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17β-Estradiol modulates the prolactin secretion induced by TRH through membrane estrogen receptors via PI3K/Akt in female rat anterior pituitary cell culture

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Sosa LdV, Gutiérrez S, Petiti JP, Palmeri CM, Mascanfroni ID, Soaje M, De Paul AL, Torres AI. 17β-Estradiol modulates the prolactin secretion induced by TRH through membrane estrogen receptors via PI3K/Akt in female rat anterior pituitary cell culture. *Am J Physiol Endocrinol Metab* 302: E1189–E1197, 2012. First published February 21, 2012; doi:10.1152/ajpendo.00408.2011.—Considering that estradiol is a major modulator of prolactin (PRL) secretion, the aim of the present study was to analyze the role of membrane estradiol receptor-α (mERα) in the regulatory effect of this hormone on the PRL secretion induced by thyrotropin-releasing hormone (TRH) by focusing on the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway activation. Anterior pituitary cell cultures from female rats were treated with 17β-estradiol (E_2, 10 nM) and its membrane-impermeable conjugated estradiol (E_2-BSA, 10 nM) alone or coincubated with TRH (10 nM) for 30 min, with PRL levels being determined by RIA. Although E_2, E_2-BSA, TRH, and E_2/TRH differentially increased the PRL secretion, the highest levels were achieved with E_2-BSA/TRH. ICI-182,780 did not modify the TRH-induced PRL release but significantly inhibited the PRL secretion promoted by E_2 or E_2-BSA alone or in coincubation with TRH. The PI3K inhibitors LY-294002 and wortmannin partially inhibited the PRL release induced by E_2-BSA, TRH, and E_2/TRH and totally inhibited the PRL levels stimulated by E_2-BSA/TRH, suggesting that the mER mediated the cooperative effect of E_2 on TRH-induced PRL release through the PI3K pathway. Also, the involvement of this kinase was supported by the translocation of its regulatory subunit p85α from the cytoplasm to the plasma membrane in the lactotroph cells treated with E_2-BSA and TRH alone or in coincubation. A significant increase of phosphorylated Akt was induced by E_2-BSA/TRH. Finally, the changes of ERα expression in the plasmalemma of pituitary cells were examined by confocal microscopy and flow cytometry, which revealed that the mobilization of intracellular ERα to the plasma membrane of lactotroph cells was only induced by E_2. These finding showed that E_2 may act as a modulator of the secretory response of lactotrophs induced by TRH through mER, with the contribution by PI3K/Akt pathway activation providing a new insight into the mechanisms underlying the nongenomic action of E_2 in the pituitary.

lactotrophs; thyrotropin-releasing hormone

ESTROGEN ACTS AS AN IMPORTANT MODULATOR of many lactotroph functions, including the synthesis and secretion of prolactin (PRL), cell proliferation, and apoptosis (44, 46). In addition, estrogen sensitizes lactotrophs to respond to neuropeptides and to growth factors by inducing differential secretory activity (10, 40) and stimulating PRL cell proliferation (11, 22), respectively.

The biological actions of estradiol are classically mediated through the estrogen receptors (ER) α or β, which function as ligand-dependent transcription factors, with both isoforms being localized in different pituitary cell types (32). The presence of the classical α and β ERs and the signaling pathways involved in estrogen regulation of hormone secretion, cell proliferation, and cell death have been reported in pituitary gland (47). In addition to cytoplasmic/nuclear localization of ER, in several cell types there are also small pools of ERα and -β at the plasma membrane (mER) (28, 34) that can be activated and rapidly trigger multiple signal transduction cascades through direct interactions of ER with various proteins, including growth factor-dependent kinases and adaptor proteins (30, 43). This multiprotein complex leads to the activation of many downstream signaling molecules, such as protein kinase C (PKC) (38), protein kinase A (3) , nitric oxide (NO) (24), mitogen-activated protein kinase (5), phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) (12), and increased calcium levels in different tissues (6) . It has been reported that, in the presence of estradiol, ERα interacts with the p85α regulatory subunit of PI3K, thus triggering activation of the p110 catalytic subunit, increasing intracellular production of phosphoinositides and leading to Akt activation (8).

In previous studies, we demonstrated the participation of the membrane-bound ER in the regulatory action of 17β-estradiol (E_2) on the secretory and proliferative lactotroph cell activities from primary pituitary cell cultures. Estradiol through membrane ER reversed the proliferative effect of insulin by inhibition of the PKCϵ/extracellular signal-regulated kinase (ERK) 1/2/Pit-1 cascade and stimulation of the NO/soluble guanylate cyclase/cGMP signaling pathway (19, 20). Moreover, we showed the presence of mERα in pituitary cells, specifically in lactotrophs, which mediated the PRL secretion stimulated by E_2 through PKCs/ERK1/2 activation (19). Related to this, nongenomic effects of E_2 on PRL secretion were previously reported in normal pituitary tissue (9) and GH3/B6 cells, with the effects of E_2 on PRL release being described to be dependent on the mERα levels (6).

Another report described that the PI3K/Akt pathway regulated PRL secretion by an ERK-dependent mechanism in insulin-like growth factor (IGF)-I-stimulated pituitary cells (37). However, to date, the participation of the PI3K/Akt pathway in membrane-initiated estrogen actions on the regulation of PRL release has been little explored.

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The control of PRL secretion involves a complex interrelationship between neurohormones, neurotransmitters, and the plasmatic levels of ovarian steroids. PRL is tonically inhibited by dopamine, the main PRL-inhibiting factor, and can be stimulated by a number of neuropeptides, with thyrotropin-releasing hormone (TRH) being the principal physiological factor stimulating the fast release of PRL (16).

In female rats, PRL secretion shows marked changes in different reproductive states, such as the estrous cycle (39), pregnancy (1), and lactation (42). During the estrous cycle, the serum PRL levels fluctuate, reaching a peak on the proestrous afternoon depending on the increase in the plasma estradiol levels (33), which is coincident with the highest ERα expression at proestrus (17). This event is associated with a significant TRH release into hypophysial portal blood (14, 15) and plays an important role in the timing and initiation of the PRL surge in the proestrous (23).

The stimulatory effects of estrogens are exerted at the hypothalamic level, thereby eliciting the release and activity of stimulatory hypophysiotropic factors such as TRH (31) and at the pituitary level by increasing the transcription rate of the PRL gene (25). Furthermore, in lactotroph cells, it has been demonstrated that E_2 upregulates the mRNA expression levels of the TRH receptor by an increase in both the transcription rate and stability of this receptor (27), thus modulating the fast actions of TRH on PRL secretion.

Bearing in mind that E_2 participates as the main modulator of the secretory response of lactotroph cells to TRH, it is of great interest to analyze the contribution of membrane-associated ERα by focusing on the PI3K/Akt pathway activation in this effect. A better knowledge of the molecular mechanisms involved could contribute to understand how rapid changes in PRL levels occur in different physiological processes.

MATERIALS AND METHODS

Reagents and antibodies. E_2, 17β-estradiol 6-*O*-carboxymethyloxime-BSA (E_2-BSA), TRH, ICI-182,780, wortmannin, and LY-294,002 were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies recognizing ERα MC-20 (directed to the COOH-terminal of the ERα), total Akt1/2, phosphorylated Akt1/2/3 (Ser^{473}), and the PI3K-p85α subunit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-PRL was provided by Dr. A. Parlow (National Hormone and Pituitary Program, Torrance, CA). The β-actin mouse monoclonal antibody was obtained from Sigma-Aldrich.

