSA (E_2-BSA), PPT (ERα agonist), and DPN (ERβ agonist) alone or combined with FGF2 (10 ng/ml) for 30 min or 4 h. Although our results showed that the uptake of BrdU into the nucleus of lactotrophs was not modified by E_2 or FGF2 alone, a significant increase in the lactotroph uptake of BrdU was observed after E_2/FGF2 coincubation, with this effect being mimicked by PPT/FGF2. These proliferative effects were blocked by ICI 182,780 or PD-98059. The involvement of membrane ER in the proliferative response of prolactin cells induced by the steroid and FGF2 coincubation was confirmed using E_2-BSA, and the association between ERα and FGF receptor was observed after E_2/FGF2 treatment by immunoprecipitation. A significant increase in the ERK1/2 expression was noted after E_2, E_2-BSA, PPT, and FGF2 alone, which was more noticeable after E_2-BSA/FGF2, E_2/FGF2, or PPT/FGF2 treatments. This study provides evidence that E_2 and FGF2 exert a cooperative effect on the lactotroph proliferation principally by signaling initiated at the plasma membrane triggering a genomic effect mediated by MEK/ERK1/2, a common signaling pathway, that finally regulates the lactotroph population, thus contributing to pituitary plasticity.

17β-estradiol; fibroblast growth factor 2; membrane estrogen receptor; lactotroph proliferation; mitogen-activated kinase effector kinase/extracellular signal-regulated kinase 1/2.

LACTOTROPH CELLS REPRESENT A DYNAMIC POPULATION in the pituitary gland that exhibits significant changes in the cell number under different physiological reproductive states, such as the estrous cycle, pregnancy, and lactation, in response to prolactin requirements resulting from the stimulatory action of neuropeptides, steroid hormones, and growth factors (3, 13, 31, 38, 62). Pituitary lactotrophs are typical estrogen-responsive cells, with it being well known that estrogen treatment in vivo and in vitro induces lactotroph proliferation (15, 47, 50). Additionally, the mitogenic action of estrogen on lactotroph cells has been suggested to be mediated by paracrine growth factors secreted from neighboring estrogen receptor-positive cells (19).

The physiological functions of estrogen can be mediated by the classical cytoplasmatic/nuclear estrogen receptors (ERα and ERβ, which work as ligand-activated transcription factors and are localized in different pituitary cell types (45). In addition, it has been described that estradiol effects are exerted through a pool of ER at the plasma membrane [membrane estrogen receptor (mER)] in different tissues (24, 39), including anterior pituitary endocrine cells (14, 26, 30, 63, 64). The identity of these membrane receptors has been quite controversial, but to date, the presence of classical ERα and ERβ and various truncated ERs (ERα46, ERα36), as well as other proteins such as GPR30, has been described in this subcellular localization (21, 32, 39). The mER activation rapidly triggers multiple signal transduction cascades such as protein kinase C (PKC) (54), protein kinase A (4), nitric oxide (36), mitogen-activated protein kinase (MAPK) (6), phosphatidylinositol 3-kinase (PI3K)-Akt (58), and increased calcium levels in different tissues (7). By definition, the rapid effects of estrogen that involve nongenomic mechanisms are independent of the transcriptional activation mediated by the intracellular ER. However, it has been reported that signaling initiated in the plasma membrane can produce the activation of genomic events (44, 61, 64), with the interaction between estradiol and growth factors such as EGF or IGF-I stimulating cell proliferation in different tissues (12, 37, 55). This effect might be mediated through direct interactions between ER with kinase-dependent growth factor receptors or scaffolding proteins (23, 57). Even though the main effect of estradiol and growth factors is related with the enhanced lactotroph proliferation, we demonstrated that 17β-estradiol (E_2) was able to reverse the proliferative effect of IGF-I or insulin on lactotroph cells (28, 29). These apparently contradictory actions may contribute to the fine adjustment of the size of the pituitary cell populations through intracellular mechanisms that have not yet been fully clarified.

Another important regulator of lactotroph cell population is the basic fibroblast growth factor 2 (FGF2) (10), a potent mitogen synthesized by adenohypophysis (5), with the folliculo-stellate cells being the main source of this growth factor (20, 34). In previous studies, we reported an increase in FGF2 expression in estrogen-induced experimental prolactinomas (48). The biological effects of FGF2 are mediated by high-affinity transmembrane receptors called FGF receptors (FGFR), which have intrinsic tyrosine kinase activity (46). These receptors can activate multiple signaling pathways, including PKC, Src kinases, Ras, and MAPK p44/42 (9, 11). Previous studies have shown dysregulation of the FGF/FGFR function. In human pituitary adenomas, alterations have been demonstrated in both the FGFR subtype and isoform expression (1), whereas increased FGFR levels were reported in the tumoral pituitary of dopamine D2 receptor-knockout mice (8).

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The interdigitation of folliculo-stellate and prolactin cells indicates a dynamic action of intercellular communication between the two cell types. In this way, a paracrine role for FGF2 has been described in mediating the estrogen-proliferative action on lactotroph cells (33). However, another report has suggested that the estrogen-induced mitogenic and antimitogenic actions on lactotrophs do not require paracrine signals from other pituitary cell types, thus directly influencing lactotroph proliferation (35).

To investigate how estradiol and growth factors regulate the prolactin cell population size in the pituitary gland, we evaluated the E_2 and FGF2 combined effects on the lactotroph cell-proliferative response and determined the involvement of membrane ER and the MEK/ERK1/2 signaling pathway in this effect.

MATERIALS AND METHODS

Reagents and antibodies. E_2, E_2 6-*O*-carboxymethyloxime-BSA (E_2-BSA), FGF2, ER antagonist ICI 182,780, and anti-diphosphorylated ERK1/2 monoclonal anti-MAPK activate (M8159) were purchased from Sigma-Aldrich (St. Louis, MO). Selective ERα agonist 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-tryl) trisphenol (PPT) and β-agonist 2,3-bis(4-hydroxyphenyl)-propio-nitrile (DPN) were acquired from Tocris Cookson, and the inhibitor of mitogen-activated kinase effector kinase (MEK) PD-98059 was obtained from Calbiochem (San Diego, CA). Antibodies recognizing ERα MC-20 (directed to the COOH terminus of the ERα-sc542), FGFR (Flg C-15-121), total (sc-8312) and phosphorylated Akt 1/2/3 (Ser^{473}, sc-33437), and total ERK1 (C-16, sc-93) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-prolactin (PRL) was provided by Dr. A. Parlow (National Hormone and Pituitary Program, Torrance, CA).

