Molecular and Cellular Probes xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Differential expressions of estrogen and progesterone receptors in endometria and cyst walls of ovarian endometrioma from women with endometriosis and their responses to depo-medroxyprogesterone acetate treatment

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ARTICLE INFO

Keywords:
Endometriosis
Estrogen receptors
Progesterone receptors
Expression
Depo-medroxy progesterone acetate (DMPA)

ABSTRACT

Background: Depo-medroxyprogesterone acetate (DMPA) is an injectable progestin contraceptive that provides a highly effective reduction of pelvic pain in women with endometriosis. Despite its wide use to treat pain associated with endometriosis, its precise mechanisms of action remain unclear. The aims of this study were to investigate the differential expressions of estrogen receptors (ERs), and progesterone receptors (PRs) in endometria and ovarian endometrioma cyst walls of women with endometriosis with and without DMPA treatment.

Methods: Endometria and cyst walls of endometrioma were obtained from 25 to 45 year-old women who suffered from endometriosis and had ovarian endometrioma with the size ≥3 cm. The expression levels of ERs and PRs and the numbers of ER- and PR-positive cells before and after treatment with DMPA were evaluated by Western blot, real-time PCR, and immunohistochemistry.

Results: The levels of ERα and ERβ expression, their corresponding mRNAs, and numbers of ERα- and ERβ-immunoreactive cells in stroma and glands of endometria of the DMPA group were significantly decreased when compared with those of the untreated groups ($p < 0.05$). In contrast, the levels of PRA/B expression and numbers of PRA/B positive cells in stroma and number of PRB positive cells in stroma and endometrial glands were significantly increased in endometria of the DMPA group when compared with those of the untreated groups. However, in cyst wall the expression levels of these proteins, their corresponding mRNAs, and immonoractive cells were low compared to those in endometria, and DMPA-treatment did not cause any significant changes in these parameters.

Conclusion: These data indicated that DMPA could upregulate the expressions of PRA/B and down-regulate ERα and ERβ in endometria but not in cyst walls from women with endometriosis.

1. Introduction

Endometriosis is an abnormal growth of endometrial tissue at ectopic sites in the peritoneal cavity that sometime infiltrates into the bowel, uterus, and ovary, which often results in severe pain and infertility [1–3]. This disease is a major health problem that afflicts 7–10% of women [4]. Although the exact pathogenesis mechanisms of endometriosis are still unclear, it is well known that estradiol stimulates the growth of endometriotic tissue [5], and there is a 7-fold increase of estradiol in women with endometriosis when compared with normal persons [6]. The actions of estradiol are mediated within target cells by two isoforms of estrogen receptors (ERs): estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β) [5,7]. High level of estradiol also causes the reduction in the levels of progesterone receptors (PRs),

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https://doi.org/10.1016/j.mcp.2018.07.001
Received 27 November 2016; Received in revised form 21 June 2018; Accepted 2 July 2018
0890-8508/

which leads to progesterone resistance and the down–regulation of 17 β-hydroxysteroid dehydrogenase type 2 (17β-HSD-2), the enzyme that converts estradiol (E2) to estrone (E1) which is less potent than estradiol. Thus, the level of mitogenic estradiol is elevated in women with endometriosis [7]. The modified steroid receptor coactivator-1 (SRC-1) promotes endometriosis progression by facilitate proliferation and invasion of ectopic endometrium [8].

Endometriosis can be treated with medications and/or surgery. Surgery provides temporary relief for women with severe endometriotic pain. However, seventy five percent of the treated women have recurrent endometriosis and pain within 2 years [9], and frequently further operation is required in many cases. Drug treatments include contraceptive steroids, gonadotropin-releasing hormone agonist (GnRH-a), Danazol, and Progestin [10,11]. GnRH-a is effective in treating symptomatic endometriosis, limits growth and activity of endometriotic masses, and suppresses ovarian estradiol production [12,13]. Unfortunately, the most serious side effect of GnRH-a treatment is a rapid bone loss, so that GnRH-a therapy is limited to a short period of time (3–6 months) [11]. Depo-medroxyprogesterone acetate (DMPA) is an injectable progestin-based contraception that is highly effective in reducing pelvic pain in women with endometriosis [11]. In addition, the advantages of DMPA treatment include high therapeutic efficacy, low-cost, safety, and only a single injection is required every 3 months [14]. Despite its wide usage in treatment for pain-related endometriosis [15,16], the precise mechanisms of DMPA actions remain unclear. In this report, we have investigated the effects of DMPA on the levels of expressions of ERs and PRs in the endometria as well as ovarian cyst walls of endometrioma from women with endometriosis by using Western blot analyses, qPCR, and immunohistochemistry.

2. Materials and methods

2.1. Subjects

The women in this study were categorized into 3 groups: endometriotic-untreated with proliferative phase (n = 10), endometriotic-untreated with secretory phase (n = 11), and DMPA-treated (n = 16) groups. All women, aged between 25 and 45 year-old, were admitted at Department of Obstetrics and Gynecology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand. All women in 3 groups had ovarian endometrioma with the size ≥3 cm. The biopsied eutopic endometria and cyst walls of endometrioma from endometriotic-untreated group were collected from women who had not received any hormonal treatment before biopsy for at least 6 months. The biopsied endometria and cyst wall from DMPA-treated group were collected from women who had received 150 mg DMPA during previous 12 ± 2 weeks before biopsies. The protocol for tissue biopsy was submitted and approved by the Ethical Clearance Committee on Human Rights Related to Researches Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (No.MURA2011/215/S6May14). Informed consents were obtained from all participants included in the study.

2.2. Western blot analyses of ER and PR expressions

The endometrial and cyst wall samples were homogenized in a lysis buffer and incubated on ice for 10 min with gentle shaking. The homogenates were centrifuged at 10,000 × g, 4 °C for 20 min. The supernatants were collected and protein contents were measured by a modified Lowry method (DC Protein Assay Kit; Bio-Rad, Hercules, CA, USA). Pooled samples from the same group as well as individual samples were analyzed by Western blot. Twenty micrograms of protein from each sample was loaded and separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% separating gel and transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies overnight at 4 °C at the following dilutions: 1:100 of anti-ERα (sc-7207; Santa Cruz Biotechnology, CA), 1:100 of anti-ERβ (sc-8974; Santa Cruz Biotechnology), 1:500 of anti-PRA/B (sc-7208, Santa Cruz Biotechnology), and 1:500 of anti-β-actin (sc-130656, Santa Cruz Biotechnology). The membranes were then incubated with the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL), for 1 h at room temperature. The color was developed by incubating with NBT/BCIP solution (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The experiments were done in triplicate and the intensities of immunoreactive bands were evaluated by using ImageJ software. The ratio between target protein and β-actin intensity in each lane was determined.

2.3. Real time PCR analyses of ER and PR expressions

2.3.1. RNA preparation and reverse transcription

Total RNAs were isolated from endometrial and cyst wall samples by using Trizol® reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Genomic DNA was then removed by using DNase I (Invitrogen) to remove genomic DNA, following the manufacturer's instructions. The iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., CA, USA) containing the unique blend of oligo (dT) and random hexamer primers was used to synthesis cDNA according to the manufacturer's protocol.