Animals. Three-month-old female virgin rats of the Wistar strain were used. Large pools of these animals were assigned to each culture taken at random cycle stages (n = 25–30). 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 (14:10-h light-dark cycle) conditions, having free access to commercial rodent food and tap water. Animals were kept in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the United States National Institutes of Health (1996), and the experiments were approved by the local Institutional Animal Care Committee.

Anterior pituitary cell culture. 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 pituitary cells has been described previously (10). 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 1.5–2 × 10^6/pituitary, and the cell viability, tested with Trypan blue exclusion, was always better than 90%. The final suspension was adjusted to 1 × 10^6 cells/ml of medium. For PRL secretion analysis and the other techniques, the cells were plated on six-well culture plates (Corning) at a density of 7 × 10^5 cells/well. For immunofluorescence by Confocal Laser Scanning Microscopy, the cells were seeded on glass cover slips (13 mm) at a density of 1.5 × 10^5 cells/well on 24-well culture plates (Corning). Next, the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4% FCS and 8% horse serum (GIBCO) in an oven 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). The cell culture grade reagents were obtained from Sigma Chemical.

Cell treatment conditions and use of inhibitors. After 3 days of culture, the cells were maintained in DMEM without Phenol Red and serum 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. Next, the cells were stimulated for 30 min with vehicle (0.1% BSA), E_2 (10 nM), E_2-BSA (10 nM), and TRH (10 nM), either alone or in combination. In addition, pituitary cells were preincubated with a range of concentrations of the competitive inhibitor of E_2 binding ICI-182,780 (100, 500, or 1,000 nM) and with specific PI3K inhibitors: LY-294002 (10 μM) and wortmaninn (100 nM). These inhibitors were applied for 30 min before the stimulation with E_2, E_2-BSA, and TRH alone or in combination for another 30 min. The culture media were collected and stored frozen at −20°C before determining the PRL levels by radioimmunoassay. The anterior pituitary cells were processed to confocal laser scanning microscopy, Western blot, and flow cytometry.

A stock solution of E_2-BSA was made according to our previous report (19), 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) 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.

PRL determination. The PRL concentration in the culture medium was measured by a double-antibody radioimmunoassay (RIA), with materials supplied by Dr. A. F. Parlow from the National Hormone and Pituitary Program. The PRL was radioiodinated using the chloramine T method and purified by passage through Sephadex G-75 and polyacrylamide agarose (ACA 54; LKB, Bromma, Sweden) columns. The assay sensitivity was 1 ng/ml medium, and the inter- and intra-assay coefficients of variation were <10%.

Confocal laser scanning microscopy. Cover slips containing attached pituitary cells from different experimental groups were fixed with 4% formaldehyde, permeabilized with 0.25% Triton X-100 in PBS, and incubated for 1 h with 5% PBS-BSA to block nonspecific binding. Slides were incubated overnight with ERα rabbit polyclonal antibody (1:50) in a humidified chamber at 4°C, which were then washed three times with PBS and further incubated with Alexa 594 antirabbit secondary antibody (1:1,000; Invitrogen) for 1 h. Concanavalin A Alexa Fluor 488 conjugate (100 μg/ml; Invitrogen, Eugene, OR), a lectin that specifically binds to the α-mannosyl saccharides expressed in the core structures of plasma membrane glycoproteins, was used as a plasma membrane marker.

For double inmunostaining, slides were incubated for 1 h with anti-p85α (1:50 rabbit polyclonal), followed by 1 h with Alexa 594 antirabbit as the secondary antibody, before being washed and then incubated with anti-PRL antibody using fluorescein isothiocyanate (FITC) antirabbit as the secondary antibody (Santa Cruz Biotechnology), as explained above.

To validate the specificity of the immunostaining, controls were performed by applying the same protocol but replacing primary antibody with 1% PBS-BSA.

Images were then obtained using an inverted confocal laser scanning microscope (FluoView FV 1,000; Olympus, Tokyo, Japan). An

Ar-ion 488-nm laser was used for excitation of green fluorescence and a 543-nm one for excitation of red fluorescence. Serial *z*-axis sections were collected using a ×60 or ×100 objective. An analysis of the confocal microscopy images was performed using the software FV10-ASW 1.6 Viewer.

Preparation of cell lysates for Western blot 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 EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. 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 were separated using 12% acrylamide gel (Sigma Chemical), and, to estimate the corresponding molecular weights, the 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 antibodies rabbit polyclonal antitotal Akt1/2 (1:300) and antiphosphorylated Akt1/2/3 (Ser^{473}; 1:300). The blots were incubated with a peroxidase-conjugated (HRP) antirabbit secondary antibody (1:5,000; Jackson Immunoresearch Laboratories, West Grove, PA) diluted in blocking buffer and then thoroughly rinsed in PBS/0.1% Tween 20, and the HRP-coupled secondary antibody was revealed with enhanced chemiluminescence Western blot detection reagents (Inmuno Star HRP Substrate Kits; Bio-Rad Laboratories) following the manufacturer's instructions. Emitted light was captured on Hyperfilm (Amersham-Pharmacia-Biotech, Bucks, UK), and signals were scanned and quantified with Scion Image software (version beta 4.0.2; Scion Image, Frederick, MD) at three different exposure times. The expression of β-actin (1:5,000) was used as an internal control to confirm an equivalent total protein loading.

Flow cytometry. Living dispersed cells were incubated with anti-ERα (1:50) or an appropriate isotype control (BD Biosciences) for 30 min at 4°C and then incubated with secondary antibody Alexa Fluor 647 (1:1,000; Invitrogen) for 30 min at 4°C. Next, the cells were fixed (CITOFIX; BD Biosciences Pharmingen, San Diego, CA) for 20 min at 4°C and permeabilized with Perm/Wash (BD Biosciences Pharmiogen), before being incubated with primary antibody (1:20,000, anti-PRL) for 30 min at 4°C followed by incubation with secondary FITC antibody (1:300; Santa Cruz Biotechnology) for 30 min at 4°C. As isotype control, the cells were incubated with rabbit normal serum instead of PRL or ERα antibody. Finally, the cells were washed, resuspended in filtered PBS, and analyzed by flow cytometry (1×10^5

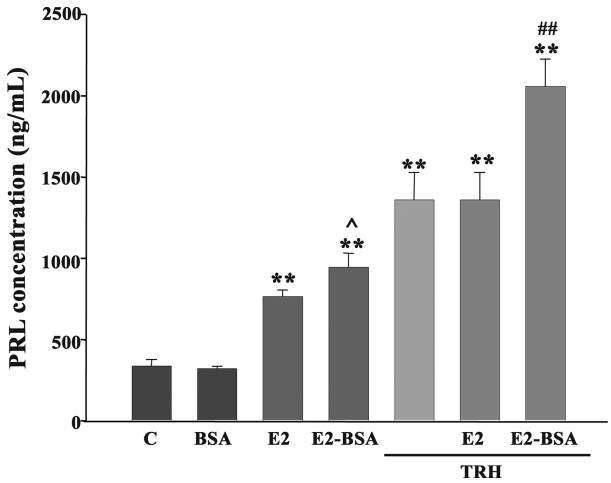

Fig. 1. Effect of 17β-estradiol (E_2) and thyrotropin-releasing hormone (TRH) combined treatments on prolactin (PRL) secretion. The presence of E_2 (10 nM), 17β-estradiol 6-*O*-carboxymethyloxime-BSA (E_2-BSA) (10 nM), or TRH (10 nM) alone or in combination for 30 min induced a significant increase in the PRL release, attaining the highest values after E_2-BSA/TRH treatment. Results are given as means ± SE of 6 wells from three independent experiments, and data were evaluated by the ANOVA followed by Tukey's posttest: $^{**}P < 0.01$ vs. control; $^{\wedge}P < 0.05$ vs. E_2; and $^{\#\#}P < 0.01$ vs. TRH.

