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LPAR2 and LPAR4 are the Main Receptors Responsible for LPA Actions in Ovarian Endometriotic Cysts

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Abstract

Endometriosis has been considered as an estrogen (E2)-dependent and progesterone (P4)-resistant disease. On the other hand, lysophosphatidic acid (LPA) has been suggested as a significant modulator of ovarian pathology, acting via both LPA levels and LPA receptor (LPAR) upregulation. Therefore, the objective of the present study was to evaluate LPA concentration as well as LPARs, autotaxin (ATX), and phospholipase A2 (PLA2) expression in ovarian endometriotic cysts and normal endometrium with correlation of the expression of E2 and P4 receptors in endometriotic cysts. The analyses were carried out using the tissues derived from 37 patients with ovarian endometriosis and 20 endometrial samples collected from women without endometriosis were used as a control. We found that ovarian endometriotic cysts are a site of LPA synthesis due to the presence of enzymes involved in LPA synthesis in the tissue. Additionally, when we compared endometriotic cysts versus normal endometrium, we were able to show overexpression of 3 from 6 examined LPARs and both enzymes responsible for LPA synthesis in endometriotic cysts. Finally, we found the correlations between LPARs, ATX, and PLA2 and the expression of E2 and P4 receptors in endometriotic cysts. Owing to the high LPAR2 and LPAR4 transcript and protein expression in endometriotic ovarian cysts and positive correlations of both these receptors with the PR-B and ERβ, respectively, those receptors seem to be the most promising predictors of the endometriotic cysts as well as the main receptors responsible for LPA action in the ovarian endometriosis.

Keywords

ovarian endometriotic cysts, LPA, LPAR, human

Introduction

One of the most common forms of endometriosis is the ovarian endometrioma. The ovarian endometriotic cyst (OEC) wall is thick and fibrotic, and the cyst contains endometriotic glands, endometriotic stroma, multiple hemorrhagic lesions, and hemosiderin-laden macrophages in the cyst wall and stroma.[1]

The growth of endometrial and endometriotic tissue is regulated by estrogen (E2) and progesterone (P4) via activation of corresponding steroid receptors. Estrogen receptors ERα and ERβ are present in the normal endometrium, but ERα is the primary mediator of estrogenic actions in this tissue.[2] Estrogen receptor ERβ levels in endometriosis are more than 100 times higher than in the levels in endometrial tissue. At high levels, ERβ suppresses ERα expression.[3] The effects of P4 are mediated through intracellular progesterone receptors, which are expressed as 2 predominant PR isoforms, namely, PR-A (a 94-kDa protein) and PR-B (an ~114-kDa protein), which are transcribed from the same gene by 2 distinct promoters.[4,5]

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Attia et al[6] showed that the receptor PR-B mRNA and protein levels were significantly reduced in endometriosis lesions, whereas PR-A isoforms were generally spared. P4 and other progestins have been used for a long time in the treatment of endometriosis to relieve pain by limiting growth and inflammation associated with endometriosis. The molecular basis of P4 resistance in endometriosis may be related to the reduction or even lack of PRs, especially PR-B. The presence of the inhibitory isoform A and the absence of the stimulatory isoform B provide a possible explanation for the P4 resistance of endometriotic implants.[6,7] Endometriosis is considered an estrogen (E2)-dependent and progesterone (P4)-resistant disease, but the etiology and pathogenesis of endometriosis remain unclear.

Several medical therapies exist for the treatment of endometriosis, such as treatments with gonadotropin-releasing hormone agonists, aromatase inhibitors, and selective progesterone and estrogen receptor modulators, but these treatments have short-term effects on the symptoms of endometriosis. Additionally, ovarian endometriosis is not entirely susceptible to pharmacological treatment[8] and conservative medical treatment may lead to a reduction in volume rather than complete regression of the endometriotic cyst.[9,10] Resistance to conventional therapy critically raises the need for novel treatment options that target specific, dysregulated underlying molecular mechanisms. Lysophosphatidic acid (LPA), which is implicated in cell proliferation in various types of hypertrophies, may be a potential target for alternative new therapies in ovarian endometriosis.