2.3.2. qPCR

Quantitative expression of ERs and PRs genes were performed by using the iTaq™ universal SYBR® Green supermix (Bio-Rad Laboratories, Inc). The ERα and ERβ genes' expressions were determined by using primers as follows: ERα forward, 5′-CCTCGGACACCTTGCCTGAA-3′; ERα reverse, 5′-CATGCCCTCTACACATTTTCCC-3′; ERβ forward, 5′-GGGTGCAAGTCCTCCATCAC-3′; ERβ reverse, 5′-GACACACTGGAGT TCACGCT-3′. The PRA/B primers were used to determine the expression of both PRA and PRB mRNA because PRA mRNA cannot be distinguished from PRB mRNA. The PRA/B primers are as follows: PRA/B forward, 5′-CAGCCAGAGCCCACAATACA-3′, PRA/B reverse, 5′-GCTC CCACAGGTAAGGACAC-3′. GAPDH was used as the internal controls with primer sequences as follows: GAPDH forward, 5′- GGACTGACCT GCCGTCTAG-3′; GAPDH reverse, 5′-TAGCCCAGGATGCCCTTGAG-3′. Each qPCR run was performed using cDNA from individual women comprising of endometriotic-untreated in proliferative phase (n = 10), endometriotic-untreated in secretory phase (n = 11), and DMPA-treated (n = 16). To determine the levels of mRNAs of ERs, PRs and GAPDH, each 10 μl reaction mixture containing 5 μl iTaq™ universal SYBR® Green supermix, 1.5 μl forward primer, 1.5 μl reverse primer, and 2 μl of 10 ng cDNA template was prepared. The PCR reaction was performed using the CFX96 real-time PCR detection system ((Bio-Rad Laboratories, Inc.). An initial denaturation step was set at 95 °C for 3 min, 44 cycles at 95 °C for 5 s, and 65 °C for 30 s. Each experiment was performed in duplicate for each individual sample. Relative quantification was analyzed by $2^{-\Delta\Delta Cq}$ method as described previously [17,18]. The relative expressions were shown as change in folds of mRNAs for target genes, ERα, ERβ, and PRA/B, relative to the control condition (endometriotic-free samples), and all of which were normalized with mRNA of the housekeeping gene, GAPDH. The Cq data were imported from Bio-Rad CFX manager 3.1 software (Bio-Rad Laboratories, Inc). The mean of fold change of each target gene expression was calculated using the following equation: relative gene expression = $2^{-\Delta\Delta Cq}$ [17], where $\Delta\Delta Cq = (C_{q,Target} - C_{q,GAPDH})-(C_{q,control} - C_{q,GAPDH})$ [17].

2.4. Immunohistochemical detections of ER- and PR-positive cells

Endometrial and cyst wall samples were fixed in a 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 24 h at 4 °C and embedded in Paraplast blocks. The embedded tissues were cut using rotary

microtome at 5 μm thickness and the sections were placed onto the silane-coated slides. The sections were deparaffinized with xylene and then rehydrated in a serial concentrations of ethanol. The endogenous peroxidase was quenched with 3% H_2O_2 and the sections were incubated for 2 h in a blocking solution and then incubated with the primary antibodies overnight with the following dilutions: 1:50 of anti-ERα (sc-7207; Santa Cruz Biotechnology), 1:50 of anti- ERβ(sc-8974; Santa Cruz Biotechnology), 1:100 of anti-PRA/B (sc-7208, Santa Cruz Biotechnology), and 1:100 of anti-PRB (ab97801; Abcam). After that, the sections were washed and incubated with the secondary antibody, HRP-conjugated goat anti-rabbit IgG (Southern Biotech) for 45 min. The liquid DAB-Plus Substrate Kit (Invitrogen, Camarillo, CA, USA) was added onto the tissue sections to develop the color for 15 min, and the reaction stopped by washing in tap water. The stained tissue sections were observed and photographed under a Nikon E600 microscope equipped with a Nikon digital DXM1200 camera, using an ACT-1 software package.

Each slide was examined by two observers who did not know the designated groups of the tissue sections. Clear immunohistochemically stained cells were counted per 1000 cells in five high power fields (examined with 40 × objective lens). The numbers of ERα-, ERβ-, PRA/B-, PRB-labeled stromal or epithelial cells per 1000 cells were counted separately and the results were reported as means from data gathered by the two observers.

Apart from the numbers of immunopositive cells, intensities of the immunoreactivities that reflect the levels of ERs and PRs expressions in cells were analyzed by semi-quantitative H-score (Nap et al., 2004) which was calculated by the following formula: 3 × percentage of strongly staining cells + 2 × percentage of moderately staining cells + 1 × percentage of weakly staining cells. The intensities of immunoreactivities in cells were graded as 0 (no staining), 1 (weak), 2 (moderate) or 3 (strong).

2.5. Statistical analyses

The data were reported as means ± standard errors of means (SEM). Non-parametric Kruskal-Wallis test was used to compare data between untreated proliferative phase, untreated secretory phase, and DMPA-treated groups. One-way ANOVA and the Tukey's HSD Test were used to compare the data between groups. The level of significance was set at $p < 0.05$.

3. Results

3.1. Western blot analyses of ER and PR expressions

In Western blot analyses, the blotted membranes of pooled endometrial samples incubated with anti-ERα (sc-7207) and anti-ERβ (sc-8974) showed immunoreactive bands at ~66 and ~56 kDa, respectively. It was found that the intensities of ERα and ERβ bands were highest in the pooled endometria from women with endometriosis in proliferative phase which were significantly higher than those in the pooled samples from women with endometriosis in secretory phase and DMPA-treated women with endometriosis (Fig. 1A and B). When individual endometrial samples were examined it was found that samples from most untreated women showed high expression of ERα and low expression of ERβ while only few samples showed low expressions of both, and the expression of ERα was consistently higher than ERβ in most women. In contrast, most DMPA-treated samples showed decreased expressions of both receptors while only few still showed high expression of ERα (Fig. 1C). When the intensities of individual samples were summed together it was found that the patterns of expressions followed the same trend as found in the pooled samples (Fig. 1D). When using anti-PRA/B (sc-7208) as a probe, the immunoreactive bands of PRA and PRB were detected at ~81 and ~116 kDa, respectively, with PRA appearing more prominent than PRB in both pooled and most individual samples. The intensities of immunoreactive PRA and PRB bands were highest in the pooled endometrial samples from DMPA-treated women with endometriosis, especially for PRA (Fig. 1A and B). When individual samples were examined it was found that most untreated samples showed low expressions of PRA and PRB while few samples still showed high expression of PRA. In contrast, most samples from DMPA-treated women showed increased expressions of both receptors while only few still showed low expressions (Fig. 1C). When the intensities of individual samples were summed together it was found that the pattern of expressions followed the same trend as found in the pooled samples (Fig. 1D). Moreover, in the DMPA-treated individual samples the increased intensities were more marked for PRA than PRB (Fig. 1C).