Animals. Three-month-old female virgin rats of the Wistar strain were used. A pool of these animals ($n = 20–25$) was assigned to each culture taken at random cycle stages. All rats were bred and housed at the Animal Research Facility of the National University of Córdoba under controlled temperature (21 ± 3°C) and lighting conditions (14:10-h light-dark cycle), having free access to commercial rodent food and tap water. Animals were kept in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (1996), and the experiments were approved by the Institutional Animal Care Committee of School of Medicine, National University of Córdoba.

Anterior pituitary cell cultures. The rats were decapitated within 10 s of removal from their cage, thus avoiding any stress or external stimuli. The protocol for the dissociation of the pituitary cells has been described elsewhere (16). Briefly, anterior pituitaries excised from female rats were placed in minimal essential medium for suspension culture before being minced, digested with 0.4% trypsin, and dispersed with Pasteur pipettes. The cell yield was 1.5×10^6 per pituitary, and the cell viability, tested with trypan blue exclusion, was always >90%. The final suspension was adjusted to 1×10^6 cells/ml medium. To analyze the protein expression by Western blotting, immunoprecipitation, and ultrastructural immunocytochemistry, the cells were plated on six-well culture plates (Corning) at a density of 1×10^6 cells/well. For evaluating lactotroph proliferation and immunofluorescence by confocal laser-scanning microscopy, the cells were seeded on glass coverslips (13 mm) at a density of 1.5×10^5 cells/well on 24-well culture plates (Corning) before being maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4% fetal calf serum and 8% horse serum (Gibco) in an incubator with a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 3 days.

All culture media were filtered through 0.2-μm Nalgene membranes (Nalge), and the cell culture grade reagents were obtained from Sigma Chemical (St. Louis, MO).

Cell treatments and use of inhibitors. After 3 days of culture, the cells were maintained in DMEM without phenol red or serum and supplemented with hydrocortisone (100 μg/l), 3,3′-triiodothyronine (400 ng/l), transferrin (10 mg/l), and sodium selenite (5 μg/l) for 24 h before treatments. Then the cells were stimulated for 30 min or 4 h with vehicle (0.1% BSA), E_2 (10 nM), E_2-BSA (10 nM), PPT (10 nM), DPN (10 nM), and FGF2 (10 ng/ml) either alone or in combination. In some experiments, the cells were preincubated with a competitive inhibitor of E_2 binding (ICI 182,780; 100 nM) (29, 58) or with the specific MEK inhibitor PD-98059 (100 μM) for 30 min (51). At the end of each experimental condition, the anterior pituitary cells were processed by different techniques.

A stock solution of E_2-BSA was made according to our previous report (26, 58) by dissolving the powder in buffer (50 mM Tris·HCl, pH 8.5) at 1 mg/ml. An aliquot of the solution was added to a centrifugal filter unit with a molecular weight cutoff of 3,000 (Millipore, Bedford, MA) and subjected to centrifugation at 13,000 *g* for 30 min. The filtrate was recovered, and the retentate was washed three times with 400 μl of Tris·HCl buffer.

Immunocytochemical detection of lactotroph proliferation. Cells at the DNA-synthesizing stage and lactotrophs were identified by the dual-immunocytochemical detection of bromodeoxyuridine (BrdU) and PRL, according to Oomizu et al. (49), with modifications (28). After 30 min or 4 h of stimulation with the different reagents, culture medium was replaced, and BrdU (100 nM) was added for an additional 24 h. The cells attached to coverslips were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature (RT), washed in PBS, and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Then, nonspecific immunoreactivity was blocked with 5% PBS-BSA for 30 min at RT, and cells were incubated overnight with monoclonal antibody to BrdU (Amersham, Buckinghamshire, UK) at 4°C in a wet chamber. After being washed in PBS, cells were incubated in biotinylated anti-mouse IgG (1/100) for 1 h at RT, and coverslips were washed again in PBS and incubated in avidin-biotin-peroxidase complex. The immunoreactivity of BrdU was visualized using 3,3-diaminobenzidine tetrahydro-chloride as the chromogen to leave the nuclei of proliferating cells stained brown. PRL immunocytochemistry for lactotroph detection was then performed on the same coverslip. Cells were incubated with rabbit anti-rat PRL (1/3,000) in a wet chamber for 1 h at 37°C, washed in PBS, and incubated in biotinylated anti-rabbit IgG (1/150) for 1 h. Next, avidin-biotin-peroxidase complex was applied for 30 min at RT, and immunoreactivity for PRL was detected using chloronaphthol, which immunostained the acquired lactotrophs a purplish blue color. Finally, coverslips were mounted on glass slides using glycerol. Controls were also performed by applying the same protocols, but BrdU or PRL antibodies were omitted. A total of 1,000 PRL-immunoreactive cells were examined by light microscopy using a systematic process, standardized in our laboratory, on each glass slide to establish the proportion of double-positive BrdU-PRL cells in the total PRL-positive cells. Three slides were analyzed for each experimental condition.

