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**Participation of the mPRα in the inhibitory effect of progesterone on prolactin secretion**

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**Abbreviated Title**: Novel role of mPRα in the lactotroph population

**Key words:** pituitary, prolactin, lactotrophs, membrane progesterone receptors

**ABSTRACT**

The membrane progesterone receptors (mPRα, -β, -γ, -δ, -ε) are known to mediate rapid non-genomic progesterone functions in different cell types. However, the functions of these receptors in the pituitary have not been reported to date. Here we show that the expression of *mPRα* was the highest among the *mPRs* in the rat anterior pituitary gland. Immunostaining of mPRα was detected in somatotrophs, gonadotrophs and lactotrophs. Interestingly, 63% of mPRα-positive cells within the pituitary were lactotrophs suggesting that mPRα is involved in controlling prolactin (PRL) secretion in the pituitary. To test this hypothesis, rat pituitaries were incubated (1h) with either progesterone (P4) or the mPRα specific agonist Org OD 02-0. PRL secretion was then measured by radioimmunoassay (RIA). Results of this experiment revealed that both P4 and Org OD 02-0 decreased PRL secretion. Moreover, results from the GH3 cell line (CCL-82.1™) showed that P4 and Org OD 02-0 inhibited PRL release, but the nuclear PR agonist R5020 was ineffective. Our investigation of the cellular mechanisms behind mPRα activity indicated that both P4 and Org OD 02-0 decreased cAMP accumulation, while R5020 was ineffective. In addition, the Org OD 02-0-effect on PRL release was blocked by pretreatment with pertussis toxin, an inhibitor of Go/Gi proteins. Because TGFβ1 is a potent inhibitor of PRL secretion in lactotrophs, we lastly evaluated whether TGFβ1 was activated by progesterone and whether this effect was mediated by mPRα. Our results showed that P4 and Org OD 02-0, but not R5020 increased active TGFβ1 levels. This effect was not observed when cells were transfected with mPRα-siRNA. Taking together, these data provide new evidence that mPRα mediates the progesterone inhibitory effect on PRL secretion through both: the decreases in cAMP levels and the activation of TGFβ1 in the lactotroph population.

**INTRODUCTION**

The role of progesterone in regulating reproductive physiology in animals is complex and difficult to evaluate because the direction and extent of progesterone effects depend on the endogenous hormonal milieu. Progesterone actions typically require previous exposure to estradiol-17β (E2) since E2

regulates nuclear progesterone receptor expression (1). In the pituitary gland the role of progesterone in controlling lactotroph proliferation and prolactin (PRL) secretion is controversial (reviewed in (2)). Progesterone can facilitate the E2 effect, but can also prevent estradiol induced PRL surges and PRL gene expression during prolonged progesterone treatment (1). This can also be observed in the experimental model of prolactinoma generated in female rats by chronic treatment with diethylstilbestrol (DES), a synthetic estrogen, where co-treatment with progesterone antagonizes the proliferative effects of DES on lactotrophs, thereby decreasing tumor size and serum PRL levels (3). Therefore, it appears that progesterone serves an anti-proliferative and protective role in the pituitary gland (3,4). On the other hand, in E2 primed-animals, acute progesterone treatment increases PRL release (5-7) and, may stimulate proliferation depending on the hormonal environment and type of receptor involved (8,9).

However, it is important to recognize that progesterone also exerts an indirect impact on PRL secretion through regulating the activity of tyrosine hydroxylase (TH) in the hypothalamus, the main enzyme involved in the biosynthesis of dopamine (10-13), the major inhibitory regulator of PRL secretion.

Aside from other well-documented genomic effects, progesterone exerts rapid 'non-genomic' actions through activation of membrane progesterone receptors (mPRs). These actions are relatively unaffected by inhibitors of transcription, mimicked by steroids coupled to non-permeant-cell-membrane molecules, and persist in cells that do not express the classic genomic progesterone receptor (reviewed in (14)). The mPRs belong to the progestin and adipoQ receptor (PAQR) family and were first identified in teleost fish (15), but it was latter discovered that five subtypes (mPRα, -β, -γ, -δ and -ε) are found in tissues of several mammalian species including humans (16-24). These receptors contain seven trans-membrane domains, display high-affinity progesterone binding and rapidly activate G proteins during their downstream signaling pathways following progestin binding (20). Several studies have demonstrated that mPRα, mPRβ, and mPRγ activation induces inhibitory G protein (Gi) signaling pathways, thereby decreasing adenylyl cyclase activity and cellular cAMP accumulation (18,19,25). mPRδ and mPRε, on the other hand, are coupled to stimulatory to G proteins (Gs) and increase cAMP accumulation after activation (16). Moreover, mPRα and mPRβ are

expressed more strongly in different tissues of mammals such as the rat and mouse brain (22,23) as well as in reproductive tissues (17). mPRγ is largely expressed in the lung, liver, kidney and fallopian tube (26) meanwhile mPRδ and mPRε have been described in the human brain and pituitary (16). Among the mPRs subtypes, mPRα is the most extensively characterized in vertebrates. Studies have revealed that mPRα is involved in progesterone regulation of numerous functions (27), including uterine function in humans (17) and the inhibition of GnRH release in rodents (25). mPRα is also thought of as an intermediary of progesterone anti-tumorigenic effects in ovarian cancer cells, which may provide a new treatment option for patients undergoing advanced stage ovarian cancer therapy (28). Although there is substantial evidence that progesterone initiates rapid, cell-surface actions in several cell types through mPRs, there are no published studies reporting the exact role of these receptors in the pituitary gland.