In the pooled ovarian cyst walls, the intensities (expressions) of all receptors were generally lower by a half compared to endometria. ERβ was more intense than ERα in both proliferative and secretory phases, and PRA intensity also appeared high in proliferative phase and dropped down in secretory phase while PRB appeared low throughout (Fig. 2A). Treatment with DMPA caused the decreases of intensities of both ERα and ERβ and increases of the intensities of PRA and PRB over the levels at secretory phase, but the differences were not significantly (Fig. 2A and B). Western blotting of individual samples from ovarian cyst walls showed that most samples exhibited the expression patterns of ERα, ERβ, PRA and PRB similar to the pattern observed in pooled samples but at lower levels, and their summations showed the same profiles as the pooled samples. However, in comparison to endometria, treatment with DMPA caused only small decreases of ERα, ERβ and increases of PRA, PRB, and the differences were not significantly different from the untreated groups (Fig. 2C and D).

3.2. qPCR analyses of ER and PR expressions

Real-time PCRs were performed to examine the relative expression levels of mRNAs encoding ERα, ERβ, and PRA/B normalized by mRNA of GAPDH in endometrial and ovarian cyst wall samples. The mRNA expression levels of ERα was higher than ERβ in endometria during both proliferative and secretory phases, and DMPA-treatment caused significantly lower expressions of both receptor mRNAs (Fig. 3A). The expression levels of combined PRA/B mRNAs in endometria from untreated women in both proliferative and secretory phases were comparable and relatively high, and DMPA-treatment caused significantly higher expression levels of the combined PRA/B mRNAs (Fig. 3A). In contrast, in the cyst walls, the expression levels of mRNAs encoding ERα was lower than in endometria while that of ERβ was higher. The expressions of PRA/B in the ovarian cyst walls were comparable during both proliferative and secretory phases and lower than those in endometria. Treatment with DMPA did not cause any significantly differences in the expression levels of mRNAs for ERα, ERβ, and PRA/B in the cyst walls (Fig. 3B).

3.3. Immunohistochemical analyses of cellular expressions of ERs and PRs

Immunohistochemistry was performed to investigate the cellular expressions of ERs and PRs. In endometria, the immunoreactivities which reflected the expressions of ERα, ERβ, PRA/B and PRB were found in both epithelial and stromal cells. The expressions of ERα and ERβ were found to be more intense in epithelial cells whereas PRA/B and PRB expressions were more intense in stromal cells. The ERα tissue expression was more intense than the ERβ and the PRA/B tissue expression was also more intense than the PRB (Fig. 4). The numbers of cells containing ERα-immunoreactivity (ERα-ir), ERβ-immunoreactivity (ERβ-ir), PRA/B-immunoreactivity (PRA/B-ir), and PRB-immunoreactivity (PRB-ir) per 1000 cells were counted by including both the stromal and epithelial cells (Fig. 5). The number of ERα-ir cells in endometria of women with endometriosis during proliferative phase was higher than during secretory phase, and the

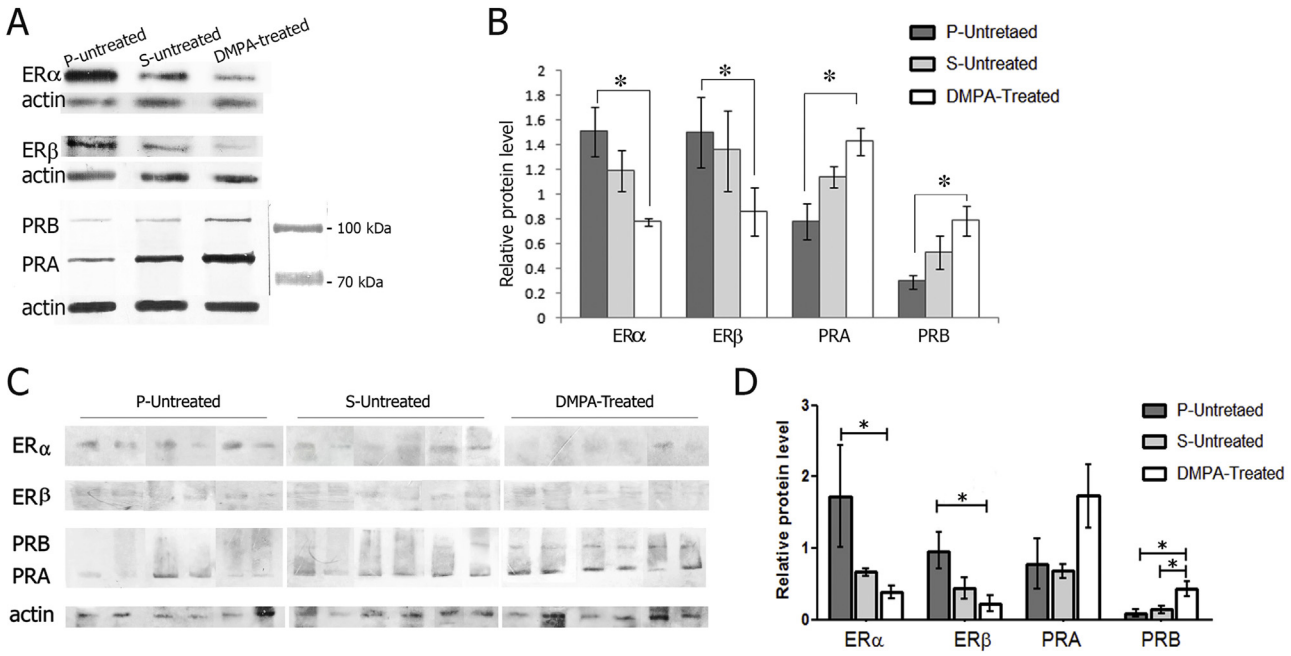

Fig. 1. (A) Western blot analyses of pooled samples of eutopic endometria from women with endometriosis. Lanes 1–2 are the pooled eutopic endometria from untreated women with endometriosis in proliferative and secretory phases, respectively. Lane 3 is the pooled eutopic endometria from DMPA-treated women with endometriosis. Using anti-ERα and anti-ERβ as probes, the immunoreactive bands are revealed at molecular weights of 66 kDa and 56 kDa, respectively, and the intensities of these bands in endometria of the DMPA-treated group were significantly reduced when compared with those in the untreated groups. Using anti-PRA/B as a probe, the two immunoreactive bands are revealed at molecular weights of ~81 kDa representing PRA and ~116 kDa representing PRB. These bands appear to be more intense in the DMPA-treated endometria when compared to the untreated samples. (B) Ratios of the intensities of the target protein bands from Fig. 1A normalized with actin band as estimated by ImageJ software. (C) Western blot analyses of individual samples of eutopic endometria from women with endometriosis using anti-ERα, -ERβ, and -PRA/B antibodies as probes. Lanes 1–6 are individual eutopic endometria from untreated women with endometriosis in proliferative phase. Lanes 7–12 are individual eutopic endometria from untreated women with endometriosis in secretory phase. Lanes 13–18 are individual eutopic endometria from DMPA-treated women with endometriosis. (D) Average intensities from individual samples normalized with their corresponding actin bands.

treatment with DMPA significantly decreased the number of positive cells. The number of ERβ-ir cells was lower than ERα-ir cells and they tended to be more numerous in endometria of women with endometriosis during secretory phase than during proliferative phase. DMPA treatment also caused significant decrease in number of ERβ-ir cells. The numbers of both PRA/B and PRB-ir cells were relatively low during both proliferative and secretory phases of untreated endometria, and treatment with DMPA increased the numbers of positive cells (Fig. 5).

