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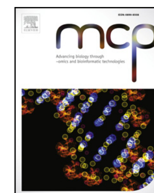

Differential expressions of estrogen and progesterone receptors in endometria and cyst walls of ovarian endometrioma from women with endometriosis and their responses to depo-medroxyprogesterone acetate treatment

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A R T I C L E I N F O

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A B S T R A C T

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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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

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