Ultrastructural immunocytochemistry for FGFR in lactotroph cells. To identify the presence of FGFRs in the plasma membrane of lactotroph cells, a procedure for immune labeling in intact cells was performed (27). This technique, designed for antigen labeling on the cell surfaces prior to fixation, allows an excellent preservation of the antigen and cellular fine structure, thereby improving the immunocytochemistry and the ultrastructural morphology. After 96 h of culture, the pituitary cells were rinsed with Hanks' solution, with the unspecific endogenous antigens being blocked with 1% PBS-BSA for 15 min at 37°C. Cells were incubated with anti-FGFR (1/50) for 1 h at 37°C before being rinsed with Hanks' and treated with 1% PBS-BSA for 15 min at 37°C. Subsequently, the immunoreactive sites were incubated with anti-rabbit secondary antibody conjugated to 15 nm of colloidal gold particles (15 nm-1/20; Electron Microscopy, Hatfield, PA) for 1 h at RT. To validate the specificity of the immunostaining,

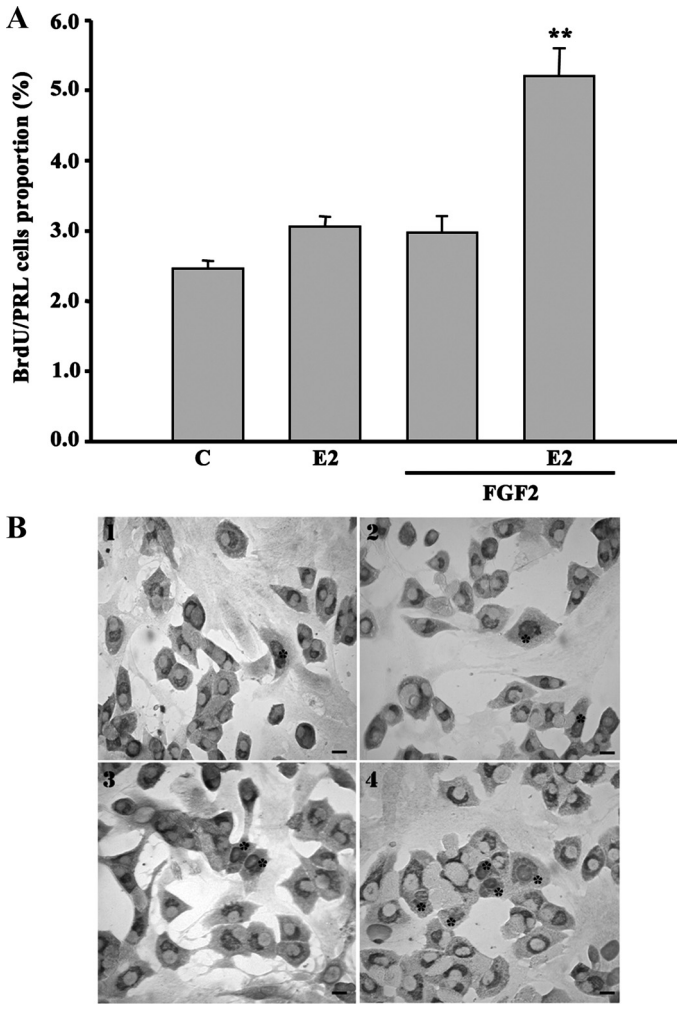

Fig. 1. Effect of 17β-estradiol (E_2) and fibroblast growth factor 2 (FGF2) combined treatments on lactotroph proliferation. *A*: primary pituitary cell cultures were treated with E_2 (10 nM) and FGF2 (10 ng/ml) alone or in coincubation for 4 h, and then bromodeoxyuridine (BrdU) was added for an additional 24 h. The data represent the proportion of double-positive BrdU-prolactin (PRL) cells in the total PRL-positive cells. The data are shown as means ± SE of 3 wells from 3 independent experiments ($n = 9$), and data were evaluated by the ANOVA-Tukey test. $^{**}P < 0.01$ vs. E_2 and FGF2. *B*: representative light microscope photomicrograph of double-immunocytochemical detection of BrdU and PRL from control (*image 1*) and treated cells: E_2 (*image 2*), FGF2 (*image 3*), and E_2/FGF2 (*image 4*). The PRL exhibits a paranuclear distribution (dark gray), with the BrdU label being localized in the nucleus (medium gray) of positive lactotroph cells (*). Scale bar, 10 μm.

both negative and absorption controls (blocking peptide, sc-121 P; Santa Cruz Biotechnology) were performed by applying the same protocol but replacing primary antibody with 1% PBS-BSA or antibody preabsorbed with purified antigens.

After the immunocytochemical reaction, the cells were scraped from the wells, centrifuged, and fixed in Karnovsky fluid (1.5% glutaraldheyde and 1.5% formaldehyde in 0.1 M cacodylate buffer). These were then centrifuged, and the pellet was treated with 1% OsO_4 for 1 h before being stained in bloc with 1% uranyl acetate in 0.1 M acetate buffer, pH 5.2, for 20 min. After dehydration using a series of graded cold acetones, the cells were embedded in Araldite. Then, thin sections were cut using a JEOL ultramicrotome with a diamond knife.

After that, and to identify the lactotroph cells, the same cell culture sections were etched for 7 min with 10% hydrogen peroxide, incubated on a drop of 1% normal goat serum for 45 min, and then transferred overnight to a drop of anti-rat PRL (1/5,000) and labeled with goat anti-rabbit IgG-colloidal gold complex (5 nm-1/30) for 1 h at 37°C. Finally, the grids were stained with uranyl acetate and examined in a Zeiss LEO 906-E electron microscope.

Immunoprecipitation. Aliquots from the protein extract of pituitary cells (1 mg of protein) were subjected to immunoprecipitation using specific rabbit antiserum against ERα (10 μg/ml). The immune complexes were adsorbed and precipitated using protein A-Sepharose beads (Sigma Aldrich), washed three times with lysis buffer, and denatured by boiling for 5 min in sample buffer. Parallel immunoprecipitations were performed using pituitary cell lysate as the positive control to detect ERα with the known interactor IGF-I receptor, (40) using the total extract from the Hela ER-negative cell line as negative control.