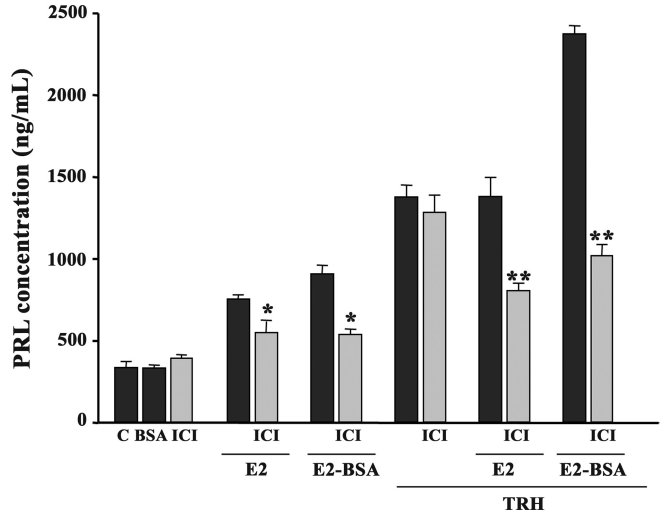

Fig. 2. Effect of ICI-182,780 (100 nM) on PRL secretion induced by E_2 and TRH combined treatment in pituitary cell cultures. The specific antagonist of estrogen receptors (ERs) reversed the PRL release induced by E_2 (10 nM) or E_2-BSA (10 nM) alone or in coincubation with TRH (10 nM) and was unable to modify the rise in PRL levels promoted by TRH. The data are expressed as means ± SE of 6 wells from three independent experiments, and data were evaluated by the ANOVA followed by Tukey's posttest: $^{*}P < 0.05$ vs. E_2 or E_2-BSA and $^{**}P < 0.01$ vs. E_2/TRH or E_2-BSA/TRH.

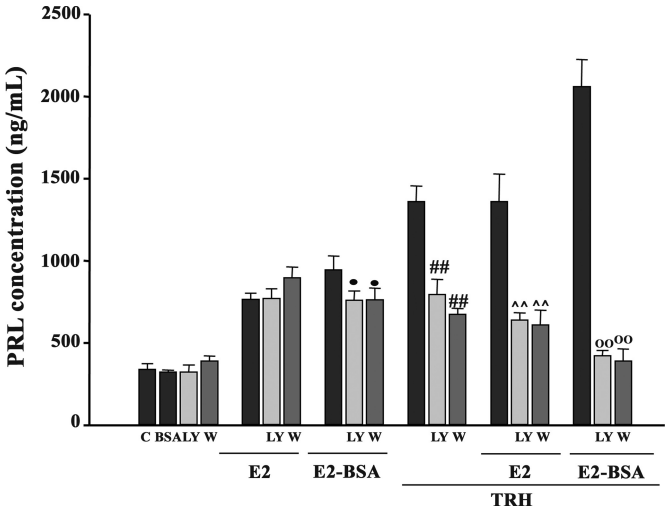

Fig. 3. Effect of phosphatidylinositol 3-kinase (PI3K) inhibitors on PRL secretion stimulated by E_2, E_2-BSA, and TRH combined treatments in pituitary cell cultures. The cells were pretreated with LY-294002 (10 μM, LY) or wortmannin (100 nM, W) for 30 min before application of E_2 (10 nM), E_2-BSA (10 nM), or TRH (10 nM) alone or in combination for an additional 30 min. The data are shown as means ± SE of 6 wells from three independent experiments, and the data were evaluated by the ANOVA followed by Tukey's posttest: $^{\#}P < 0.05$ vs. E_2-BSA; $^{\#\#}P < 0.01$ vs. TRH; $^{\wedge\wedge}P < 0.01$ vs. E_2/TRH; and $^{oo}P < 0.01$ vs. E_2-BSA/TRH.

events/experimental treatment; Ortho Diagnostic System, Raritan, NJ). A data analysis was carried out using the FlowJo software (Tree Star, Ashland, OR), the percentage of mERα-positive lactotrophs was calculated by the equation [(mERα$^+$PRL$^+$)/total PRL$^+$ cells] × 100, and the variations of intensity levels of mERα were determined using the geometric mean fluorescence intensity.

Statistical analysis. The statistical analysis was carried out on three replicates measured on three independent cell cultures using an ANOVA with Tukey's posttest (InfoStat software package). Significance levels were chosen at $P < 0.05$.

RESULTS

E_2 acting through membrane-associated ER increased the PRL release induced by TRH. We investigated whether E_2 was able to modulate the PRL release induced by TRH and, if so, the involvement of the membrane-associated ER in this effect. Primary cell cultures were incubated with E_2, E_2-BSA (E_2 covalently linked to membrane-impermeable BSA), or TRH alone or using a combination of the neuropeptide with E_2 or E_2-BSA. As shown in Fig. 1, E_2 stimuli caused a significant