In our present work, we hypothesize that mPRs mediate rapid progesterone actions in the pituitary gland. To test this, we (1) studied the expression of mPRs in rat pituitaries and in rat pituitary tumor-derived GH3 cells and (2) investigated the cell-type localization as well as the role of the mPR most likely involved in mediating progesterone actions in the rat pituitary.

## MATERIALS AND METHODS

### *Chemicals and reagents*

Chemicals were purchased from Sigma-Aldrich (St. Louis, Mo) unless otherwise stated: Diethylstilbestrol (DES): or (E)-3,4-Bis(4-hydroxyphenyl)-3-hexene, a synthetic estrogen; and progesterone was purchased from Steraloids (Newport, RI); Org OD-02-0 was obtained from Organon (Oss, The Netherlands) and R5020 was purchased from GE Healthcare (Piscataway, NJ). Although Org OD 02-0 was initially described as a human mPRα agonist, its selectivity on mPRs in rat cells has been also demonstrated in the rat brain and hypothalamus (23,24). [2,4,6,7-3H]progesterone ([3H]P4; ~84 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, MO). Antibodies against ERK (p42/44) and phosporylated ERK (p-p42/44) were purchased from Cell Signaling Technology (Danvers, MA).

***Animals***

Adult female Sprague-Dawley (SD) rats (3-month old, 250 ± 30 g) were maintained at 22 ± 2°C and 12 h light – dark cycle, lights on 07:00 – 19:00 h. Cycling rats were daily monitored, by vaginal smears, during 4–5 day estrous cycles, and euthanized in diestrus. Anterior pituitary glands were removed within minutes after decapitation.

The animals were provided with food and water *ad libitum*. The Institutional Animal Care and Use Committee of the Institute of Biology and Experimental Medicine (IByME) approved all animal procedures, and the studies were conducted in accordance with National Institutes of Health guidelines for animal research (8th ED. 2010, NRC, USA) and in accordance with the European Communities Council Directive of November 2010 (2010/63/UE).

***Quantitative real-time PCR***

Total RNA was isolated from anterior pituitaries of female adult Sprague-Dawley rats in diestrus with TRIzol® Reagent (Ambion, Life Technologies) according to the manufacturer´s protocol. RNA purity and quantity was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). Purity was determined by 260/280nm absorbance ratios, and absorbance ratios above 1.8 were considered acceptable. Random primers (Tecnolab, Buenos Aires, Argentina) and Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Promega) were used for cDNA synthesis. Reverse transcription was performed with 1ug RNA at 70°C for 5 min, 37°C for 60 min, and 95°C for 5 min, as the minimum specification of first-strand cDNA made from 1µg of RNA is 120ng. Then, a working solution of cDNA (6 ng/ul) was prepared by diluting samples 1:20 with DEPC-treated water. Five microliters of cDNA working solution was added to a 5 µl master mix containing 2 µl EVA green qPCR mix (Solis BioDyne, Estonia), and 0.5 µl of each forward primer (0.25 µM) and reverse primer (0.25 µM) as recommended by manufacturer's protocol. Specific primers (Table 1), located on separate exons, were designed using Primer3 and synthesized by Tecnolab S.A (Buenos Aires, Argentina). Quantitative real-time PCR analysis was carried out using a Bio-Rad real-time PCR detection system and CFX Manager Software (Bio-Rad laboratories, Hercules, CA, USA). Standard thermocycler conditions were as follows: 95°C for 5 min, followed by

40 cycles of 95°C for 15 sec, 60°C for 20 sec, and 72°C for 1 min. All cDNA samples were assayed in duplicate for each gene, and melt curve analyses were performed to ensure specificity of amplification. qPCR efficiency of each pair of primers was previously tested using serially diluted samples. All primers showed similar efficiencies, approximately 95-100%. A housekeeping gene, *Cyclophilin B* (*Cyp B*), was used as an internal control to normalize differences in the amount of starting template between samples, as previously described (29). Target gene expression was quantified using the comparative CT method (ΔCT). To analyze the relative mRNA abundance among all the receptor types, we compared $2^{-\Delta Ct}$ ($2^{-(Ct\ mPR\text{-}Ct\ Cyb\ B)}$) values of each receptor. The expression of *nPRs* was analyzed using one primer set to amplify total *PRs*, and a second primer set to amplify only *PR-B*.

***Ex vivo assay***

Female Sprague Dawley rats in diestrus were sacrificed by decapitation and anterior pituitaries were collected. Anterior pituitaries were cut in pieces, and incubated in 500 µl of Dulbecco Eagle's Modified Medium (DMEM) supplemented with 15% horse serum, 2.5% fetal bovine serum, penicillin/streptomycin (20µg/ml, Gibco) and amphotericin B (0.25µg/ml, PAA, Linz, Austria) for 2h at 37°C. Then, tissues were washed and stimulated for 1h at 37°C with 100nM progesterone (P4); or with the selective mPR agonist, 10-ethenyl-19-norporgesterone Org OD 02-0 (02), in serum-free medium (vehicle).

At the end of the treatment period, secreted medium and pituitaries were collected and PRL levels were measured by radioimmunoassay (RIA). Serum PRL levels were determined by RIA using a primary antibody of defined specificity raised in rabbit against rat PRL, with synthetic PRL as a reference preparation and [$^{125}$I]PRL as tracer (all reagents provided by National Hormone and Pituitary Program). The inter- and intra-assay coefficients of variation were 6.7% and 12.6%, respectively.