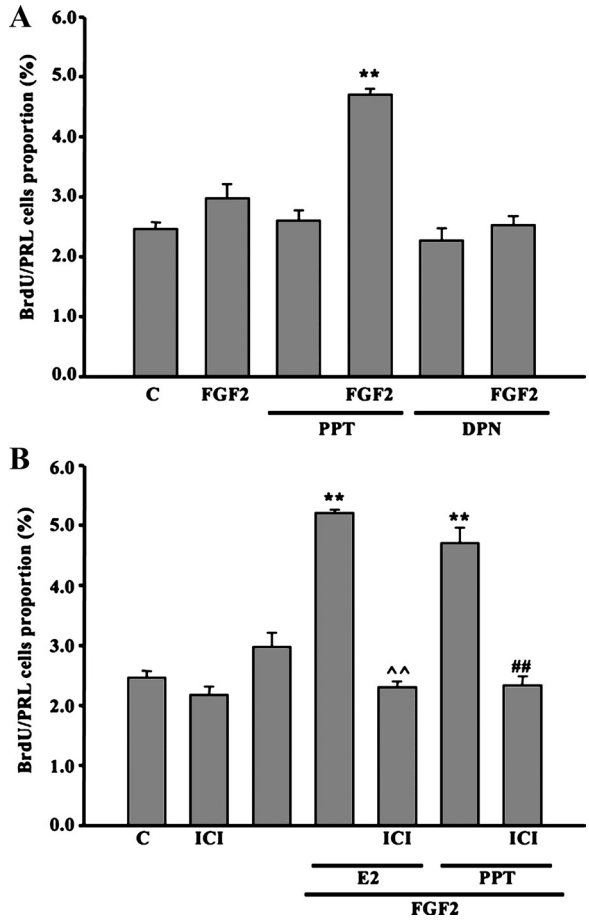

Fig. 2. E_2 acts through estrogen receptor-α (ERα) to exert a proliferative effect on lactotroph cells in interaction with FGF2. *A*: primary pituitary cell cultures were treated with 10 nM E_2, 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-tryl) trisphenol (PPT), 2,3-bis(4-hydroxyphenyl)-propio-nitrile (DPN), or 10 ng/ml FGF2 either alone or in coincubation for 4 h, and then BrdU was added for an additional 24 h. The data represent the proportion of double-positive BrdU-PRL cells in the total PRL-positive cells. Results are given as means ± SE of 3 wells from 3 independent experiments ($n = 9$), and data were evaluated by ANOVA, followed by Tukey's posttest. $^{**}P < 0.01$ vs. PPT and FGF2. *B*: primary pituitary cell cultures were pretreated with ICI 182,789 (ICI) for 30 min, and then the cells were stimulated with E_2 (10 nM), PPT (10 nM), and FGF2 (10 ng/ml) alone or in coincubation for 4 h. Finally, BrdU was added for an additional 24 h. The data represent the proportion of double-positive BrdU-PRL cells in the total PRL-positive cells. Results are given as means ± SE of 3 wells from 3 independent experiments ($n = 9$), and data were evaluated by ANOVA, followed by Tukey's posttest. $^{**}P < 0.01$ vs. control (C); $^{\wedge\wedge}P < 0.001$ vs. E_2/FGF2 without ICI; $^{\#\#}P < 0.01$ vs. PPT/FGF2 without ICI.

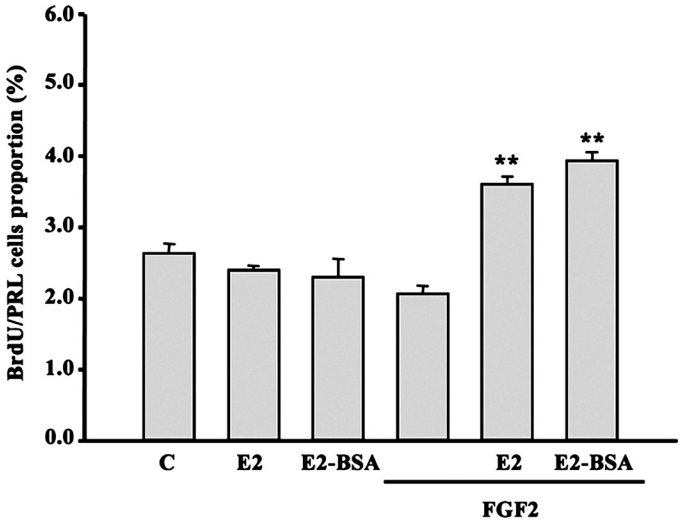

Fig. 3. E_2 in interaction with FGF2 enhanced the lactotroph cell proliferation through membrane ER. Cells were treated with E_2 (10 nM), E_2-BSA (10 nM), and FGF2 (10 ng/ml) alone or in coincubation for 30 min, with BrdU being added for an additional 24 h of factor incubation. The data represent the proportion of double-positive BrdU-PRL cells in the total PRL-positive cells. Results are given as means ± SE of 3 wells from 3 independent experiments ($n = 9$), and data were evaluated by ANOVA, followed by Tukey's posttest. $^{**}P < 0.01$ vs. E_2, E_2-BSA, and FGF2.

The membranes were blocked with 5% nonfat dry milk diluted in 0.1% Tween-20 Tris-buffered saline and incubated overnight with the primary antibodies to FGFR (1/200–1 μg/ml). Then the membranes were processed as described in *Preparation of cell lysates for Western blotting analysis*.

Preparation of cell lysates for Western blotting analysis. Once the experimental protocols were completed, the pituitary cells were rinsed with PBS and lysed on ice by the addition of 120 μl of cold PBS containing 1.25% Igepal CA-630, 1 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM NaF, and 20 mM sodium pyrophosphate. This was followed by the scraping and transfer of the lysate to a centrifuge tube placed on ice. After 30 min, these lysates were centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatants were collected and stored in aliquots frozen at −40°C until required.