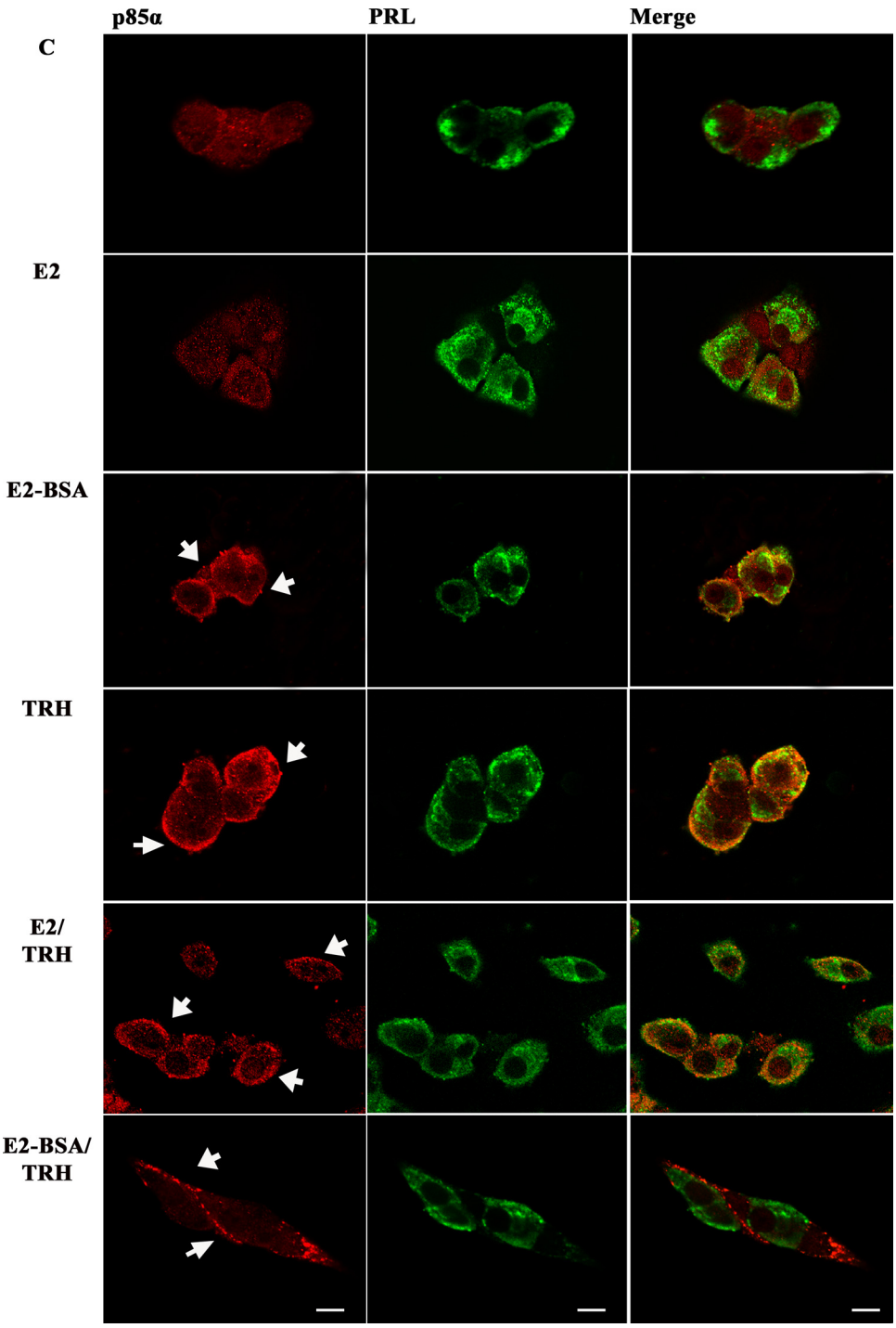

Fig. 4. Translocation of p85α regulatory subunit of PI3K to the plasma membrane of lactotroph cells. Pituitary cell cultures, nonstimulated or stimulated with E_2, E_2-BSA, and TRH alone or combination for 30 min, were immunolabeled for p85α (red) and PRL (green). A notable p85α translocation from the cytoplasm to the plasma membrane (arrows) of lactotroph cells (merge) was observed when the cells were exposed to TRH alone or in coincubation with E_2 or E_2-BSA. The images correspond to a representative experiment from a total of three with similar results. Bar = 10 μm.

increase of around 120% in the PRL levels, with E_2-BSA incubation inducing an even greater effect and reaching values of 180% compared with nonstimulated cells. As was expected, a noticeable PRL secretion of about 300% was observed when the pituitary cells were treated with TRH. Moreover, the highest PRL levels in culture media were quantified after E_2-BSA/TRH incubation, reaching values of 500% compared with control and with approximately a 60% increase with respect to the levels promoted by TRH. These results suggest that E_2 acting through membrane ER may have exerted a cooperative effect with TRH to promote PRL release.

To examine the specificity of estrogen action on PRL secretion, we studied the effect of ICI-182,780, the pure antagonist of the ER. Pituitary cells were preincubated using a range of ICI concentrations (100, 500, and 1,000 nM) for 30 min before exposition to specific factors for an additional 30 min (data not shown). For all pretreatments, ICI significantly inhibited the PRL secretion promoted by E_2 or E_2-BSA. Next, based on the responses obtained and those from previous work (21), in this study we selected the lower dose (100 nM), which reduced the PRL secretion induced by TRH coincubated with E_2 or E_2-BSA. As was expected, ICI did not modify the PRL release induced by TRH (Fig. 2).

PI3K participated in the stimulatory effect induced by E_2 and TRH on PRL secretion. To analyze whether PI3K was involved in the secretory activity of lactotroph cells induced by E_2 or E_2-BSA coincubated with TRH in lactotroph cells, the two PI3K inhibitors LY-294,002 and wortmannin were used. The preincubation of pituitary cells with LY-294,002 (10 μM) or with wortmannin (100 nM) did not modify the PRL secretion induced by E_2. However, when the pituitary cells were preincubated with PI3K inhibitors before the addition of E_2-BSA, TRH, or E_2/TRH, the PRL release was partially blocked. Furthermore, both inhibitors were able to block completely the secretory activity of lactotroph cells promoted by E_2-BSA/TRH, reaching levels similar to control (Fig. 3). These results suggest that the ER membrane mediated the cooperative effect of E_2 on TRH-induced PRL release through the PI3K pathway.

E_2-BSA/TRH induced both p85α translocation at the membrane in lactotroph cells and Akt phosphorylation. To evaluate the activation of PI3K induced by E_2, E_2-BSA, and TRH, we analyzed the translocation of the PI3K regulatory subunit p85α in lactotroph cells by confocal laser scanning. In unstimulated PRL cells, the immunofluorescence corresponding to the p85α subunit was mainly observed in the cytoplasm compartment, which could have been indicative of an inactive state. E_2-BSA and TRH alone or in coincubation induced translocation of the p85α subunit from the cytoplasm to the plasma membrane of the lactotroph cells, which may be interpreted as a hallmark of activation. On the other hand, free E_2 was unable to modify the subcellular distribution of this subunit, although, when coincubated with TRH, p85α mobilization to the plasma membrane was detected (Fig. 4).

In addition, we analyzed the Akt expression, considered to be the principal downstream kinase of the PI3K pathway. The total and phosphorylated Akt were detected by immunoblotting using specific antibodies as bands of ~56 kDa. As shown in Fig. 5, the incubation of E_2-BSA/TRH for 30 min in serum-free culture medium induced a significant increase of phosphorylated Akt compared with respective control. Total Akt expression was similar for all lines. The expression of β-actin was used as an internal control to confirm equivalent total protein loading.

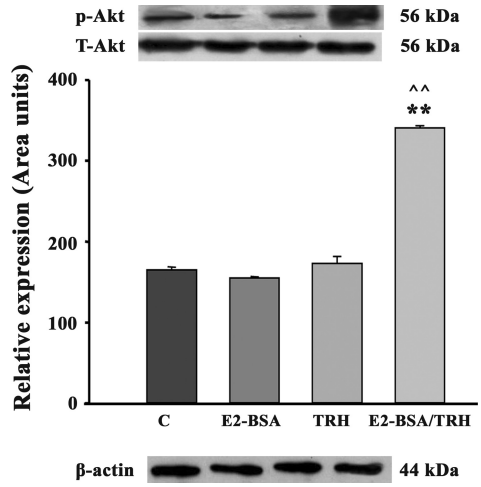

Fig. 5. Western blot analysis of protein kinase B (Akt) expression from total pituitary cell extracts. E_2-BSA/TRH stimuli for 30 min induced a significant increase of phosphorylated Akt (p-Akt, Ser^{473}) expression. The total Akt (T-Akt) signals were similar for all lines, and β-actin expression was used to confirm equivalent total protein loading. A representative panel of three independent experiments is shown. The graph represents the p-Akt-to-T-Akt ratio of the means ± SE of three independent experiments, and the data were evaluated by the ANOVA followed by Tukey's posttest: $^{**}P < 0.01$ vs. C and $^{\wedge\wedge}P < 0.01$ vs. TRH.

In summary, both p85α translocation to the plasma membrane of the lactotrophs and Akt phosphorylation might indicate activation of the PI3K pathway in response to E_2-BSA/TRH stimuli, leading to the highest PRL release.