When the two types of cells (stromal and epithelial) were analyzed separately it was found that the endometria from untreated women with endometriosis showed higher number of ERα-ir cells in stroma and glands during proliferative phase than secretory phase. Treatment with DMPA decreased the number of ERα-ir positive stromal cells (44.88 ± 5.84 cells/1000 cells) when compared with the numbers of positive cells in the untreated endometria during proliferative (106.80 ± 34.40 cells/1000 cells) and secretory phases (63.89 ± 9.57 cells/1000 cells). In the uterine glands, the number of ERα-ir cells in DMPA-treated endometrium was significantly lower (63.19 ± 13.61 cells/1000 cells) when compared with untreated endometria at proliferative (187.40 ± 29.97 cells/1000 cells, $p < 0.01$) and secretory phases (164.11 ± 45.94 cells/1000 cells, $p < 0.05$) (Fig. 6A). When applying a semi-quantitative method, H-score, to estimate immunoreactive cells with different levels of intensities, it was confirmed that the ERα expression in epithelial cells was significantly decreased in DMPA-treated group when compared with untreated groups (Fig. 6B).

The number of ERβ-ir stromal cells in the endometria of DMPA-treated group was decreased significantly (16.00 ± 2.57 cells/1000 cells) when compared with untreated endometria of women with endometriosis during proliferative (32.00 ± 4.56 cells/1000 cells, $p < 0.05$) and secretory phases (36.60 ± 6.58 cells/1000 cells, $p < 0.01$). In the uterine glands, the number of ERβ-ir cells in DMPA-treated endometria (13.00 ± 2.84 cells/1000 cells) was also decreased significantly when compared with untreated endometria in secretory phase (106.90 ± 26.94 cells/1000 cells, $p < 0.01$). The semi-quantitative H-score also confirmed that the numbers of both ERβ-ir positive stromal and epithelial gland cells significantly decreased in DMPA-treated group when compared with untreated groups during both proliferative ($p < 0.01$) and secretory phases ($p < 0.01$) (Fig. 6C). As well, H-score confirmed the expressions of ERβ in both stromal and epithelial cells of DMPA-treated group were significantly lower than that in untreated group during both proliferative and secretory phases ($p < 0.01$) (Fig. 6D).

When using anti-PRA/B (sc-7208, Santa Cruz Biotechnology) the number of PRA/B-ir stromal cells of DMPA-treated endometria (277.31 ± 44.55 cells/1000 cells) was increased significantly when compared with the endometria of untreated women with endometriosis during proliferative (79.00 ± 11.51 cells/1000 cells, $p < 0.05$) and secretory phases (62.00 ± 15.37 cells/1000 cells, $p < 0.01$). In uterine glands, the number of PRA/B-ir cells in DMPA-treated endometria was not significantly different from untreated endometria (Fig. 6E). The semi-quantitative H-score confirmed that PRA/B expression in stromal cells was significantly increased in the endometria of DMPA-treated group when compared with those of untreated women during proliferative and secretory phases ($p < 0.05$) (Fig. 6F).

When using anti-PRB (ab97801; Abcam), which is specific to PRB, only PRB-ir cells were detected. The number of PRB-ir cells in the stroma from DMPA-treated endometria (199.36 ± 39.15 cells/1000 cells) was increased significantly when compared with the untreated endometria during proliferative (78.09 ± 18.33 cells/1000 cells, $p < 0.05$) and secretory phases (49.70 ± 12.49 cells/