***Immunostaining analysis of mPRα receptor and pituitary hormones (PRL; LH and GH) by confocal laser scanning microscopy***

Pituitaries from 3-month-old female SD rats in diestrus were removed immediately after euthanasia and the pituitary cells were dispersed and seeded on glass coverslips (13 mm) at a density of 105 cells/well. Then, the cells were maintained in DMEM supplemented with 4% fetal calf serum and 8% horse serum (Gibco, NY) in an incubator with a humidified atmosphere of 5% CO2 and 95% air at 37 ºC for 3 days, and finally were fixed in 4% formaldehyde.

For mPRα receptor detection, dispersed pituitary cells in coverslips were blocked for 1h in 5% PBS–BSA, incubated overnight in primary antibody (anti-rat mPRα 306t, Dr. Thomas, 1:50) and exposed to Alexa594 anti-rabbit secondary antibody (Invitrogen, 1:1000) for 1 h. Then, the cells were permeabilized with 0.50% Triton X-100/PBS, blocked for 1h in 5% PBS–BSA, incubated with guinea pig antiserum directed against rat PRL; or rat LH; or rat GH (1:1000, Dr. A.Parlow, National Hormone and Pituitary Program, Torrance, Calif., USA) and further incubated with Alexa488 anti-guinea pig secondary antibody (Invitrogen, 1:1000) for 1 h. Then, the glass coverslips were mounted with fluoromount (Sigma, St Louis, MO) containing DAPI.

Control cells were incubated with the corresponding normal serum or IgG subtype instead of primary antibody. Images were obtained using the inverted confocal laser scanning microscope FluoView FV 1000 (Olympus; Tokyo, Japan). The analysis of confocal microscopy images was performed using the software FV10-ASW 1.6 Viewer.

***Flow cytometry***

Control rats were sacrificed and anterior pituitary glands were removed after decapitation and collected in 1000 µl of DMEM. First, anterior pituitary glands were washed with DMEM-BSA containing 3 mg/ml bovine serum albumin. Then, anterior pituitaries were cut into small fragments and dispersed enzymatically by successive incubations in DMEM-BSA containing 0.75% trypsin, 10% charcoal-dextran-adsorbed fetal calf serum (FCS) and 45 U/ml deoxyribonuclease type I (DNAse). Finally, the cells were dispersed by extrusion through a Pasteur pipette in Krebs buffer without $Ca^{2+}$ and $Mg^{2+}$. Dispersed cells were washed and re-suspended in PBS (30). Cell viability, as

assessed by trypan blue exclusion, was over 95%. Cells were fixed using PFA 0.2% 15 min at RT, then permeabilized with saponine 0.05%, washed and centrifuged. Immunostaining of mPRα positive cells and of PRL (lactotrophs) was performed using mPRα antibody (1:50, anti-rat mPRα 306t, Dr. Thomas) and a guinea-pig antiserum directed against rat PRL (1:1000, Dr. A. Parlow, National Hormone and Pituitary Program, Torrance, Calif., USA) 1 h at 37°C. Then, cells were washed in PBS and incubated with a PE-conjugated anti-rabbit (Chemicon International, Temecula, CA., USA) (1:67, 40min at 37°C) and FITC-conjugated anti-guinea pig antibody (Chemicon International, Temecula, CA., USA) (1:75, 40min at 37ºC). For isotype controls, cells were incubated with guinea pig serum instead of PRL antiserum and rabbit IgG instead of specific anti- rabbit antibody. Cells were washed, re-suspended in PBS and analyzed by FACS (31). Fluorescence intensity of ≥ 10,000 gated-cells/tube was analyzed using a FACScalibur (BD). Data was analyzed using WinMdi and FlowJo Softwares.

***Culture of GH3 cells***

GH3 cells (ATCC® CCL-82.1™) were grown as monolayer culture in DMEM supplemented with 5% fetal bovine serum and 5% horse serum, 2mM glutamine, 1% MEM amino acids and 1% penicillin/streptomycin (pH 7.4). The cells were cultured in 15-cm culture dishes and replicated every 3-5 days in the exponential phase. Cells were harvested once a week by treatment with a phosphate-buffered saline containing trypsin (2.5 mg/ml, Gibco) and reseeded at 20% the original density, and 4 subcultures were made before each experiment. In some experiments, the culture media was replaced with serum-free DMEM before experimentation.

***Preparation of plasma membranes***

GH3 cells, sub-cultured in 15-cm culture dishes until they were 90% confluent, were harvested with a cell scraper and collected in ice-cold HAED buffer (25mM HEPES, 10 mM NaCl, 1 mM EDTA, 1mM ditioerythritol, pH 7.6) with protease inhibitors cocktail (Thermo Scientific). Cells were rinsed twice followed by 5-min centrifugation at 1000 × g for at 4°C and cell pellets resuspended in freshly prepared ice-cold HAED buffer. Cells were lysed by sonication for 10 sec and then centrifuged at 1000 × g 4°C for 7 min to remove any nuclear and heavy mitochondrial material (nuclear fraction).

The supernatant was centrifuged at 20000 × g at 4°C for 20 min to obtain the plasma membrane fraction which was resuspended with HAED buffer.