The protein concentration of the cell lysates was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Fifty micrograms of total homogenate was separated using 12% acrylamide gel (Sigma Chemical), and to estimate the corresponding molecular weights a full-range rainbow molecular weight marker was run in parallel (Amersham-Life Science, Bucks, UK). The proteins were transferred to the nitrocellulose membrane, and nonspecific binding was blocked with PBS containing 5% nonfat dried milk and 0.1% Tween-20 (blocking buffer) at room temperature. The membranes were rinsed and incubated for 2 h with the appropriate primary antibody: rabbit anti-phosphorylated Akt (1/300–0.70 μg/ml), rabbit anti-total Akt (1/300–0.70 μg/ml), mouse anti-diphosphorylated ERK 1/2 (1/700–0.2 μg/ml), or rabbit anti-total ERK1 (1/700–0.28 μg/ml). The blots were incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1/5,000) or anti-mouse secondary antibody (1/2,500 Jackson Immunoresearch Laboratories, West Grove, PA) diluted in blocking buffer before being thoroughly rinsed in PBS-0.1% Tween-20. The HRP-coupled secondary antibody was revealed with ECL Western blotting detection reagents (Inmuno Star HRP Substrate Kits; Bio-Rad Laboratories, Hercules, CA), following the manufacturer's instructions. Finally, the emitted light was captured on Hyperfilm (Amersham-Pharmacia-Biotech, Bucks, UK), and signals were scanned and quantified with Scion Image software (V. Beta 4.0.2; Scion Image, Frederick, MD) at three different exposure times.

Statistical analysis. The statistical analysis was carried out on three replicates measured from three independent cell cultures, using an analysis of variance with Tukey's posttest (InfoStat version 2004; Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Cordoba). The results were given as means ± SE, and the significance levels were chosen at $P < 0.05$.

RESULTS

E_2/FGF2 coincubation increased the lactotroph cell proliferation. The effect of E_2 and FGF2 on lactotroph cell proliferation was evaluated. To carry this out, the uptake of BrdU into the nucleus of cells immunolabeled for PRL was analyzed.

Under basal conditions, 2.5% of lactotroph cells showed uptake of BrdU. Although treatment with E_2 (10 nM) or FGF2 (10 ng/ml) alone for 4 h did not induce modifications in the uptake of BrdU in lactotroph population in serum-free conditions, the interaction of E_2/FGF2 triggered about a twofold

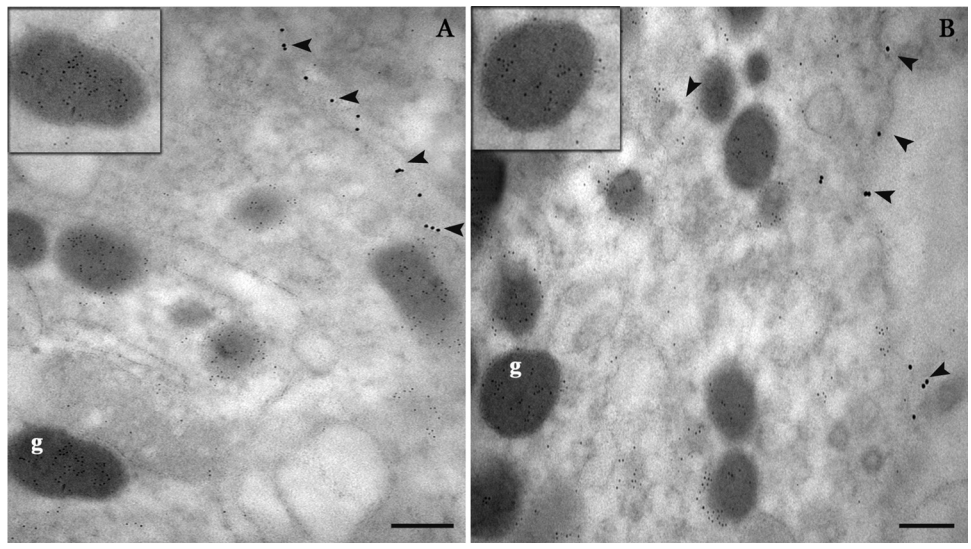

Fig. 4. The lactotroph cells expressed FGF receptor at the plasma membrane. Electron micrographs: type I [secretory granules (g), diameter 500–900 nm; *A*] and type II lactotroph (g, diameter 300–500 nm; *B*) immune-labeled with 5-nm gold particle exhibiting the FGF receptor adhered to the plasma membrane and some apparently located over an endocytic vesicle (arrows, 15-nm gold particle). *A* and *B*, *insets*: g identified for PRL. Bar = 500 nm.

increase in the lactotroph cell number, taking up BrdU with respect to the baseline level ($P < 0.01$) (Fig. 1, *A* and *B*).

ERα mediated the proliferative effect exerted by E_2/FGF2. To investigate whether the mitogenic effect of E_2 interacting with FGF2 was mediated by ERα or -β, the ERα and -β agonists PPT and DPN were used. Anterior pituitary cell cultures were either incubated with PPT (10 nM), DPN (10 nM), or FGF2 (10 ng/ml) alone or coincubated with the E_2 agonists for 4 h. Figure 2*A* shows that the number of lactotrophs undergoing mitosis did not change with PPT or DPN alone. However, PPT/FGF2 coincubation induced a significant increase in BrdU-labeled lactotrophs, which almost duplicated the values obtained with PPT or FGF2. This proliferative response was similar to that achieved after the E_2/FGF2 combined treatment, as shown in Fig. 1. In contrast, DPN/FGF2 failed to increase the number of lactotroph cells taking up BrdU. These results suggest that ERα contributed to the E_2/FGF2 combined stimulatory effects on lactotroph cell proliferation. Also, the anterior pituitary cells were preincubated with ICI 182,780 (100 nM) for 30 min prior to exposition to the specific factors for an additional 4 h, and the uptake of BrdU was analyzed. As shown in Fig. 2*B*, ICI 182,780 blocked the proliferative effects promoted by E_2/FGF2 or PPT/FGF2 ($P < 0.01$), thus confirming the participation of specific ERs in the proliferative effects.

mER was involved in the lactotroph-proliferative effects induced by E_2/FGF2. To analyze the involvement of mER in the mitogenic activity of the lactotrophs induced by E_2/FGF2, the pituitary cells were stimulated with E_2-BSA (E_2 covalently linked to BSA) and FGF2 for 30 min. Previously, the plasma membrane has been shown to be impermeable for steroids conjugated to BSA, which have been widely used as tools to investigate nongenomic, cell surface steroid effects (59). Our results showed that the percentage of BrdU-positive lactotroph cells after E_2-BSA/FGF2 coincubation was similar to that of E_2/FGF2 stimuli (Fig. 3). These results suggest that mER participated in the lactotroph cell mitogenic activity induced by E_2 in interaction with FGF2.