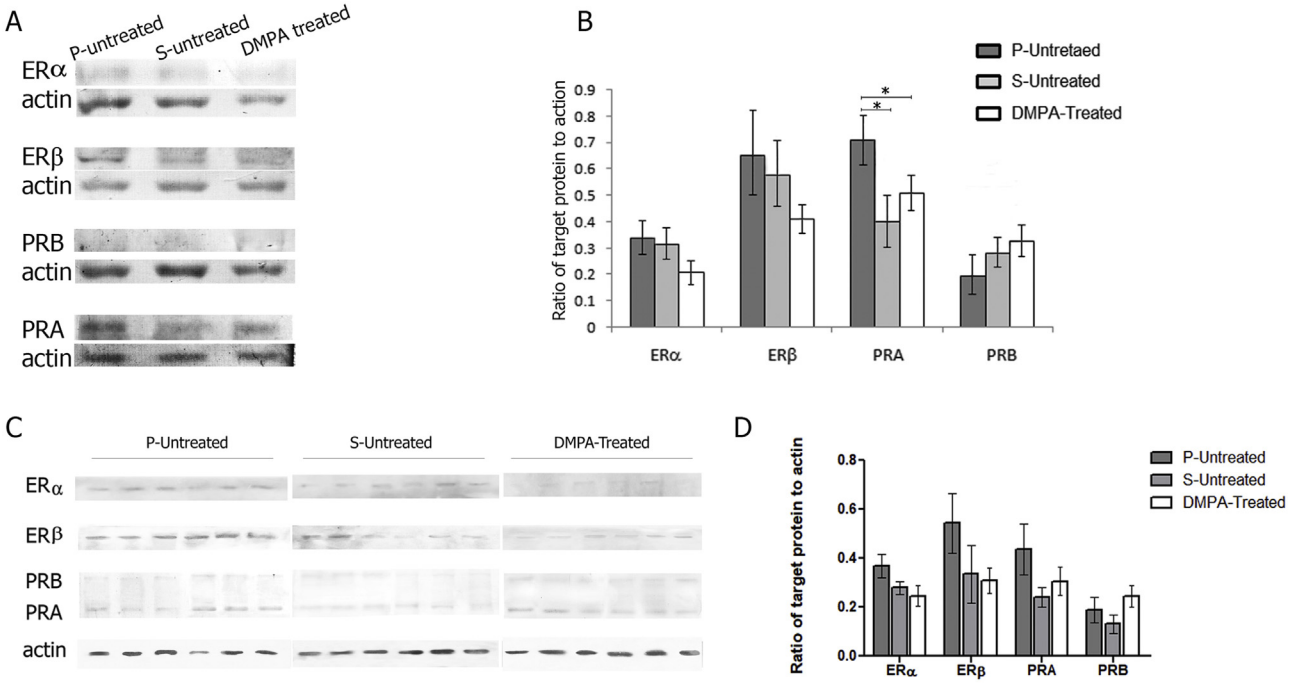

Fig. 2. (A) Western blot analyses of pooled samples of ectopic endometrioma (ovarian cyst walls) from women with endometriosis. Lanes 1–2 are the pooled ovarian cyst walls from untreated women with endometriosis in proliferative and secretory phases, respectively. Lane 3 is the pooled ovarian cyst wall from DMPA-treated women with endometriosis. Using anti-ERα and anti-ERβ as probes, the difference of intensities of these bands in the three groups were not distinct. Using anti-PRA/B as a probe shows the two immunoreactive bands of PRA/B and PRB in the cyst walls of untreated women with endometriosis. The intensities of PRA/B and PRB bands of DMPA-treated endometria were not significant different from those in the cyst walls of untreated women with endometriosis in secretory. (B) Ratios of the intensities of the target protein bands from Fig. 2A normalized with actin band as estimated by ImageJ software. (C) Western blot analyses of individual samples of cyst walls from untreated and DMPA-treated women with endometriosis using anti-ERα, -ERβ, and -PRA/B antibodies as probes. Lanes 1–6 are individual cyst walls from untreated women with endometriosis in proliferative phase. Lanes 7–12 are individual cyst walls from untreated women with endometriosis in secretory phase. Lanes 13–18 are individual cyst walls from DMPA-treated women with endometriosis. (D) Average intensities from individual samples were normalized with their corresponding actin bands.

1000 cells, $p < 0.01$). In uterine glands, the numbers of PRB-ir cells in DMPA-treated endometria (318.29 ± 56.52 cells/1000 cells) were also increased significantly when compared with the untreated endometria during proliferative (97.36 ± 30.84 cells/1000 cells, $p < 0.01$) and secretory phases (85.40 ± 29.20 cells/1000 cells, $p < 0.01$) (Fig. 6G). The semi-quantitative H-score confirmed that the PRB expressions in stromal and epithelial cells were significantly increased in DMPA-treated women when compared with untreated groups during both proliferative and secretory phases ($p < 0.05$) (Fig. 6H).

Immunohistochemistry was also performed in the ovarian cyst walls. The expressions of ERα, ERβ, PRA/B and PRB were found in stromal cells which are the predominant cell type in the ovarian cyst walls (Fig. 7A–L). When numbers of immunoreactive cells per 1000 cells were counted, the numbers of positive cells were generally lower by two to three folds when compared to endometria (Fig. 8A). The number of ERα-ir cells was relatively high in both proliferative and secretory phases of untreated women while the number of ERβ-ir was lower in both phases. The numbers of PRA/B-ir and PRB-ir cells in the cyst walls of untreated women with endometriosis were moderately high during proliferative but lower in secretory phase. Unlike in the

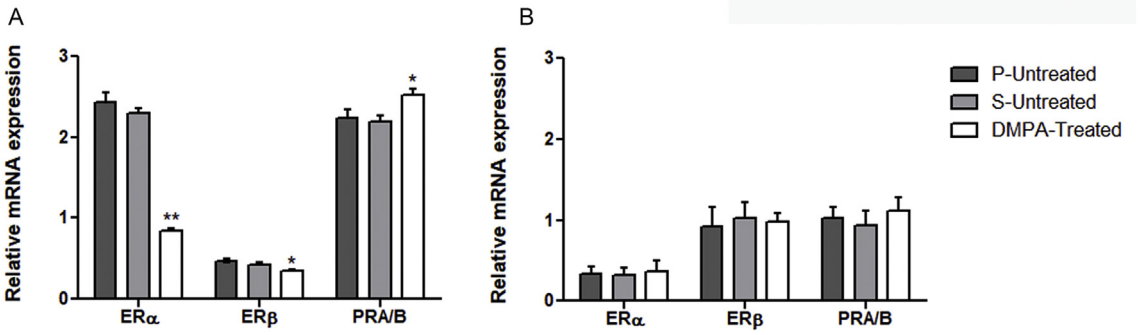

Fig. 3. The relative expression levels (expression fold change) of mRNAs of ERα, ERβ, and PRA/B by real-time PCR (qPCR) normalized by the expression of mRNA of GAPDH. (A) Relative expressions levels of mRNAs of ERα, ERβ, and PRA/B in eutopic endometria from untreated women with endometriosis in proliferative (P-untreated), (S-untreated) secretory phases, and DMPA-treated women with endometriosis (DMPA-treated). The relative expression levels of ERα and ERβ in the DMPA-treated women were significantly lower than those in P-untreated and S-untreated groups. While the relative expression level of PRA/B was significantly higher in the DMPA-treated group than in the P-untreated and S-untreated groups. (B) Relative expression levels mRNAs of ERα, ERβ, and PRA/B in ovarian cyst walls from P-untreated, s-untreated, and DMPA-treated groups which show no significant difference. The results are expressed as means ± standard errors of means (SEM). $*p < 0.05$; $**p < 0.01$; error bars represent SEM.