***Single-point competitive binding assay***

Single-point competitive assay was conducted as described previously (20) using GH3 cells. Briefly, plasma membranes were incubated with 4 nM [2,4,6,7-$^{3}$H]-progesterone ([$^{3}$H]-P4) alone (total binding) or in the presence of 1 μM P4, 02 or R5020 for 30 min at 4°C. Bound [$^{3}$H]-P4 was separated from free by rapid filtration over Whatman GF/B filters with a 36-well cell harvester (Brandel, Gaithersburg, MD), and radioactivity bound to the filters was measured by scintillation counting (Beckman LS6000). The displacement of radiolabeled steroid binding by the progestin competitors was expressed as a percentage of the maximum specific [$^{3}$H]-P4 binding.

***Western Blot analysis of membrane progesterone receptors***

GH3 plasma membranes were solubilized and mixed with 4× Lane Marker Reducing Sample Buffer (Thermo) boiled for 10 min and run on a 15% SDS-PAGE gel (15ug protein/lane). Proteins were then transferred onto nitrocellulose membranes at 4°C and 1 h-blocked in PBS-T with 5 % non-fat dried milk. Membranes were incubated overnight at 4°C in PBS + Odyssey® Blocking Buffer containing the previously validated polyclonal antibodies (16,17,26,32): mPRα (1:500); -β, -γ and -ε (1:1000) and antibodies directed against PGRMC1 (1:1000) or nPR (1:1000) (Table 2). The nitrocellulose membranes were then incubated with fluorescent-conjugated secondary antibodies (1:10000) (LI-COR Biosciences) for 1 h at room temperature washed three times with PBS and scanned with the Odyssey® Infrared Imaging System (LI-COR Biosciences).

***Immunocytochemical analysis of progesterone receptors***

The presence of mPRs in GH3 cells was evaluated by immunofluorescent staining. GH3 cells (~2 X $10^{5}$cells/well) were grown on glass coverslips and fixed with 4% paraformaldehyde (PFA) in PBS for 20 min. Then, the cells were washed with PBS, blocked with 2% bovine serum albumin for 1.5 h and then incubated with mPRα (1:50), mPRβ, -γ, -δ, –ε or PGRMC1 antibodies (1:100) (see table 2) overnight at 4°C. After that, cells were washed and incubated with an Alexa Fluor 488 donkey anti-rabbit secondary antibody or an Alexa Fluor 488 donkey anti-goat secondary antibody (1:1000)

(Thermo Fisher Scientific, Waltham, MA) for 1 h at room temperature. The cells were washed and the coverslips were mounted on glass slides with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Grand Island, NY). Localization of fluorescent-labeled mPRs was assessed using a Nikon inverted microscope with a MetaVue Research Imaging System.

***Measurement of prolactin secretion in GH3 collected medium after progestin treatments***

GH3 cells were cultured in 12-well plates (~$1.10^6$ per well) and then incubated with 20 nm P4, 02, R5020 or vehicle in supplemented medium at 37°C for 30 min. At the end of the treatment period, the supernatants were collected and PRL levels were measured using a specific Rat Prolactin EIA kit (Cayman Chemicals, Ann Arbor, MI) following the manufacturer's instructions.

***Pertussis toxin assay***

Pertussis toxin (List Biological Laboratories, Inc., Campbell, California) was activated by incubation with 15 mM DTT at 35°C for 30 min (PTXa). For control groups, an aliquot of PTX was heat-inactivated by further incubation for 10 min at 99°C (PTXhi). Cells were pretreated with PTXa and PTXhi (2.5 ug/mL, List Biological Laboratories Inc) 30 min and then incubated with 02 (20 nM) 4 h at 37°C. PRL concentration in cell supernatants was measured using a specific Rat Prolactin EIA kit following the manufacturer's instructions.

***cAMP assay***

GH3 cells were cultured in 12-well plates (~1 X $10^6$ cells/well) and serum-starved for 72 h prior to a 10 min-treatment with progestins (20 nM) or vehicle. After treatments, cells were washed twice with ice-cold PBS and lysed by a 20 min-incubation with 100 µL 0.1M HCl at room temperature, followed by scrapping and pipetting to homogenize samples. The cell suspension was clarified by a 10 min-centrifugation at 12000 × g and the supernatant diluted 3X. Cellular cAMP concentration was determined with a Cyclic AMP EIA Kit (Cayman chemical, Ann Arbor, MI) following manufacturer´s instructions.

***ERK activation assay***

GH3 cells grown in 12-well plates (~1 X $10^6$ cells/well) and serum-starved for 48 h were incubated with P4, 02, R5020 (20 nM) or vehicle for 15 min. EGF (100nM) was used as positive control (data not shown in the figure). After treatments, 100 µl of RIPA buffer (EMD Millipore, Billerica, MA) containing phosphatase and protease inhibitors cocktail (Thermo Scientific) was added and cells were collected and lysed by vortexing for 30 min. Then lysates were centrifuged at 15000 x g for 5 min at 4°C to remove the cell debris. An aliquot of 5µl of the supernatant was taken from each sample to quantify proteins by Bradford assay. Samples containing ~15 µg were prepared for western blot analysis as described above. Total ERK and phosphorylated ERK were detected using monoclonal antibodies directed against total p42/44 and phospho-p42/44 respectively (p42/44 #9102; p-p42/44 #9106; Cell Signaling Technology (Danvers, MA)) (1:2000). Membranes were then incubated with fluorescent-conjugated secondary antibodies (LI-COR Biosciences) and scanned with the Odyssey® Infrared Imaging System (LI-COR Biosciences). Phosphorylated ERK was normalized to total ERK using ImageJ software.