The FGFR was identified in lactotroph cells. Considering the cooperative effect of E_2/FGF2 on the proliferative response of lactototroph cells, we investigated the presence of FGFRs at the plasma membrane in this cell population. To carry this out, the preembedding immune gold electron microscopy technique was used, following a protocol established in our laboratory (18). The lactotroph cells revealed colloidal gold particles (15 nm) attached to the cell surface as well as others that adhered to the membranes of cytoplasmic vesicles, indicating the presence of FGFR. By applying double-immune gold labeling, it has been possible to identify the type I lactotroph containing large, irregularly shaped electron-dense secretory granules (diameter 500–900 nm) and type II cells with medium-sized spherical granules (300–500 nm) labeled with small colloidal gold particles (5 nm) that coexpressed the FGFR in the plasma membrane (Fig. 4).

ERα and FGFR coimmunoprecipitation was detected after E_2/FGF2 stimuli. To assess a possible interaction between ERα and FGFR, immunoprecipitation assays were carried out. Endogenous ERα was immunoprecipitated from the pituitary cell culture stimulated with E_2 alone, FGF2 alone, or a combination of both for 30 min. Treatment with E_2 was unable to induce interaction between ERα and the FGFR, whereas the FGF2 treatment induced a significant association between both receptors, which was greater after E_2/FGF2 stimulus for 30 min, as shown in Fig. 5*A*. In the same lysate, we observed immunoprecipitated ERα associated with IGF-I receptor as a positive control. Extracts from the Hela ERα-negative cells were used as negative control (Fig. 5*B*).

ERK1/2 mediated the proliferative effect induced by E_2/FGF2. To investigate the membrane-initiated signaling involved in the lactotroph cell proliferation induced by the E_2 and FGF2 interaction, total and phosphorylated Akt and ERK1/2 expression were analyzed by immunoblotting using specific antibodies.

Figure 6*A* shows that the Akt phosphorylation did not exhibit any significant variation after the use of E_2, E_2-BSA, or FGF2 alone or in coincubation treatments for 30 min. However, a significant increase in phosphorylated ERK1/2 expression was observed after both E_2-FGF2 and E_2-BSA-FGF2 treatments for 30 min (Fig. 6*B*).

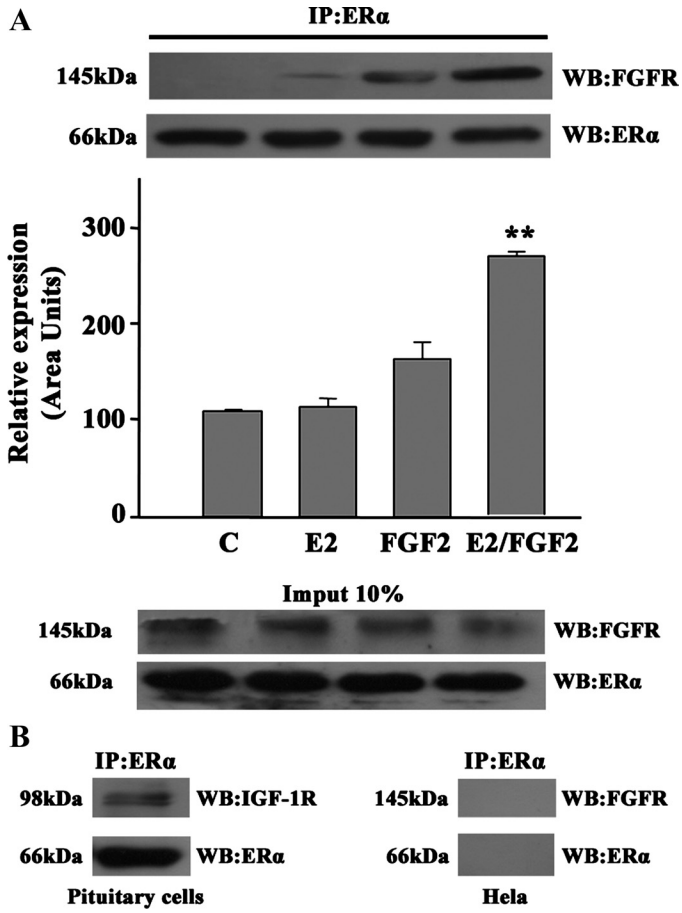

Fig. 5. The association between ERα and FGFR was observed after E_2/FGF2. *A*: primary pituitary cell cultures were treated with E_2 (10 nM) and FGF2 (10 ng/ml) alone or in coincubation for 30 min. The total cell extracts were used for immunoprecipitation (IP), with anti-ERα and coimmunoprecipitates then being probed with anti-FGFR. A representative panel of the mean ± SE of 3 independent experiments ($n = 3$) is shown, and the graph represents the relative expression of FGFR. The data were evaluated by ANOVA-Tukey test. $^{**}P < 0.01$ vs. E_2 at 30 min. In total anterior pituitary cell culture lysates, both antibodies recognized the antigens (imput). *B*: positive control. ERα interacts with IGF-I receptor (IGF-IR) in pituitary cell lysate. Negative control: total extract from Hela ERα-negative cell line. WB, Western blot.