ERα translocation to the plasma membrane in pituitary cells was induced by E_2. Considering that the ER membrane participated in the regulatory effects of E_2 on the PRL secretion induced by TRH, we analyzed the changes of ERα expression in the plasma membrane of pituitary cells after E_2/TRH stimuli by confocal microscopy and flow cytometry. In unstimulated pituitary cells, the endogenous ERα specific immunostaining was mainly observed in the nucleus and cytoplasm by confocal microscopy. However, E_2 and E_2/TRH treatment for 30 min increased ERα expression at the plasma membrane of pituitary cells besides the cytoplasmic and nuclear localizations (Fig. 6*A*).

The presence of ERα within the plasma membrane was confirmed using Alexa 488-labeled concanavalin A, a lectin that specifically binds to α-manosyl saccharides expressed in the core structures of plasma membrane glycoproteins. ERα and concanavalin A colocalization at the membrane plasmatic level was observed after E_2/TRH stimulation for 30 min (Fig. 6*B*).

To evaluate the changes in the ERα expression on the surface of lactotroph cells induced by E_2, E_2-BSA, and TRH alone or in combination, a flow cytometry analysis was carried out. Figure 7*A* shows that ~69% of all scanned cells were lactotrophs identified using a PRL antibody, with simultaneous labeling with a specific ERα antibody indicating that 6.1 ± 0.6% of these cells also expressed ERα at the cell surface. Moreover, this percentage did not change for any condition tested. Although the ERα basal expression at the cell surface did not alter after TRH, E_2-BSA, or E_2-BSA/TRH stimuli, the E_2 and E_2/TRH treatments for 30 min induced a significant increase in the geometrical mean of $ERα^+$

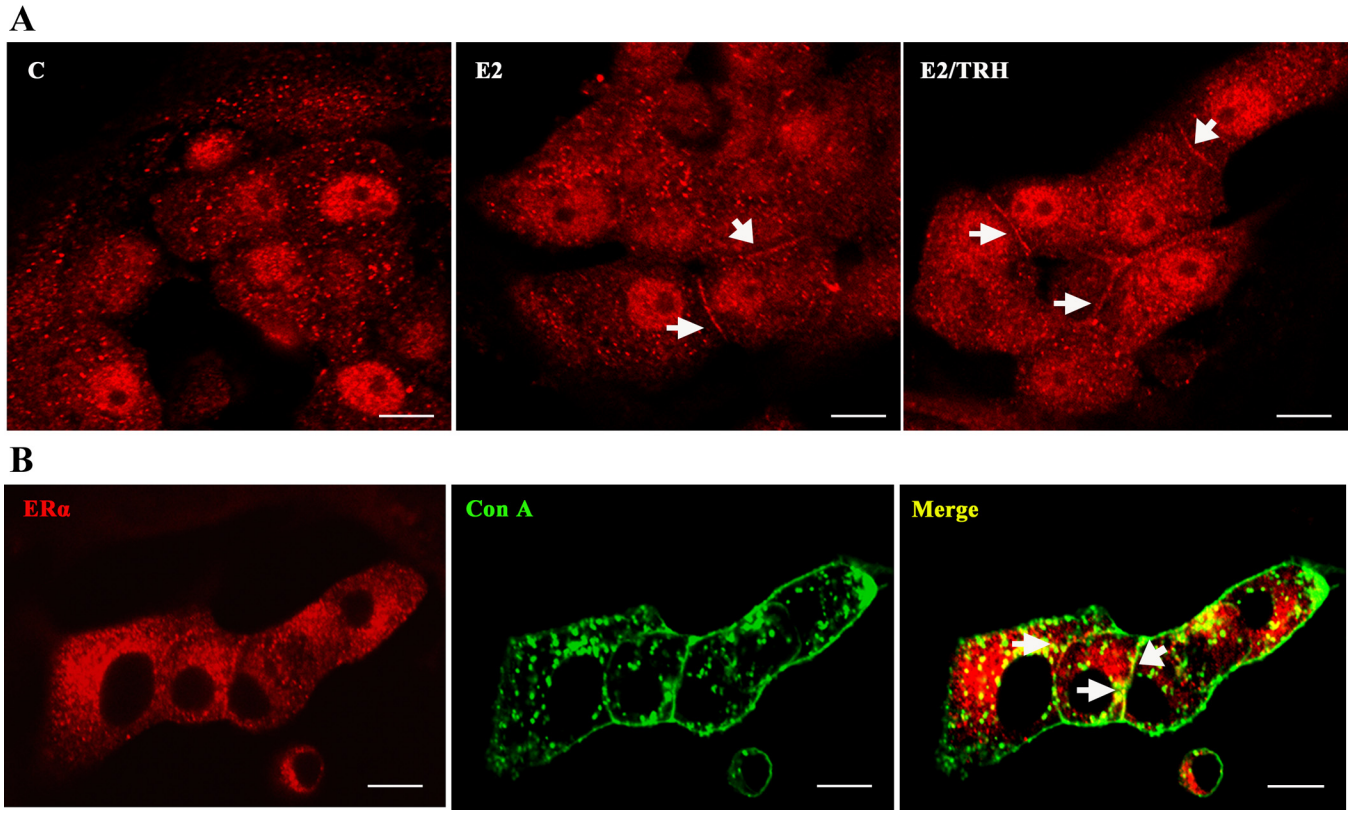

Fig. 6. Translocation of ERα in normal anterior pituitary cells. The cell cultures were treated with E_2 or E_2/TRH for 30 min and then immunostained for ERα. *A*: in control cells (C), ERα expression was detected in the nucleus and cytoplasm. The E_2 or E_2/TRH stimuli induced a redistribution of ERα from the nucleus/cytoplasm to the plasma membrane (arrows). *B*: colocalization of ERα (red) and concanavalin A (Con A) (green) at the plasma membrane of pituitary cells. After E_2/TRH stimuli, ERα was detected in or near the plasma membrane of the pituitary cells (yellow, arrows). The photographs correspond to a representative experiment from a total of three that had similar results. Bar = 10 μm.

fluorescence intensity on the surface of lactotroph cells compared with the basal state ($P < 0.01$; Fig. 7*B*). No significant differences in mERα expression between the E_2 and E_2/TRH treatments were detected. These data indicate that the mobilization of intracellular ER to the plasma membrane of lactotroph cells was only induced by E_2.

DISCUSSION

It is well known that estrogen acts as an important modulator of the neuroendocrine signals that classically regulate the PRL secretion by a transcription-dependent mechanism (2, 16). However, since the participation of different ER pools and the signaling pathways involved in this effect have not been fully elucidated, we investigated the role played by mER in the modulatory effect of E_2 on PRL secretion in response to TRH in primary cultures of female rat anterior pituitary cells. It was found that the incubation with E_2, E_2-BSA, and TRH differentially increased PRL secretion in a rapid fashion, attaining the highest levels after E_2-BSA/TRH stimuli. Steroids conjugated to BSA have been shown to be impermeant to the plasma membrane and therefore cannot access the cytoplasmic receptors. Accordingly, they have been used increasingly as tools to investigate nongenomic, cell-surface steroid effects (41). In this study, the PRL-secretagogue actions of E_2-BSA and E_2-BSA/TRH strongly suggest an effect on the membrane E_2-binding sites. In a previous work, the rapid PRL-secretagogue effect of estrogens mediated by mER was achieved independently of DNA transcription and did not seem to involve a nuclear receptor (9).