***Active TGFβ1 detection***

GH3 cells were cultured in 12-well plates (~1 X $10^6$ per well) and incubated with DMEM-BSA 2% without serum 24 h before treatments. Cells were then treated, as previously described, with P4, 02 and R5020 (20 nM) or vehicle for 15 min. After treatments, cell supernatants were collected and the content of active TGFβ1 (pg/ml medium) of each sample was measured using a TGFβ1 DuoSet ELISA Kit (DY1679-05, R&D Systems) following the manufacturer's instructions.

***Knockdown of mPRα with siRNA***

GH3 cells were transfected twice with antisense siRNA oligos for mPRα (ON-TARGET plus Rat Paqr7 siRNA, Dharmacon, CO) or non-targeting oligos (CTL) (100 nM) at 0 and 16 h, using Lipofectamine 2000® (Invitrogen) as transfection reagent. After 48 h of incubation, the siRNA mix was replaced with normal medium and then cells were cultured for additional 24 h with DMEM-BSA 2% before their use in experiments.

***Inhibitor treatments***

Cells were pretreated with Pertussis toxin (2.5 µg/ml, PTXa) for 30 min prior to treatment with 02 (20 nM, 15 min at 37°C). In other experiments, cells were co-treated with a cAMP analog (10µM; 8-Br-cAMP, Sigma-Aldrich) and 02 for 15 min. Likewise; a specific inhibitor of ERK (10µM, PD98059, Biomol) was used to pre-treat cells for 1h prior to treatment with 02. At the end of the treatment periods, active TGFβ1 levels were measured by ELISA as previously described.

***Statistical Analysis***

Results are expressed as mean ± SEM. Experiments were repeated between 3-5 times, with at least three replicates. P4, 02 and R5020 treatments results were evaluated by one-way ANOVA followed by Tukey´s *post hoc* test. Two-way ANOVA was performed when the effects of two factors (progestin treatments and PTX, inhibitors treatments or siRNA treatment) were evaluated, followed by Bonferroni´s *post hoc* multiple comparison test. $P < 0.05$ was considered significant. Data set was transformed when required, and analyzed using Graphpad Prism 5 software (GraphPad Software, San Diego, CA).

## RESULTS

### Expression of *nPRs* and *mPRs* by qRT-PCR in female rat pituitary

The mRNA expression of each progesterone receptor was assayed in pituitaries from adult female rats in diestrus. The *mPRα, mPRβ, mPRγ, mPRδ* and *mPRε* genes were found expressed in the rat pituitary gland (Figure 1). According to data shown in the table, a qualitative analysis of the putative abundance of each mRNA shows that *mPRs* would represent approximately the 15% of the total progesterone receptors. Although values shown are related to the ΔCTs results from qPCR assays, they show that *mPRα* and *β* are greatly expressed in the pituitary and are probably more abundant than other mPRs.

***mPRα is expressed in lactotrophs, gonadotrophs and somatotrophs.***

With *mPRα* being probably the most abundant *mPR* found in the pituitary, we next performed double immunocytochemistry (ICC) to assay mPRα protein co-expression with PRL, LH and GH cells. Immunostaining of mPRα was detected in lactotrophs, gonadotrophs and somatotrophs (Figure 2).

To explore the percentage of mPRα-positive cells among the total anterior pituitary cells and among the lactotroph population we ran double indirect immunofluorescence assays for both mPRα and PRL. Using an approach that preserves the integrity of the cell membrane (33) we detected the percentage of anterior pituitary cells and lactotrophs expressing mPRα by flow cytometry (Figure 3). Results revealed that 42.1 ± 11.6% of total anterior pituitary cells were mPRα-positive (Fig. 3A) and 63% of the mPRα-positive cells were also PRL-positive, (37% of the mPRα-positive cells were non-lactotrophs) (Fig. 3B).

***Effect of progesterone and specific mPR agonist Org OD-02-0 (02) on PRL secretion***

In order to evaluate the role of mPRα on lactotroph function, the effect of progesterone and Org OD 02-0, a selective mPR agonist with no nPR agonist activity (23,24,34) on PRL secretion, was assayed in anterior pituitaries incubated *ex vivo* (Figure 4). The use of this model (pituitary explants) allows as to study the direct effect of progesterone (or mPR agonist) in the pituitary, avoiding the well known progesterone actions in the hypothalamus, which affects, indirectly, the pituitary function in living organisms. On the other hand, the use of explants also maintains the tissue extracellular matrix structure, the paracrine communications and the inter-cellular junctions, both involved in cell-cell communication.

Interestingly, treatment with progesterone (P4, 100 nM) for 1 h strongly inhibited PRL release (Fig. 4A), increasing PRL content in the pituitary (Fig. 4B), an effect that was mimicked by Org OD 02-0 treatment, suggesting the involvement of mPRα in this progesterone action on PRL secretion. Fig 4C shows values normalized to total PRL (pituitary content + secreted PRL) in order to observe that the increase in pituitary PRL content after P4 or Org OD 02-0 treatment is mainly due to the inhibition of hormone secretion.