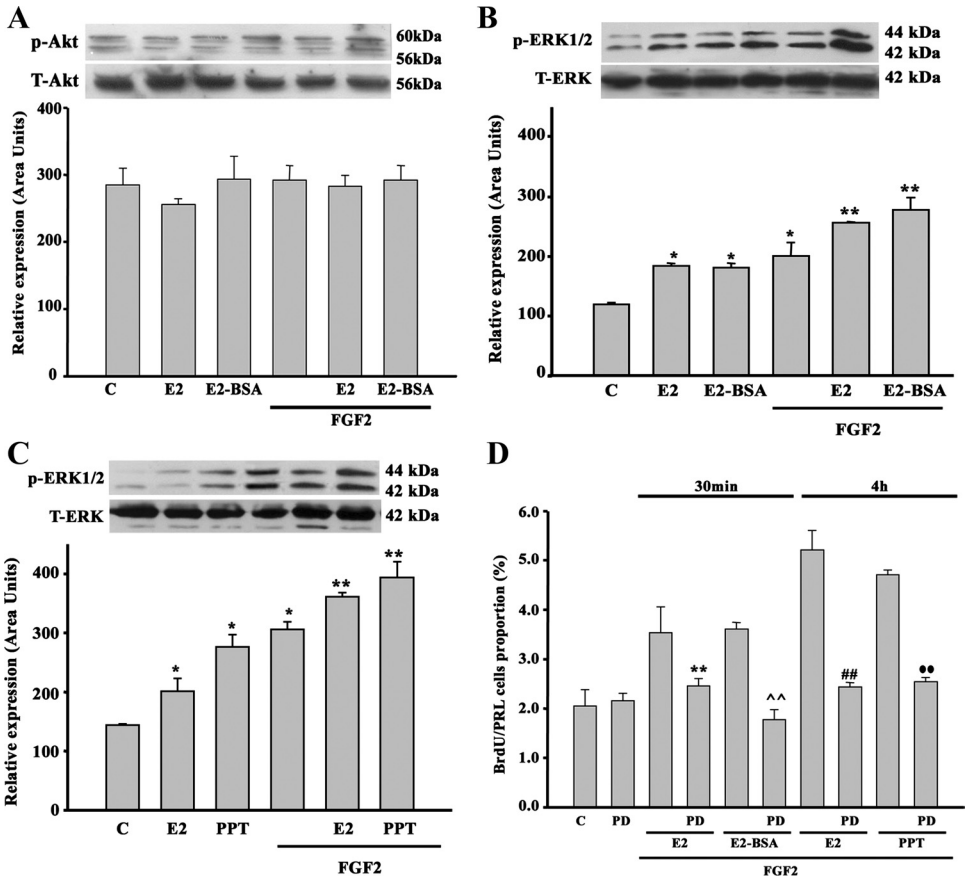

Fig. 6. ERK1/2 mediates the lactotroph-proliferative effect induced by E_2/FGF2. Western blotting of phosphorylated Akt (*A*) and ERK1/2 [30 min (*B*) and 4 h (*C*)] in total extract from primary pituitary cell cultures treated with E_2 (10 nM), E_2-BSA (10 nM), and FGF2 (10 ng/ml) alone or in coincubation for 30 min. A representative panel of the mean ± SE of 3 independent experiments ($n = 3$) is shown, and the graph represents the phosphroylated (p)-Akt/total (T)-Akt or p-ERK1/2/T-ERK ratio, and the data were evaluated by te ANOVA-Tukey test. In *B*, $*P < 0.01$ vs. C and $**P < 0.01$ vs. E_2, E_2-BSA, and FGF2 at 30 min; in *C*, $*P < 0.01$ vs. C and $**P < 0.01$ vs. E_2, PPT, and FGF2 at 4 h. *D*: the anterior pituitary cells were pretreated with PD-98059 (PD) for 30 min and were then treated with E_2 (10 nM), E_2-BSA (10 nM), and FGF2 (10 ng/ml) alone or in coincubation for 30 min or 4 h. Finally, BrdU was added for an additional 24 h. The data represent the proportion of double-positive BrdU-PRL cells in the total PRL-positive cells. Results are given as means ± SE of 3 wells from 3 independent experiments ($n = 9$), and data were evaluated by ANOVA, followed by Tukey's post-test. $**P < 0.01$ vs. E_2/FGF2 and $\wedge\wedge P < 0.01$ vs. E_2-BSA/FGF2 at 30 min without PD; $\#\#P < 0.01$ vs. E_2/FGF2 and $\cdot\cdot P < 0.01$ vs. PPT/FGF2 at 4 h without PD.

In addition, E_2 and FGF2 alone increased the ERK1/2 phosphorylation at 4 h compared with nonstimulated cells. Interestingly, coincubation with E_2/FGF2 provoked an increase of phosphorylated ERK1/2 expression at both time periods examined, showing significant differences with respect to these factors used alone ($P < 0.01$). The treatments with PPT/FGF2 promoted a similar effect as those induced by E_2/FGF2 stimuli for 4 h (Fig. 6*C*).

Finally, to verify the role of the MEK/ERK1/2 pathway on the proliferative effect induced by the steroid and FGF2, the anterior pituitary cell cultures were preincubated with PD-98059 (inhibitor of MEK) for 30 min prior the addition of E_2, E_2-BSA, PPT, or FGF2 alone or in coincubation for 30 min or 4 h. This inhibitor was found to block the proliferative effect induced by E_2-BSA/FGF2 and E_2/FGF2 at 30 min as well as that of E_2/FGF2 and PPT/FGF2 at 4 h ($P < 0.01$; Fig. 6*D*).

These results demonstrate that the MEK/ERK1/2 pathway may be involved in the proliferative effect induced by E_2/FGF2 on lactotroph cells.

DISCUSSION

In this study, we addressed the question of whether the interaction between E_2 and FGF2 is involved in the regulation of normal lactotroph proliferation. The data presented here showed that E_2 and FGF2 incubated alone were unable to increase the mitogenic activity of lactotroph cells. However, a combined treatment with both factors resulted in a significant stimulation of lactotroph proliferation. Interestingly, for these experimental conditions, we demonstrated the involvement of MEK/ERK1/2 as a convergence toward a common signaling pathway induced from the activation of membrane and intracellular ERα and FGFRs, thus triggering a mitogenic action on lactotrophs.