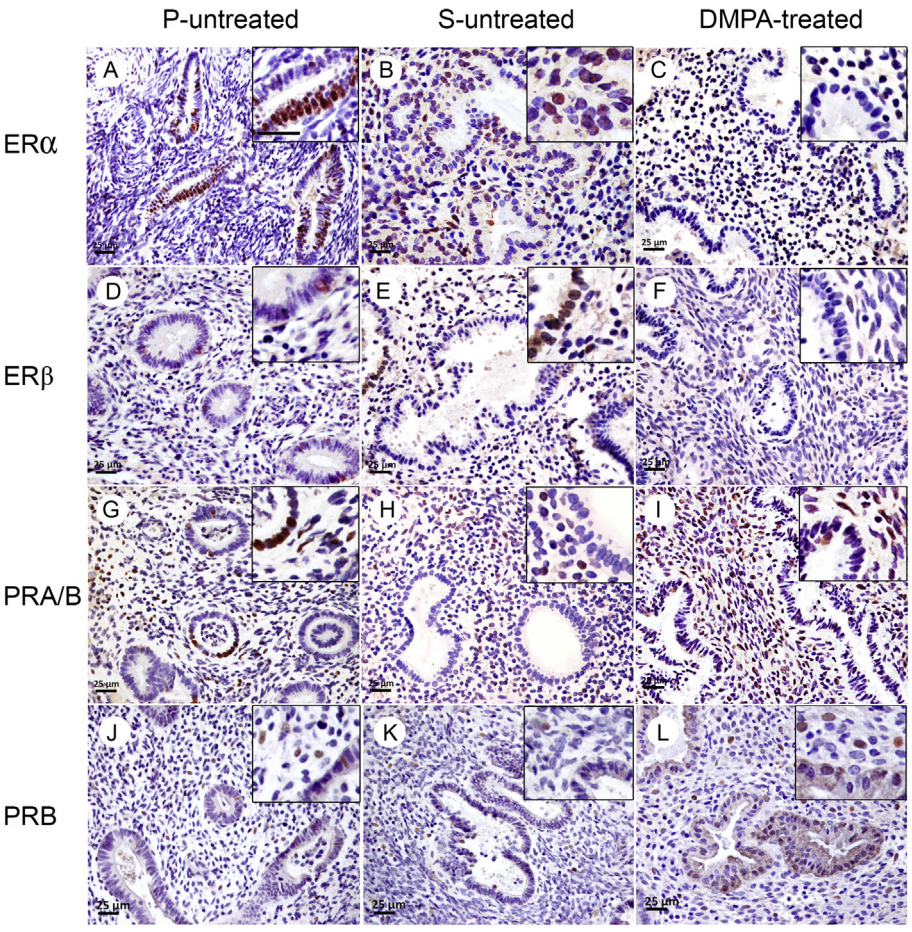

Fig. 4. Immunohistochemical stainings for ERα, ERβ, PRA/B, and PRB expressing cells in ovarian cyst wall using corresponding antibodies. (A–C) Immunostainings for ERα cells in eutopic endometria of untreated women with endometriosis during proliferative and secretory phases, and in DMPA-treated endometria, respectively. (D–F) Immunostainings for ERβ cells in eutopic endometria of untreated women with endometriosis during proliferative and secretory phases, and in endometria of DMPA-treated women, respectively. (G–I) Immunostainings for PRA/B cells in eutopic endometria of untreated women with endometriosis during proliferative and secretory phases, and in endometria of DMPA-treated women, respectively. (J–L) Immunostainings for PRB cells in eutopic endometria of untreated women with endometriosis during proliferative and secretory phases, and in endometria of DMPA-treated women, respectively. Insets show enlargements of immunohistochemically stained cells in the same figures. All insets show the same magnification and the scale bar is 25 µm.

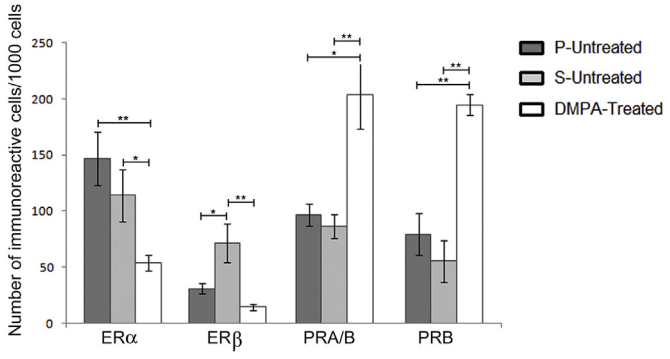

Fig. 5. The effect of DMPA on the numbers of positive cells bearing immunoreactivities (-ir) of ERs and PRs in eutopic endometria of DMPA-treated and untreated women with endometriosis. The histograms indicate cells bearing ERα-ir, ERβ-ir, PRA/B-ir, and PRB-ir per 1000 cells, inclusive of both the stromal and epithelial cells which are expressed as means ± standard errors of means (SEM). $*p < 0.05$; $**p < 0.01$; error bars represent SEM.

cases of DMPA-treated endometria DMPA-treatment did not cause any significantly changes in the numbers of all types of immunoreactive cells (Fig. 8A). A semi-quantitative method, H-score, indicating the expression levels of ERα-ir, ERβ-ir, PRA/B-ir and PRB-ir, showed that DMPA-treatment did not caused any significant difference of expression levels of all types of receptors between the treated and untreated women (Fig. 8B).

4. Discussion

In this study, we investigated the differential expressions of ERs and PRs in eutopic endometria and cyst walls of endometrioma from women with endometriosis by using Western blotting and real-time PCR to determine the levels of total expressions of these receptors and their genes in the tissues, and the changes in numbers of stromal and epithelial cells expressing these receptors by immunohistochemistry. It was found that both ERα and ERβ were expressed in the endometrium at a higher level during proliferative than secretory phase with the expression of ERα more than ERβ, and the number of ER positive cells were higher in the gland than stroma of untreated endometria at both proliferative and secretory phases. The levels of ERα and ERβ expressions and the numbers of positive cells in the endometria were significantly lower by DMPA treatment. In endometria from untreated women the expression levels of PRA and PRB were higher in secretory than proliferative phase, with more expression of PRA than PRB, and there were more PR cells in the stroma than in the glands. DMPA treatment significantly up-regulated the expressions of both PRs and the numbers of PR-positive cells in the endometria. In comparison, in ovarian cyst walls of untreated women, the protein and mRNA expression levels and the numbers of immune-reactive cells of ERα, ERβ PRA and PRB were much lower than in endometria at both phases. DMPA-treatment did not caused any significantly changes in the expressions of all types of receptors.

The effects of other progestin contraceptives on steroid receptor expressions have been widely studied. The subdermal implanted levonorgestrel, Norplant, reduced the expressions of ERs in the endometrium of the users [19]. During the first year of Norplant usage, the expressions of PRA/B-ir were increased in the stromal cells in endometrium of users [20]. Similarly, the use of another subdermal contraceptive implant containing steroidal progestin, Implanon, leaded to significant increases of PRA/B expressions after a 12 month treatment [21]. Administration of levonorgestrel intrauterine system (LNG-