***mPRs are expressed in the GH3 cell line***

To elucidate the cellular mechanisms behind mPRα activity, the following studies were performed using the GH3 cell line (CCL-82.1™). The GH3 cell line derives from a pituitary tumor carried in a 7-month-old female Wistar-Furth rat, and produces both growth hormone and prolactin providing an excellent tool to study lactotroph-like function and signaling (35). First, mRNA expression of mPRs was examined in the GH3 cell line by qRT-PCR (Figure 5). Based on ΔCT data obtained, mPRα mRNA appears to have the highest expression among mPRs (Fig. 5A), followed by mPRε and mPRβ. Then, using GH3-cells plasma membrane extracts, the protein expression of mPRs, nPRs and the progesterone receptor membrane component-1 (PGRMC1) was detected (Fig. 5B) using specific mPRs, nPRs and PGRMC1 antibodies (Table 2) by Western Blot. Immunoreactive bands of mPRβ (≈40 kDa) and PGRMC1 (≈28 kDa) were observed. However, although denaturing conditions were used (SDS-PAGE), mPRα, mPRγ and mPRε bands were detected at ≈80 kDa. These molecular weights were previously described (16, 23, 24, 32), which authors suggested could be due to dimer formation. Although further work is required in order to establish whether this is the case, the reduction observed in the staining for this band after siRNA to mPRα in GH3 cells (Figure 8), is consistent with it being mPRα. The presence of two bands of predicted sizes for nPRs (88-110kDa) was detected in cell lysates (c), and weak bands were observed in membrane extracts (m). In addition, mPRs expression and localization were examined by immunocytochemical analysis (ICC), (Fig. 5C). We found protein expression of mPRα, -β, -γ, -ε and PGRMC1 in GH3 cells but not mPRδ, probably reflecting its low mRNA levels.

***Involvement of second messengers in mPRα mediated progestin effects on PRL secretion***

The specific binding of mPRα agonist Org OD 02-0 to GH3 cells membranes was demonstrated by a ~~In~~ single-point ligand competition assay~~s~~. A significant displacement of [$^3$H]-P4 binding to GH3 plasma membranes was observed by both progesterone and Org OD 02-0, whereas the nuclear PR agonist R5020 was ineffective (Figure 6A). This result shows the specificity of progestin binding sites in GH3 cells-membrane extracts, characteristic of mPRs.

Since it was observed that progesterone and Org OD 02-0 inhibit PRL secretion in *ex vivo* pituitary incubations, this effect was studied in the GH3 cell line (Figure 6B). Results showed that PRL was significantly decreased by 20 nM progesterone and Org OD 02-0 treatments, but not with R5020 treatment suggesting the involvement of mPRα in this progesterone action. Hence, the involvement of second messengers in mPRα-mediated effects was examined. mPRα has been shown to alter second messenger pathways in a variety of cells (i.e. MCF-7 and SK-BR-3 human breast cancer cells, and human umbilical vein endothelial cells) through activation of a pertussis toxin-sensitive inhibitory G protein. To test this in our model, GH3 cells were pretreated with activated pertussis toxin (PTXa, 2.5μg/ml) or heat-inactivated pertussis toxin (PTXhi) 30 min prior to Org OD 02-0 treatment (20 nM, 4 h) and then PRL levels were measured. PTXa treatment completely abolished the inhibition of PRL secretion induced by Org OD 02-0 (Fig. 6C) whereas PTXhi was ineffective, suggesting the involvement of an inhibitory G protein in this effect. The role of cAMP signaling was also investigated. Treatment with either progesterone or Org OD 02-0 resulted in a significant decrease in cAMP accumulation after 10 min (Fig. 6D), probably due to progesterone activation of an inhibitory G protein (Gαi), with a consequent decrease in adenylyl cyclase activity and cAMP cellular levels. Finally, the involvement of the MAP kinase cascade in mPRα-mediated progestin effects on PRL secretion was evaluated by Western Blot analysis of ERK1/2 phosphorylation after treatments with P4, Org OD 02-0 and R5020. Both, progesterone and Org OD 02-0 stimulation induced a significant increase in ERK phosphorylation while R5020 did not, (Fig. 6E) indicating the involvement of mPR, but not nPR in this effect.

***TGFβ1 involvement in mPRα mediated effects***

Transforming growth factor beta 1 (TGFβ1) is a multifunctional and ubiquitous cytokine, well known for its ability to inhibit cell proliferation in epithelial cells. In the pituitary, TGFβ1 and its receptor TβRII are expressed in lactotrophs, and TGFβ1 is a known inhibitor of lactotroph function (36,37). Stimulatory effects of progesterone on TGFβ1 activation have been previously reported in different tissues,(38-40); however the receptors and mechanisms involved were not investigated in those studies. In order to explore the possible TGFβ1-mediated action in PRL inhibition induced by mPRα

stimulation, active TGFβ1 levels after progestin stimulation in GH3 cells were measured by specific ELISA (Figure 7). Treatment with either P4 or Org OD 02-0 significantly increased active TGFβ1 levels after 15 min, whereas the nPR agonist R5020 had no effect (Fig 7A), suggesting that the rapid progesterone effect on TGFβ1 activation was mediated by mPRα.

In addition, signaling pathways involved in this effect were investigated using pertussis toxin (2.5 μg/ml), a cAMP analog (10 μM, 8-Br-cAMP) and a specific ERK inhibitor (10 μM, PD98059). Pretreatment with PTX completely blocked the activation of TGFβ1 by Org OD 02-0 (Fig. 7B), suggesting the involvement of the inhibitory G protein Gαi in this effect. Likewise, co-treatment with 8-Br-cAMP, which increases cAMP levels, also abolished Org OD 02-0-induced TGFβ1 activation, indicating the involvement of decreased cAMP levels in this Org OD 02-0 effect (Fig 7C). However, Org OD 02-0 treatment was able to increase active TGFβ1 levels even in the presence of ERK inhibitor, PD98059 (Fig 7D), suggesting that the MAP kinase pathway may not be involved in Org OD 02-0-activation of TGFβ1 through mPRα.