The PRL release induced by E_2-BSA was significantly higher than that of free E_2, inferring the possibility that this effect may have originated because the conjugated steroid persistently stimulated the mER for 30 min. Free E_2 also binds to mER but readily diffuses inside the cells after application and activates the intracellular receptors. These data are in agreement with our previous report (19) and also with Bulayeva and coworkers (6), who demonstrated the more pronounced Ca^{2+} elevation and PRL secretion caused by E_2-BSA compared with free estradiol in pituitary cell lines. Although no effect of free E_2 on TRH-induced PRL release was detected for our experimental conditions, the ICI-182,780 pretreatment resulted in a reduction of the PRL secretory levels induced by both factors, suggesting the ER participation in the E_2 effect on TRH-promoted PRL secretion. One possible explanation could be that the rapid stimulation of the mER induced by free E_2 may be overlapped or hidden by the powerful secretory stimulus exerted by TRH. Therefore, the contribution of mER in the cooperative effect of E_2-BSA/TRH was confirmed by the significant reduction of PRL secretion promoted by ICI-182,780 pretreatment.

In an attempt to achieve a better understanding of the signaling pathway underlying the effects of E_2 modulating the TRH-induced PRL secretion, we analyzed the participation of the PI3K/Akt pathway. Our results showed that the PI3K

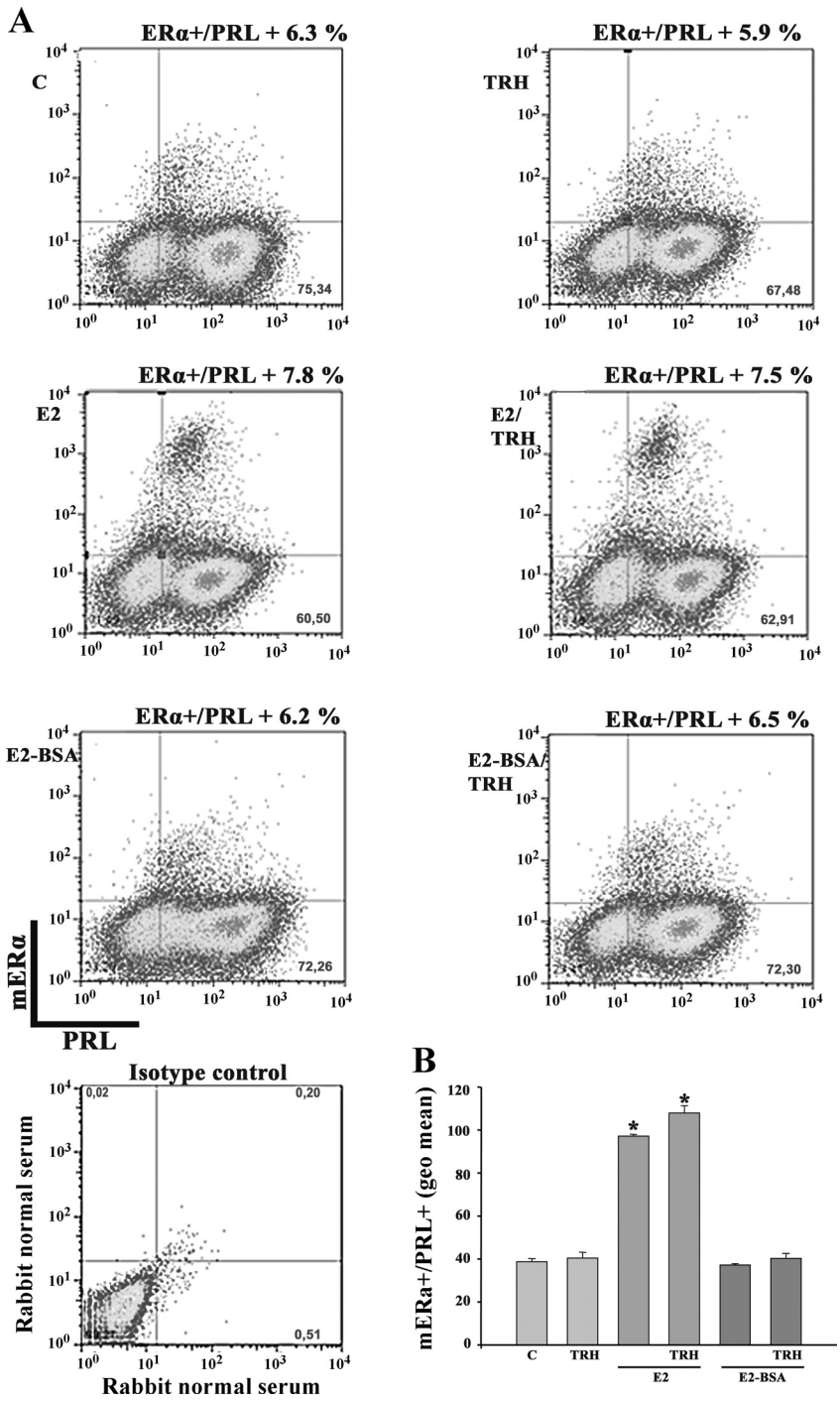

Fig. 7. Expression of membrane-associated ERα on the cell surface of lactotroph cells by flow cytometry. *A*: representative dot-blot diagrams of double-negative cells for both PRL and mERα (*bottom left*) and of basal, TRH, E_2, and E_2/TRH treatments. The *x*-axis represents PRL expression, and the *y*-axis represents the surface marker mERα. The variations of positive cell percentages are shown in the superior quadrant. The plots correspond to a representative experiment from a total of three with similar results (10^5 events for each experimental condition). *B*: the E_2 and E_2/TRH treatments promoted a significant increase of the geometrical mean fluorescence intensity for ERα in lactotroph cells compared with controls (*$P < 0.01$). The TRH, E_2-BSA, or E_2-BSA/TRH stimulus were unable to modify the fluorescent pattern, showing a similar intensity to control.

inhibitors were unable to modify the PRL secretion stimulated by E_2, suggesting the contribution of other signaling cascades in the estradiol action on lactotroph secretory activity. In fact, in a previous work, we showed the activation of the PKC signaling pathway through mERα in PRL release from lactotroph cells (19). Furthermore, the activation of cAMP (26), ERK phosphorylation (5), and/or increased intracellular calcium (6, 45) has been demonstrated to be involved in regulation of PRL release.

In the present study, we observed that the E_2- the BSA-stimulated PRL release was partially inhibited by LY-294,002 and wortmannin, indicating that PI3K participated in this effect. Although it is believed that free E_2 through mER activates PI3K in a similar manner to E_2-BSA, in our experiments E_2 failed to exert this action. We hypothesize that a sustained stimulation of mER by E_2-BSA might be required for the PI3K activation under our experiment conditions. Also, the participation of PI3K in the E_2- and BSA-induced PRL release is strongly suggested by the translocation of its regulatory subunit p85α from the cytoplasm to the plasma membrane in the lactotroph cells, which is indicative of PI3K activation. Moreover, the total blockade exercised by LY-294,002 and

wortmannin on the PRL secretion promoted by E_2-BSA/TRH suggests that mER activation may potentiate the TRH effect on PRL release through the PI3K pathway. This interpretation has also been suggested for other molecules in neurons, which, acting through their membrane receptors, were able to amplify the TRH effect by positive modulation of its transduction mechanism (35, 36).