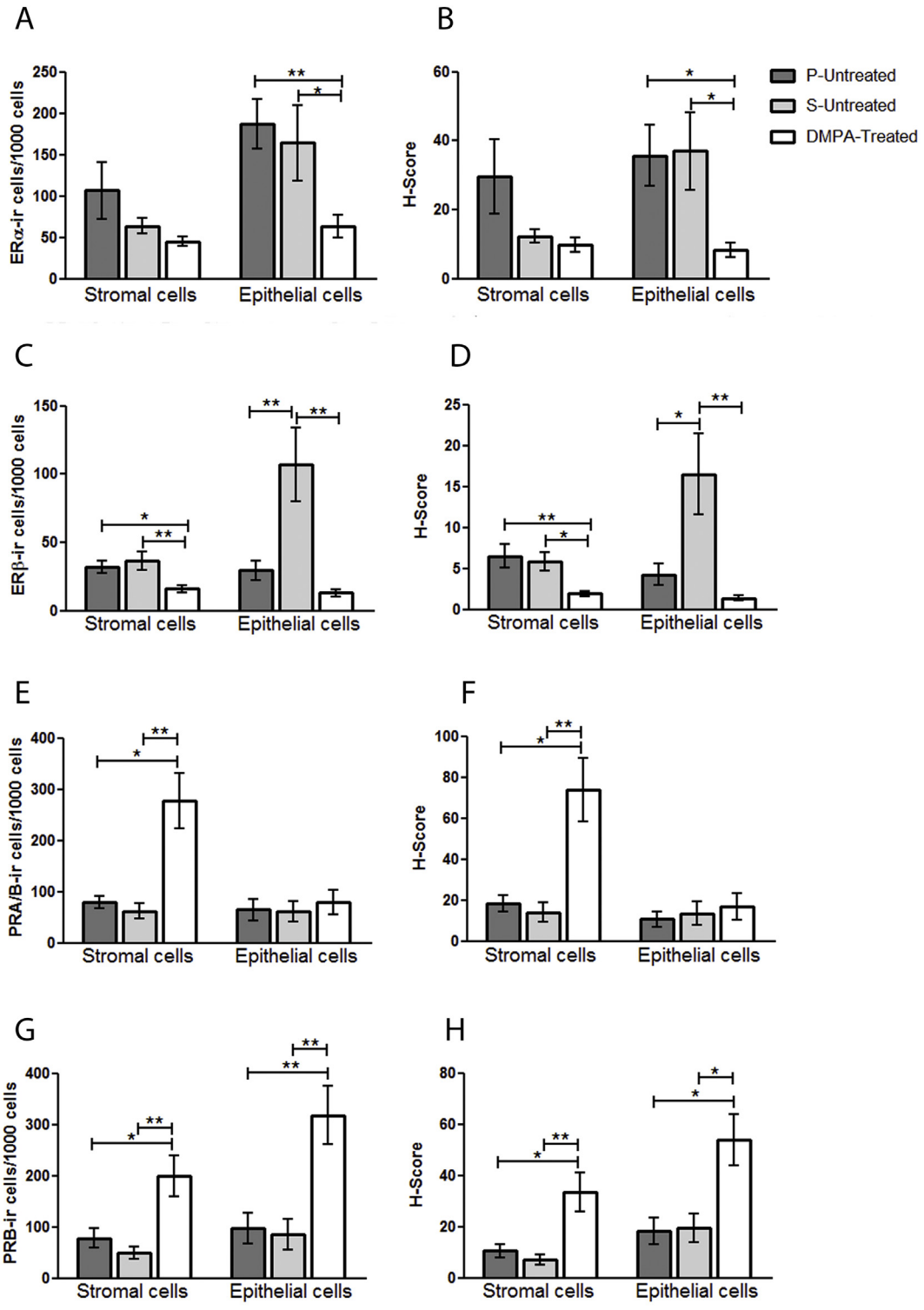

Fig. 6. The numbers of cells expressing ERα-ir, ERβ-ir, PRA/B-ir, PRB-ir in the eutopic endometria of women having endometriosis with and without DMPA treatment. The numbers of ERα-ir (A), ERβ-ir (C), PRA/B-ir (E), and PRB-ir (G) cells per 1000 cells were counted separately for both the stromal and epithelial cells. Semi-quantitative estimations of the immunostain intensity in each cell type were performed by using H-score. The H-score of ERα-ir, ERβ-ir, PRA/B-ir, and PRB-ir are shown in (B), (D), (F), and (H), respectively. The results are expressed as means ± standard errors of means (SEM). $*p < 0.05$; $**p < 0.01$; error bars represent SEM.

IUS) resulted in initial decreased expression of PRs, while a long-term exposure (6–12 months) resulted in the increase of PRA/B expressions in epithelial cells, but not PRB [22]. The authors assumed that PRA subtype is up-regulated and may mediate LNG-IUS action in the endometrium between 6 and 12 months. Long-term treatment (up to 35 months) with MPA in female long-tailed macaques (*Macaca fascicularis*) resulted in the increase immunostaining intensity and the number of PRA/B-ir positive cells in the endometrial stroma [23]. As well, it has been shown in human that MPA treatment increased PRA and PRB mRNA as well as protein expressions in endometrial stromal cells [24].

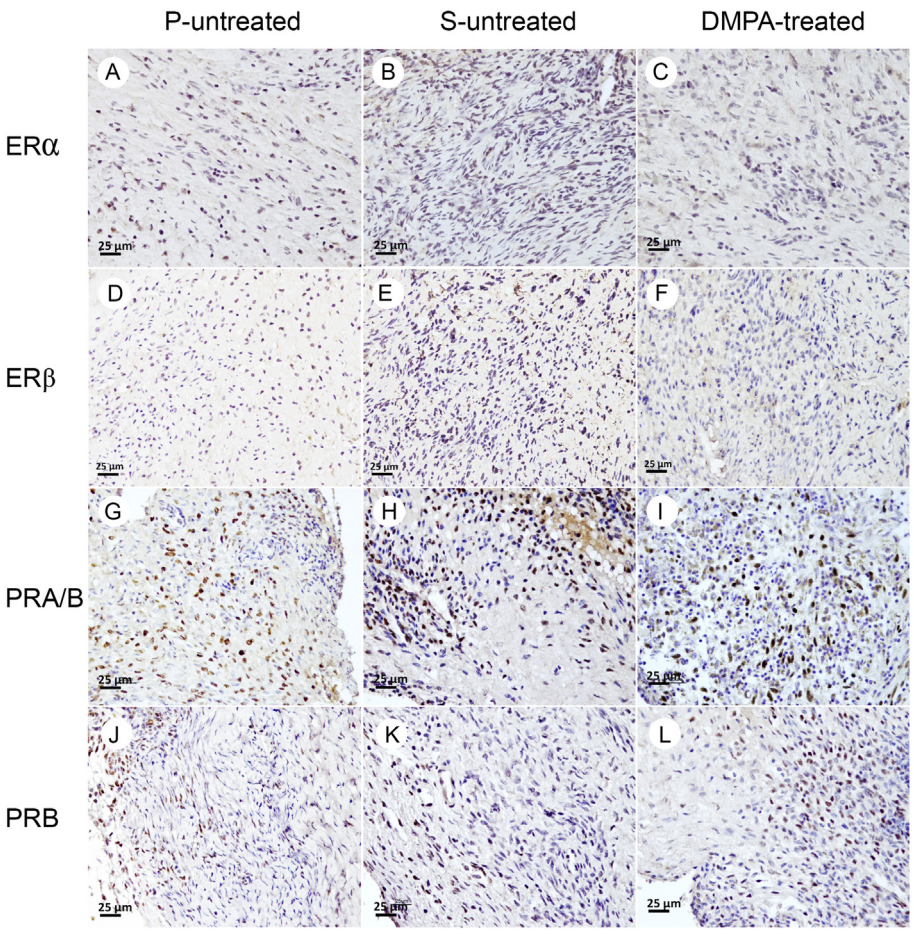

Fig. 7. Immunohistochemical stainings for ERα, ERβ, PRA/B, and PRB cells in ovarian cyst walls using corresponding antibodies. (A–C) Immunostainings for ERα cells in cyst walls of untreated women with endometriosis during proliferative and secretory phases, and in cyst walls of DMPA-treated women, respectively. (D–F) Immunostainings for ERβ cells in cyst walls of untreated women with endometriosis during proliferative and secretory phases, and in cyst walls of DMPA-treated women, respectively. (G–I) Immunostainings for PRA/B cells in cyst walls of women with endometriosis during proliferative and secretory phases, and in cyst walls of DMPA-treated women, respectively. (J–L) Immunostainings for PRB cells in cyst walls of untreated women with endometriosis during proliferative and secretory phases, and in cyst walls of DMPA-treated women, respectively.