Finally, in order to determine the specific role of mPRα in mPR-mediated TGFβ1 activation, siRNA directed against mPRα was used to knock down its expression in GH3 cells (Figure 8). Transfection with mPRα siRNA knocked down mPRα protein expression in GH3 cell membranes (Fig. 8B) and completely abolished the progesterone and Org OD 02-0-induced increase in TGFβ1 activation (Fig. 8A).

## DISCUSSION

mPRs mediate a wide variety of the non-genomic actions of progestins and are localized in the major reproductive tissues of mammals. Our present results clearly demonstrate that mPRs, protein and transcripts, are expressed in the rat pituitary gland as well as in the GH3 cell line. The observation that mPRα would appear to be the most abundant in both models is consistent with previous studies showing mPRα as a major mPR subtype expressed in the pituitary gland of other species (21,41,42). mPRα expression was found in gonadotrophs, somatotrophs and mainly in lactotrophs. The

localization in gonadotrophs emphasizes the involvement of these receptors in the control of GnRH-gonadotroph axis and deserves future investigation. In agreement, previous results showed the potential role of mPRα in the progesterone-mediated inhibitory effect on GnRH release and LH secretion in rodents, even in PR-KO mice (25). mPRα expression was also found in rat somatotrophs, but the significance of this finding is currently unknown. On the other hand, the fact that mPRα was found highly expressed in the lactotroph population suggests a physiological role for this receptor in the lactotroph function.

The role of progesterone in the regulation of PRL secretion is controversial. Some studies reported inhibition (43), whereas other reported enhancement of PRL secretion (44). Our present findings support the role of progesterone as a negative modulator. This is in agreement with previous observations of Cramer *et al*. (45) which showed that *in vivo* progesterone treatment stimulates dopamine release into the hypophysial portal blood in female rats. In accordance, Arbogast *et al.* reported that progesterone enhances tyrosine hydroxylase (TH) activity, which consequently leads to the inhibition of PRL secretion (12). On the other hand, it has been largely assumed that most of the progesterone direct or indirect effects on PRL secretion depend on the activation of the nuclear PRA/B receptors. However, our present results, using an *ex vivo* assay in order to avoid hypothalamic influence, demonstrated that either progesterone or the mPRα selective agonist significantly decreased PRL secretion suggesting the involvement of mPRα in this inhibitory effect. However, as mPRα expression was also found in both somatotrophs and gonadotrophs, we do not discard paracrine interactions.

In order to deepen our understanding of the mechanisms involved, studies in the GH3 cell line were performed. First, the expression of the five mPRs isoforms was demonstrated in GH3 cells, by real time qPCR, western blot and ICC, with mPRα being the most abundant. Second, progesterone as well as Org OD 02-0, but not R5020, were able to displace [3H]-P4 binding to GH3 cells-membrane extracts demonstrating the specificity of progestin binding sites in GH3 membranes, characteristic of mPRs. Third, prolactin secretion was inhibited by progesterone and Org OD 02-0, but not by R5020 in GH3 cells thereby validating our previous results obtained from the *ex vivo* experiment with

pituitary tissue. In addition, the fact that the Org OD 02-0 effect on PRL secretion was blocked by pretreatment with pertussis toxin indicates that this effect is dependent on the activation of an inhibitory G protein (Gi). In accordance, the treatment with either progesterone or Org OD 02-0 decreased cAMP levels probably due to a decrease in the activity of adenylyl cyclase. Interestingly, this mechanism of action, reducing intracellular cAMP levels through the inhibition of adenylyl cyclase activity, has also been described for dopamine as the primary mechanism by which dopamine suppresses PRL gene expression and PRL release (46).

Finally, a novel progesterone inhibitory mechanism mediated by mPRα in lactotrophs was described. It has been previously shown that TGFβ1 mediates, at least partially, the dopamine inhibitory action on lactotrophs. Dopamine, acting through the Drd2, up-regulates TGFβ1 expression and secretion contributing to the inhibitory effect of dopamine (36,37). Recent evidences obtained in our laboratory indicate that pituitary TGFβ1 activity is regulated by dopamine and estradiol, the main factors involved in the control of lactotroph function (47), but how progesterone regulates this cytokine in the pituitary remains unknown. Other groups have reported stimulatory progesterone effects on TGFβ1 activation in breast epithelial cells and lung cells (38-40) but the receptors and mechanisms involved were not investigated in those studies. One of the major findings of the present study is that mPRα is the main receptor mediating the rapid, nongenomic actions of progesterone resulting in TGFβ1 activation in GH3 cells. The fact that the Org OD 02-0 mimicked the stimulatory effects of progesterone on TGFβ1 activation, while the nPR agonist R5020 was ineffective, supports the involvement of mPRα in this effect. In addition, the knockdown studies with mPRα siRNA provided definitive evidence that mPRα is the principal receptor regulating this rapid effect of progesterone on TGFβ1 activation.

Moreover, the stimulatory effect of Org OD 02-0 on TGFβ1 activation was found to be caused by the activation of an inhibitory G protein with consequent reduction in cAMP levels, since these actions were blocked by pretreatment with pertussis toxin, and the co-treatment with a cAMP analog (8-Br-cAMP) respectively. This signaling pathway has been previously proposed for TGFβ1 activation in response to dopamine through D2 receptors coupled to an inhibitory G protein in rat lactotrophs (48).