Lysophosphatidic acid is a small, potent molecule that acts through specific cellular G protein-coupled receptors known as LPA receptors (LPARs) 1-6. Two general pathways of LPA production have been demonstrated. One pathway involves the generation of precursor lysophospholipids from various membrane phospholipids (PLs) by phospholipase A1 and phospholipase A2 (PLA1/2), followed by the action of autotaxin (ATX), which is also known as lysophospholipase D. A second pathway involves the formation of phosphatidic acids (PAs) from PL cleavage by phospholipase D (PLD) or from diacylglycerol by diacylglycerol kinase with subsequent deacylation of PA by PLA type enzymes.[11]

On the other hand, LPA has been suggested as a significant modulator of ovarian pathology, acting via the LPA levels and through LPAR upregulation.[12] Therefore, the main objective of the present study was to evaluate LPA concentrations and the LPAR, ATX, and PLA2 expression levels in the endometriotic cyst wall and normal endometrium and to form correlations with the expression of the E2 and P4 receptors in the OECs.

Materials and Methods

Patients and Samples

The study was approved by the local ethics committee of the Faculty of Medical Sciences, University of Warmia and Masuria in Olsztyn.

Tissue samples were collected from 37 women with ovarian endometriosis who underwent laparoscopic excision of the endometrioma. The ages of patients ranged from 24 to 48 years (mean: 36 years). The control endometrial samples (nonendometriosis) were collected from women without endometriosis ($n = 20$; mean age: 44 years; range: 35-53 years) who underwent surgical procedures for benign gynecological diseases. The control cases included patients with infertility due to causes other than endometriosis. The explants for the LPA concentrations and gene and protein expression analyses were frozen in liquid nitrogen and kept at $-80^{\circ}C$ until molecular studies were performed.

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from the tissue explants using TRIzol according to the manufacturer's instructions. RNA samples were stored at $-80^{\circ}C$. Before use, the RNA content and quality was evaluated using spectrophotometric measurements and agarose gel electrophoresis. One microgram of each sample of total RNA was reverse transcribed using a QuantiTect Reverse Transcription kit (#205311; Qiagen; Hilden, Germany). The reverse transcription (RT) reaction was performed in a total reaction volume of 20 μL, following the manufacturer's instructions, and the products were stored at $-20^{\circ}C$ until further analysis by real-time PCR amplification.

Quantitative Real-Time PCR

The mRNA quantification for the studied genes was conducted by real-time PCR using specific primers for ATX, PLA2, LPAR1, LPAR2, LPAR3, LPAR4, LPAR5, LPAR6, ER-α, ER-β, PR-A, and PR-B. The mRNA expression results were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH, an internal control) mRNA expression level and were expressed as arbitrary units. The GAPDH housekeeping gene was chosen using the NormFinder software, which compared 3 candidate genes: GAPDH, β-actin, and H2A.1. The primers were designed using an online software package (http://bioinfo.ut.ee/primer3/). The primer sequences and the sizes of the amplified fragments of all transcripts are shown in Table 1. Real-time PCR was performed with an ABI Prism 7900 (Applied Biosystems Life Technologies; Foster City, California, USA) sequence detection system using Maxima SYBR-Green/ROX qPCR Master Mix (#K0222; Fermentas, Thermo Scientific; Waltham, Massachusetts, USA). The PCR reactions were performed in 384-well plates. Each PCR reaction well (10 μL) contained 3 μL of the RT product, 5 μM forward primer, 5 μM reverse primer, and 5 μL of the SYBR-Green PCR Master Mix. Real-time PCR was performed under the following conditions: $95^{\circ}C$ for 10 minutes, followed by 40 cycles of $94^{\circ}C$ for 15 seconds and $60^{\circ}C$ for 60 seconds. Subsequently, melting curves were obtained to ensure single product amplification in each PCR reaction. To exclude the possibility of genomic DNA contamination in the RNA samples, the reactions were also performed either with blank-only

Table 1. Primers Used for Real-Time PCR.