To analyze further the contribution of the PI3K/Akt pathway, we examined the phosphorylation of the principal kinase Akt, downstream of the PI3K. Under our experimental conditions, when cells were treated with E_2-BSA or TRH, no significant increase of phosphorylated Akt expression was detected by Western blot. However, the E_2-BSA/TRH treatment induced a strong Akt phosphorylation, suggesting that the PI3K/Akt pathway was involved in the effect of both secretagoges on the PRL release. In agreement, estradiol in interaction with IGF-I increased the Akt phosphorylation in the brain, including the hippocampus, the cerebral cortex, hypothalamus, and the cerebellum (7).

The present study demonstrates for the first time that mER mediate the cooperative effect exerted by E_2 on rapid PRL release induced by TRH through the activation of PI3K/Akt, a convergent signaling pathway involved in this regulatory effect. In a physiological environment, there is a basal level of estrogen that fluctuates under different reproductive states. This variation may in turn activate the mER-triggering effects mediated in part by PI3K activation as observed with E_2-BSA in our experimental conditions.

Having demonstrated that mER play a central role in modulating the effect of E_2 on TRH-induced PRL secretion, we next focused on the analysis of the changes in mERα expression in pituitary cells after stimulation with E_2, E_2-BSA, and TRH alone or in combination using confocal microscopy and flow cytometry. Our findings showed that the increased ERα expression observed at the plasma membrane of pituitary cells, specifically on the surface of lactotroph cells after E_2 and E_2/TRH incubation, was due to intracellular ERα translocation to the plasma membrane induced by E_2. The similar rise in mERα expression detected for both E_2 and E_2/TRH allows us to infer that TRH did not cause ERα mobilization. Furthermore, the ERα basal expression at the cell surface did not alter following E_2-BSA stimuli, which allows us to infer that the resident pool of membrane ERα (stimulated steadily) could have been responsible for PRL release in experiments where E_2-BSA was added. In accordance with our results, other reports have demonstrated that E_2 regulates the endogenous ER translocation to the plasma membrane in Sertoli cells (29), hypothalamic cells (13), neurons (18), astrocytes (4), and pituitary cells (21).

In the present study, the fact that the increase in mERα expression detected in lactotroph cells after E_2/TRH treatment correlated with the highest PRL secretion observed after E_2-BSA/TRH stimuli could be a clear indicator of the role that the membrane ERα plays in mediating the cooperative effect of E_2 on the rapid PRL release induced by TRH.

In summary, our results show that estradiol may act as a modulator of the secretory response of lactotrophs induced by TRH through membrane ER, with this contribution being provided by PI3K/Akt pathway activation. It is also possible that this mechanism is implicated in the enhancement of the PRL levels observed in different reproductive states, such as the estrous cycle, pregnancy, and lactation, which are closely associated with fluctuations in the estrogen and TRH levels, thus revealing a new insight into the mechanisms underlying the nongenomic action of E_2 in the pituitary glands.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

REFERENCES

1. **Bast JD, Melampy RM.** Luteinizing hormone, prolactin, and ovarian 20 -hydroxysteroid dehydrogenase levels during pregnancy and pseudopregnancy in the rat. *Endocrinology* 91: 1499–1505, 1972.
2. **Ben-Jonathan N, Lapensee CR, Lapensee EW.** What can we learn from rodents about prolactin in humans? *Endocr Rev* 29: 1–41, 2008.
3. **Beyer C, Karolczak M.** Estrogenic stimulation of neurite growth in midbrain dopaminergic neurons depends on cAMP/protein kinase A. *signalling J Neurosci Res* 59: 107–116, 2000.
4. **Bondar G, Kuo J, Hamid N, Micevych P.** Estradiol-induced estrogen receptor-alpha trafficking. *J Neurosci* 29: 15323–15330, 2009.
5. **Bulayeva NN, Gametchu B, Watson CS.** Quantitative measurement of estrogen-induced ERK 1, and 2 activation via multiple membrane-initiated signaling pathways. *Steroids* 69: 181–192, 2004.
6. **Bulayeva NN, Wozniak AL, Lash LL, Watson CS.** Mechanisms of membrane estrogen receptor-alpha-mediated rapid stimulation of Ca^{2+} levels, and prolactin release in a pituitary cell line. *Am J Physiol Endocrinol Metab* 288: E388–E397, 2005.
7. **Cardona-Gomez GP, Mendez P, Garcia-Segura LM.** Synergistic interaction of estradiol, and insulin-like growth factor-I. in the activation of PI3K/Akt signaling in the adult rat hypothalamus. *Brain Res Mol Brain Res* 107: 80–88, 2002.
8. **Castoria G, Migliaccio A, Bilancio A, Di Domenico M, De Falco A, Lombardi M, Fiorentino R, Varricchio L, Barone MV, Auricchio F.** PI3-kinase in concert with Src promotes the S.-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO J* 20: 6050–6059, 2001.
9. **Christian HC, Morris JF.** Rapid actions of 17beta-oestradiol on a subset of lactotrophs in the rat pituitary. *J Physiol* 539: 557–566, 2002.
10. **De Paul A, Pons P, Aoki A, Torres A.** Different behavior of lactotroph cell subpopulations in response to angiotensin I.I., and thyrotrophin-releasing hormone. *Cell Mol Neurobiol* 17: 245–258, 1997.
11. **De Paul AL, Gutierrez S, Sabatino ME, Mukdsi JH, Palmeri CM, Soaje M, Petiti JP, Torres AI.** Epidermal growth factor induces a sexually dimorphic proliferative response of lactotroph cells through P.*K*.C./E.R.K.1/2/Pit-1 in vitro. *Exp Physiol* 96: 226–239, 2011.