In contrast, incubation of endometrium with MPA caused reduction of PRA and PRB mRNA and protein expressions in endometrial epithelial cells [25]. The decrease of ERs and increase in PRs in endometrium after treatment with DMPA shown in this study is consistent with previous reports on the use of other progestins.

Besides contraception, progestins contraceptives have been used to treat endometriosis [11]. In women with endometriosis, administration of LNG-IUS resulted in the reduction of ER-ir in endometrial epithelial and stromal cells [26]. An oral progestin, dienogest, has also been used to treat endometriosis. After administration of dienogest, the expression of ERβ mRNA decreased significantly in both eutopic and ectopic endometrium when compared with that in eutopic endometrial samples obtained from females who did not receive any treatment. Moreover, PRA and PRB mRNA were significantly increased in eutopic endometria of dienogest-treated women when compared with those of untreated women [27]. The results from these previous studies are consistent with our findings.

In humans, estradiol promotes while progesterone suppresses the endometrial cell proliferation [28]. As well, it has been shown that estradiol stimulates proliferation of both epithelial and stromal cell in neonatal mice [29]. In the present study we found that in endometria of women with endometriosis ERα was expressed at a higher level than ERβ as occurs in normal women. This is because ERα is the major form of estrogen receptor present in normal endometrium and hypothalamus

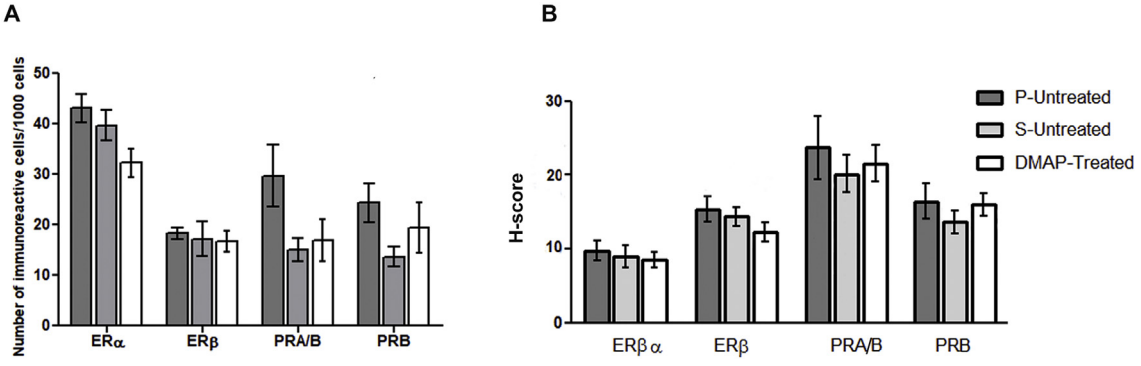

Fig. 8. The numbers of cells expressing ERα-ir, ERβ-ir, PRA/B-ir, PRB-ir in cyst walls of women having endometriosis with and without DMPA treatment. (A) The numbers of ERα-ir, ERβ-ir, PRA/B-ir, and PRB-ir cells per 1000 cells were counted from the stromal cells in the ovarian cyst wall. (B) Semi-quantitative estimations using H-score to determine the immunostaining intensity of ERα-ir, ERβ-ir, PRA/B-ir, and PRB-ir in each cell type. The results are expressed as means ± standard errors of means (SEM). Error bars represent SEM.

while ERβ is expressed more in other non-endometrial tissues such as ovary, kidney, brain and bone and other non-reproductive tissues. This corresponds with the results previously reported by Matsuzaki et al. [30] who demonstrated that ERα is the major isoform that is likely to be the primary mediator of the estrogenic action in endometrium, and its mRNA expression was higher than ERβ mRNA expression in eutopic and ectopic endometria of women with endometriosis [30]. Previous studies have also shown that higher expression of ERα than ERβ may be responsible for cellular proliferation in endometrium and endometriotic lesions [30–32]. The studies in mice demonstrated that estradiol stimulated proliferation of epithelial cells through ERα-dependent mitotic proliferative signal emanated from neighboring stromal cells that express ERα [31,32].

PRA is a truncated isoform of PRB and previous studies revealed that PRA has an inhibiting effect on many steroid receptors, including PRB [33,34] and ER [35]. Many studies demonstrated that progesterone suppresses the stimulatory effects of estradiol on endometrium [36,37] perhaps through its receptors. In the presence of PRA and PRB, treatment with progestin (agonistic ligand) and anti-progestin (antagonistic ligands) resulted in the reduction of ERs expressions in the primary culture of rat uterine cells [33]. This suggested that PRA and PRB can act as ligand-dependent repressors of ERs transcriptional activities. In the present study we found that in untreated women the expression levels of PRA and PRB were higher in secretory than proliferative phase, with slightly more expression of PRA than PRB. DMPA treatment significantly up-regulated the expressions of both PRs and the numbers of PR-positive cells in both the stroma and glands compared with untreated groups. The up-regulation of PRB in endometrium induced by DPMA as detected in this study is in agreement with the recent study by Ito et al. (2012) [38] who demonstrated that after treating adenocarcinoma Ishikawa cells with MPA for 24 h the expression of PRB was more intense when compared with the ethanol-treated control. Furthermore, the authors reported that after treatment with MPA, 14-3-3τ gene and 14-3-3 protein which regulate diverse cellular responses were up-regulated in adenocarcinoma Ishikawa cells, rat uterus, and human endometrial epithelial cells and stromal cells. From luciferase reporter assay, it was also shown that after treating Ishikawa cells with progesterone, 14-3-3τ was up-regulated, and this in turn greatly enhanced transcriptional activity of PRB gene [38]. Therefore, we suggested that DMPA may use similar pathway in inducing the upregulation of PRB, and that both PRA and PRB are involved in the suppression of ERs.

In ovarian cyst walls of untreated women, the protein and mRNA expression levels of ERα, ERβ, PRA and PRB, as well as the numbers of immunoreactive cells were much lower than in endometria at both phases and the cyst wall consisted predominantly of stromal cells which may lose control over the expressions of their receptors as evident by the aberrant expression of ERβ which is at a higher level than ERα, and PRA expression which was higher during proliferative phase than secretory phase compared to the untreated endometria. Besides, DMPA-treatment did not caused any significantly changes in the expressions of all types of receptors which may be due to the cyst wall stromal cells' resistance to this drug.

In summary, the present study showed that treatment with DMPA suppressed the expressions of both ERα and ERβ, and up-regulated the expressions of PRA/B and PRB in the endometria of women with endometriosis. On the other hand, expressions of these receptors in ovarian cyst wall were very low compared to endometria and their expressions were unaffected by DMPA treatment. However, whether there is a direct negative causal relationship between PRs and ERs suppressions needed to be investigated further.

Conflicts of interest

All authors have no conflict of interest.

Acknowledgement

This work was supported by National Research Council of Thailand and Faculty of Science and Faculty of Medicine Ramathibodi Hospital, Mahidol University (No. 2554/215).

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