In general, the proliferative response of lactotrophs is reported after prolonged treatments with estradiol and growth factors. It has been shown previously that incubation for 96 h with FGF2 (10 ng/ml) and E_2 (10 nM) with medium containing serum stimulated the proliferation of lactotrophs from ovariectomized E_2-treated Fischer 344 rats (33). Furthermore, the existence of a cooperative action of E_2 and EGF for 18–24 h has been described in pituitary tumoral GH3 cells, which resulted in a robust stimulation of cell proliferation that was greater than either ligand alone (12). In the present study performed on primary pituitary cell cultures, it is noteworthy that the combined stimulatory effect of E_2 and FGF2 on PRL cell proliferation was seen after 30 min or 4 h of treatment in serum-free conditions.

To investigate the mechanism by which E_2/FGF2 induced a mitogenic effect on pituitary cells, we first examined which ER subtypes were involved in this action by using the ERα- and ERβ-specific agonists PPT and DPN, respectively. The results demonstrated that PPT, but not DPN, mimicked the effect of E_2 on the lactotroph cell proliferation when incubated with FGF2, thus inferring that E_2 through ERα exerted a mitogenic effect on lactotrophs. ER participation in the E_2-induced effects was corroborated by the ER inhibitor ICI 182,780, which completely antagonized the proliferative effects of E_2 and PPT in coincubation with FGF2. These data are in agreement with our

previous report showing that E_2 activity, including mitogenic and antimitogenic effects as well as PRL secretion on lactotroph cells, was mediated by ERα (26, 29, 58).

To analyze the involvement of the membrane and cytoplasmic/nuclear ER pools in the proliferative response of the lactotrophs observed after E_2 and FGF2 combined treatment, we used steroids conjugated to BSA. These results showed that E_2-BSA in coincubation with FGF2 for 30 min promoted an increase in the number of lactotroph cells undergoing mitosis, suggesting that this cellular process may be mediated by membrane-initiated steroid signaling. The higher magnitude of the BrdU incorporation index in lactotroph cells observed after incubation with E_2/FGF2 for 4 h compared with E_2/FGF2 for 30 min indicates a contribution of the cytoplasmic/nuclear ER pools. Therefore, the results obtained in this study reflect an integration of the actions of different ER pools (membrane and cytoplasmic/nuclear) in the combined effect of E_2 and FGF2 on lactotroph proliferation. It has been described previously that mER activates rapid signaling cascades, thus enhancing the nuclear transcription in both the central nervous system and nonneuronal cell lines (61). Also, mER-positive breast cancer cells are an important population and have an additional impact by modulating the rate of proliferation (22). In a previous study, we demonstrated the participation of the membrane-bound ER in the regulatory action of E_2, in interaction with insulin, on lactotroph cell proliferation (26).

The interaction between estradiol and growth factors may occur at different levels of the signaling pathways (23, 60). Accumulating evidence in past years has indicated a close interdependence between the mechanism of action of IGF-I and estradiol in the brain (43). Furthermore, a transient increase found in the association between the IGF-I receptor and ERα after estradiol administration suggests that the interaction of IGF-I with ERα is one of the mechanisms involved in the signaling of both the growth factors and the hormones in the brain (41). Our results obtained by immunoprecipitation studies showed that the combined treatment with E_2/FGF2 resulted in a significant association between FGFR and ERα. However, the possible interaction via a third-party protein that binds both receptors cannot be excluded. It is probable that other proteins may interact with these two receptors as part of a multiprotein complex. The association of ERα with proteins such as MNAR, striatin, or p130Cas, which promotes ligand-dependent interactions between ERα and members of the Src and PI3K of tyrosine kinases, has been reported (53). These molecules are scaffold proteins that mediate ERα-induced activation and enhance the Src/MAPK or PI3K-Akt kinase pathways (25).

Steroids and growth factors can stimulate proliferation of several cell types by utilizing common downstream signaling intermediaries (56). Taking into account previous results obtained in our laboratory about the involvement of MEK/ERK1/2 and PI3K-Akt pathways in lactotroph functional activities (17, 28, 51, 52, 58), we evaluated the Akt and ERK1/2 phosphorylation in the effect induced by E_2/FGF2 on PRL cells. The expression of phosphorylated Akt did not exhibit any significant variation after E_2, E_2-BSA, or FGF2 alone or coincubation treatments. However, a significant increase in the ERK1/2 activation was observed after E_2-BSA/FGF2, E_2/FGF2, and PPT/FGF2. Furthermore, the results obtained with the MEK inhibitor showed a blockade of the mitogenic effects exerted by the combination of the factors mentioned above. These data suggest the convergence of the pathways initiated at the plasma membrane by the activation of mERα and FGFRs, with the contribution of intracellular ERα inducing ERK1/2 phosphorylation to finally promote lactotroph proliferation. The strongest interaction between the E_2 and FGF2 receptors detected by immunoprecipitation assays was closely associated with the synergistic activation of the ERK1/2 pathway detected in cells treated with E_2/FGF2. This molecular mechanism may provide a novel explanation for the cooperative responsiveness of lactotroph cells to growth factors and estrogen. In neurons and GH3 cells, a positive functional interaction between growth factors and estrogen receptors has been described (2, 42). In agreement with our findings, in pituitary tumour GH3 cells the convergence toward a common downstream target such as ERK1/2 has been reported by the activation of signaling cascades induced by E_2 and EGF, thus amplifying their mitogenic effects (12).

In summary, the results of this study provide evidence that E_2 and FGF2 exert a cooperative effect on lactotroph proliferation, mainly through a common signaling pathway initiated at the plasma membrane, thus regulating the lactotroph population and contributing to pituitary plasticity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.d.V.S., S.G., and A.I.T. contributed to the conception and design of the research; L.d.V.S., J.P.P., and A.M.V. performed the experiments; L.d.V.S., S.G., J.P.P., and A.M.V. analyzed the data; L.d.V.S., S.G., J.P.P., A.M.V., A.L.D.P., and A.I.T. interpreted the results of the experiments; L.d.V.S. prepared the figures; L.d.V.S., S.G., J.P.P., A.L.D.P., and A.I.T. drafted the manuscript; L.d.V.S., S.G., A.L.D.P., and A.I.T. edited and revised the manuscript; L.d.V.S., S.G., J.P.P., A.M.V., A.L.D.P., and A.I.T. approved the final version of the manuscript.

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