In addition, Pastorcic and Sarkar (49) demonstrated that treatment of anterior pituitary cells with forskolin, which activates adenylyl cyclase, significantly inhibited TGFβ1 levels in culture medium and TGFβ1 mRNA levels in cellular extracts. Thus, it is possible that inhibition of cAMP-dependent mechanisms may be involved in progestin regulation of TGFβ1. Besides the inhibition of the cAMP system, the mPRs have been shown to alter other second messenger pathways in a variety of target cells through the activation of the MAPK system (27). However, in our experimental model, a pharmacological inhibition of this pathway did not block Org OD 02-0-induced TGFβ1 activation suggesting that the MAPK cascade is not involved in this effect.

In summary, the present results demonstrate that mPRs are expressed in the female rat pituitary, with mPRα being the most abundant. We found an inhibitory effect of progesterone on PRL secretion acting through mPRα in *ex vivo* incubated female rat pituitaries as well as in the GH3 cell line. This effect was found to be mediated by a Gαi protein with a concomitant decrease in cAMP levels. Moreover, we describe for the first time, the involvement of mPRα in mediating the effect of progesterone on pituitary TGFβ1 activation. This increased level of local TGFβ1 activity is most likely involved in the direct inhibitory action of progesterone on PRL release.

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**AUTHOR CONTRIBUTIONS:** MAC, DP, JF, PT, SG and GDT conception and design of research; MAC, JF, AAM, AC, SG and EF performed experiments; MAC, JF and GDT analyzed data; MAC, JF, PT and GDT and interpret results of experiments; MAC prepared figures; MAC and GDT drafted manuscript; MAC; GDT, JF, SG and PT edited and revised manuscript; MAC, JF, PT, SG and GDT approved final version of manuscript.

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| Gene | Accession no. | Forward primer sequence 5´ - 3´ | Reverse primer sequence 5´ - 3´ |
|---|---|---|---|
| *CypB* | NM_022536 | GACCCTCCGTGGCCAACGAT | GTCACTCGTCCTACAGGTTCGTCTC |
| *mPRα* | NM_001034081 | CCGCATCATAGTGTCTCCCC | TGTCCCTGCCCAAAGATGTG |
| *mPRβ* | NM_001014099 | TACAAAGGTTGTCCTACTGAACCC | AGAATCTTGGGCAGACGACG |
| *mPRγ* | NM_001014092 | GTGCCACAGGTGTTCCATGA | ATGCGTCCAGATGTTGAGGG |
| *mPRδ* | NM_001191077 | CCACTTGGTACTTCCTGTGGC | CGAGGCGACATGGAGCTAAA |
| *mPRε* | NM_001271152 | CACTGTAGCCTGCTGCAAGA | AAGTAGCGGCGGTAGAAGTG |
| *PGRMC1* | NM_021766 | GTTCACCTTCAAGTACCATCACG | CTTCCGAGCAGCCTCATCTT |
| *PR* | NM_022847 | GACAACACAAAGCCCGACAC | CGGAAACCTGGCAGAGACTT |
| *PR B* | NM_022847 | GCATCGTCTGTAGTCTCGCCAATAC | GCTCTGGGATTTCTGCTTCTTCG |

Table 1: qPCR primers

| Peptide/protein target | Antigen sequence | Name of Antibody | Manufacturer, and/or name of individual providing the antibody | Species raised in; monoclonal or polyclonal | Dilution used |
|---|---|---|---|---|---|
| membrane progesterone receptor alpha (mPRα) | N-terminal TVDRAEVPPLFWKPC | 306t | Thomas | Rabbit, polyclonal | 1:500 for WB; 1:50 for IHC and ICC |
| membrane progesterone receptor beta (mPRβ) | N-terminal KILEDGLPKMPCTVC | A9830-4 | Thomas | Rabbit, polyclonal | 1:1000 for WB; 1:100 for ICC |
| membrane progesterone receptor gamma (mPRγ) | N-terminal TDIKNDSYSWPMLC | H833-4 | Thomas | Rabbit, polyclonal | 1:1000 for WB; 1:100 for ICC |
| membrane progesterone receptor delta (mPRδ) | N-terminal CQGGPLEGGTAKQQ | PAQR6 | Thomas | Rabbit, polyclonal | 1:1000 for WB; 1:100 for ICC |
| membrane progesterone receptor epsilon (mPRε) | N-terminal RNSHSAASRDPPASC | PAQR9 | Thomas | Rabbit, polyclonal | 1:1000 for WB; 1:100 for ICC |
| Progesterone Receptor Membrane Component 1 (PGRMC1) | Internal region of PGRMC1 of human origin | PGRMC1 (D-16) sc-82684 | Santa Cruz Biotechnology, Inc., 82684 | Goat, polyclonal | 1:1000 for WB; 1:100 for ICC |
| Nuclear progesterone receptor | amino acids 731-909 of PR of human origin | PR (2C11F11) | Santa Cruz Biotechnology, Inc., 130071 | Mouse, monoclonal | 1:1000 for WB |

Table 2: Antibodies

| gene | $2^{-\Delta CT} \pm$ SEM |
|---|---|
| *mPRα* | 0.155 ± 0.011 |
| *mPRβ* | 0.113 ± 0.008 |
| *mPRγ* | 0.025 ± 0.003 |
| *mPRδ* | 0.020 ± 0.001 |
| *mPRε* | 0.0725 ± 0.010 |
| *PR-A* | 2.298 ± 0.305 |
| *PR-B* | 0.3045 ± 0.074 |