12. **Dominguez R, Liu R, Baudry M.** 17-Beta-estradiol-mediated activation of extracellular-signal regulated kinase, phosphatidylinositol 3-kinase/protein kinase B.-Akt, and N.-methyl-D.-aspartate receptor phosphorylation in cortical synaptoneurosomes. *J Neurochem* 101: 232–240, 2007.
13. **Dominguez R, Micevych P.** Estradiol rapidly regulates membrane estrogen receptor alpha levels in hypothalamic neurons. *J Neurosci* 30: 12589–12596, 2010.
14. **Fink G, Koch Y, Ben Aroya N.** Release of thyrotropin releasing hormone into hypophysial portal blood is high relative to other neuropeptides, and may be related to prolactin secretion. *Brain Res* 243: 186–189, 1982.
15. **Franks S, Mason HD, Shennan KI, Sheppard MC.** Stimulation of prolactin secretion by oestradiol in the rat is associated with increased hypothalamic release of thyrotrophin-releasing hormone. *J Endocrinol* 103: 257–261, 1984.
16. **Freeman ME, Kanyicska B, Lerant A, Nagy G.** Prolactin: structure, function, and regulation of secretion. *Physiol Rev* 80: 1523–1631, 2000.
17. **Gonzalez M, Reyes R, Damas C, Alonso R, Bello AR.** Oestrogen receptor alpha, and beta in female rat pituitary cells: an immunochemical study. *Gen Comp Endocrinol* 155: 857–868, 2008.
18. **Gorosito SV, Lorenzo AG, Cambiasso MJ.** Estrogen receptor alpha is expressed on the cell-surface of embryonic hypothalamic neurons. *Neuroscience* 154: 1173–1177, 2008.
19. **Gutierrez S, De Paul AL, Petiti JP, Del Valle Sosa L, Palmeri CM, Soaje M, Orgnero EM, Torres AI.** Estradiol interacts with insulin through membrane receptors to induce an antimitogenic effect on lactotroph cells. *Steroids* 73: 515–527, 2008.
20. **Gutierrez S, Petiti JP, Sosa LV, Fozzatti L, De Paul AL, Masini-Repiso AM, Torres AI.** 17beta-oestradiol acts as a negative modulator of insulin-induced lactotroph cell proliferation through oestrogen receptor alpha, via nitric oxide/guanylyl cyclase/cGMP. *Cell Prolif* 43: 505–514, 2010.
21. **Gutierrez S, Sosa LV, Petiti JP, Mukdsi JH, Mascanfroni ID, Pellizas CG, De Paul AL, Cambiasso MJ, Torres AI.** 17β-estradiol stimulates the translocation of endogenous estrogen receptor α at the plasma membrane of normal anterior pituitary cells. *Moll Cell Endocrinol* 355: 186–196, 2012.
22. **Hentges S, Boyadjieva N, Sarkar DK.** Transforming growth factor-beta3 stimulates lactotrope cell growth by increasing basic fibroblast growth factor from folliculo-stellate cells. *Endocrinology* 141: 859–867, 2000.
23. **Horn AM, Fraser HM, Fink G.** Effects of antiserum to thyrotrophin-releasing hormone on the concentrations of plasma prolactin, thyrotrophin, and LH in the pro-oestrous rat. *J Endocrinol* 104: 205–209, 1985.
24. **Jaubert AM, Mehebik-Mojaat N, Lacasa D, Sabourault D, Giudicelli Y, Ribiere C.** Nongenomic estrogen effects on nitric oxide synthase activity in rat adipocytes. *Endocrinology* 148: 2444–2452, 2007.
25. **Jin L, Song JY, Lloyd RV.** Estrogen stimulates both prolactin, and growth hormone mRNAs expression in the MtT/F4 transplantable pituitary tumor. *Proc Soc Exp Biol Med* 192: 225–229, 1989.
26. **Judd AM, Login IS, Kovacs K, Ross PC, Spangelo BL, Jarvis WD, Macleod RM.** Characterization of the MMQ cell, a prolactin-secreting clonal cell line that is responsive to dopamine. *Endocrinology* 123: 2341–2350, 1988.
27. **Kimura N, Arai K, Sahara Y, Suzuki H.** Estradiol transcriptionally, and posttranscriptionally up-regulates thyrotropin-releasing hormone receptor messenger ribonucleic acid in rat pituitary cells. *Endocrinology* 134: 432–440, 1994.
28. **Levin ER.** Integration of the extranuclear, and nuclear actions of estrogen. *Mol Endocrinol* 19: 1951–1959, 2005.
29. **Lucas TF, Siu ER, Esteves CA, Monteiro HP, Oliveira CA, Porto CS, Lazari MF.** 17beta-Estradiol induces the translocation of the estrogen receptors ESR1, and ESR2 to the cell membrane, MAPK3/1 phosphorylation and proliferation of cultured immature rat Sertoli cells. *Biol Reprod* 78: 101–114, 2008.
30. **Marin R, Diaz M, Alonso R, Sanz A, Arevalo MA, Garcia-Segura LM.** Role of estrogen receptor alpha in membrane-initiated signaling in neural cells: interaction with IGF-1 receptor. *J Steroid Biochem Mol Biol* 114: 2–7, 2009.
31. **Matsushita N, Kato Y, Shimatsu A, Katakami H, Yanaihara N, Imura Effects of VIP H, TRH, GABA,.** and dopamine on prolactin release from superfused rat anterior pituitary cells. *Life Sci* 32: 1263–1269, 1983.
32. **Mitchner NA, Garlick C, Ben-Jonathan N.** Cellular distribution, and gene regulation of estrogen receptors alpha and beta in the rat pituitary gland. *Endocrinology* 139: 3976–3983, 1998.
33. **Neill JD, Freeman ME, Tillson SA.** Control of the proestrus surge of prolactin, and luteinizing hormone secretion by estrogens in the rat. *Endocrinology* 89: 1448–1453, 1971.
34. **Pietras RJ, Levin ER, Szego CM.** Estrogen receptors, and cell signaling. *Science* 310: 51–53, 2005.
35. **Rogers RC, Barnes MJ, Hermann GE.** Leptin "gates" thermogenic action of thyrotropin-releasing hormone in the hindbrain. *Brain Res* 1295: 135–141, 2009.
36. **Rogers RC, Mcdougal DH, Hermann GE.** Leptin amplifies the action of thyrotropin-releasing hormone in the solitary nucleus: an in vitro calcium imaging study. *Brain Res* 1385: 47–55, 2011.
37. **Romano D, Pertuit M, Rasolonjanahary R, Barnier JV, Magalon K, Enjalbert A, Gerard C.** Regulation of the RAP1/RAF-1/extracellularly regulated kinase-1/2 cascade, and prolactin release by the phosphoinositide 3-kinase/AKT pathway in pituitary cells. *Endocrinology* 147: 6036–6045, 2006.
38. **Sawai T, Bernier F, Fukushima T, Hashimoto T, Ogura H, Nishizawa Y.** Estrogen induces a rapid increase of calcium-calmodulin-dependent protein kinase I.I. activity in the hippocampus. *Brain Res* 950: 308–311, 2002.
39. **Smith MS, Freeman ME, Neill JD.** The control of progesterone secretion during the estrous cycle, and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* 96: 219–226, 1975.
40. **Spuch C, Diz-Chaves Y, Perez-Tilve D, Mallo F.** Fibroblast growth factor-2, and epidermal growth factor modulate prolactin responses to TRH and dopamine in primary cultures. *Endocrine* 29: 317–324, 2006.
41. **Swenson KL, Sladek CD.** Gonadal steroid modulation of vasopressin secretion in response to osmotic stimulation. *Endocrinology* 138: 2089–2097, 1997.
42. **Terkel J, Blake CA, Sawyer CH.** Serum prolactin levels in lactating rats after suckling or exposure to ether. *Endocrinology* 91: 49–53, 1972.
43. **Vasudevan N, Pfaff DW.** Membrane-initiated actions of estrogens in neuroendocrinology: emerging principles. *Endocr Rev* 28: 1–19, 2007.
44. **Watters JJ, Chun TY, Kim YN, Bertics PJ, Gorski J.** Estrogen modulation of prolactin gene expression requires an intact mitogen-activated protein kinase signal transduction pathway in cultured rat pituitary cells. *Mol Endocrinol* 14: 1872–1881, 2000.
45. **Wozniak AL, Bulayeva NN, Watson CS.** Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-alpha-mediated Ca2+ fluxes, and prolactin release in GH3/B6 pituitary tumor cells. *Environ Health Perspect* 113: 431–439, 2005.
46. **Zarate S, Jaita G, Zaldivar V, Radl DB, Eijo G, Ferraris J, Pisera D, Seilicovich A.** Estrogens exert a rapid apoptotic action in anterior pituitary cells. *Am J Physiol Endocrinol Metab* 296: E664–E671, 2009.
47. **Zarate S, Seilicovich A.** Estrogen receptors, and signaling pathways in lactotropes and somatotropes. *Neuroendocrinology* 92: 215–223, 2010.