Gene	Primer Sequence	Fragment Size (bp)
LPAR1	5′ GGCTATGTTCGCCAGAGGACTAT 3′ 5′TCCAGGAGTCCAGCAGATGATAA 3′	135
LPAR2	5′ GCTCTGTCGAGCCTGCTTGTCTTC 3′ 5′ ACAGTCTTGACCAGGCTGAGCGTG 3′	149
LPAR3	5′ AAACTTTCCTTTGGCTCTGGAC 3′ 5′ ATTCCAGCGAAGAAATCGGC 3′	458
LPAR4	5′ GGGTGACAGAAGATTCATTGACTTCC 3′ 5′ GGCCAGGAAACGATCCACACTA 3′	415
LPAR5	5′ ATGTTAGCCAACAGCTCCTCAACC 3′ 5′ GCCAGTGGTGCAGTGCGTAGTA 3′	262
LPAR6	5′ GGTAAGCGTTAACAGCTCCCACT 3′ 5′ TTTGAGGACGCAGATGAAAATGT 3′	139
ATX	5′ CGTGAAGGCAAAGAGAACACG 3′ 5′ AAAAGTGGCATCAAATACAGG 3′	776
PLA2	5′ ACATCTGCAAAAGCGCAAGG 3′ 5′ CCTGCTGTCAGGGGTTGTAG 3′	374
ERα	5′ ACCCTCCACACCAAAGCATC 3′ 5′ AGAAGGTGGACGGTTCAGG 3′	84
ERβ	5′ ATGAAGAGTGATGCCCCAAGG 3′ 5′ GGGCTTGCTCCTGTTCAAGT 3′	159
PR-AB	5′ ACATGGTAGCTGTGGGAAGG 3′ 5′ GCTAAGCCAGCAAGAAATGG 3′	202
PR-B	5′ TGTTGTATTTGTGCGTGTGG 3′ 5′ ACCTCTCGGTACAGCCCATT 3′	192
GAPDH	5′ CTGCACCACCAACTGCTTAG 3′ 5′ GGGCCATCCACAGTCTTCT 3′	120

Abbreviations: ATX, autotaxin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPAR, LPA receptor.

buffer samples or in the absence of the reverse transcriptase enzyme. The specificity of the PCR product for each examined gene was confirmed by gel electrophoresis and sequencing. The efficiency ranges for the target and internal control amplifications were between 95% and 100%. For the relative quantification of the mRNA expression levels, the previously reported real-time PCR algorithm was used.[13]

Western Blot Analysis

For immunoblotting, protein fractions were obtained from the tissue samples, and total protein was obtained from the cells. Briefly, luteal tissues were homogenized on ice in Radioimmunoprecipitation assay buffer containing 150 mM NaCl, 50 nM Tris Base, pH 7.2, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, and 5 mM EDTA in the presence of a protease inhibitor cocktail (#11697498001; Roche; Basel, Switzerland). Lysates were then sonicated and centrifuged at 10 000*g* for 15 minutes at 4°C. The protein samples were stored at −70°C for further analysis. The protein concentration was determined according to Bradford et al.[14] Equal amounts (50 µg) of the membrane fractions were dissolved in SDS gel-loading buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol and 2% β-mercaptoethanol), heated at 95°C for 5 minutes and separated by 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The resolved proteins were electroblotted using a semidry transfer method onto polyvinylidene difluoride membranes (Immobilon-P Transfer Membrane, #IPVH00010; Millipore; Darmstadt, Germany) in a transfer buffer (0.3 mM Tris buffer, pH 10.4, 10% methanol, 25 mM Tris buffer, pH 10.4, 10% methanol, 25 mM Tris buffer, pH 9.4, 10% methanol, 40 mM glycine). After blocking in 5% nonfat dry milk in a TBS-T buffer (Tris-buffered saline with 0.1% Tween-20) for 1.5 hours at 25.6°C, the membranes were incubated overnight with rabbit polyclonal anti-LPAR2, anti-ATX, anti-cPLA2, anti-ERα, and anti-ERβ antibodies (1:100; #sc-25490, #sc-66813, #sc-438, #sc-542, and #sc-8974, respectively; Santa Cruz Biotechnology; Dallas, Texas, USA), rabbit polyclonal anti-LPAR1 and anti-LPAR3 antibodies (4 µg/mL or 1:200, #10005280 and #10004840, respectively; Cayman Chemicals; Ann Arbor, Michigan, USA), rabbit polyclonal anti-LPAR5 and anti-LPAR6 antibodies (1 µg/mL or 1:300, #ab130993 and #ab113055, respectively; Abcam; Cambridge, UK), goat polyclonal anti-LPAR4 (1:100; #sc-46021; Santa Cruz Biotechnology), mouse monoclonal anti-PR-B and anti-PR-AB (1:100; #sc-811 and #sc-810, respectively; Santa Cruz Biotechnology) and mouse monoclonal anti-GAPDH antibody (0.05 µg/mL, #G8795; Sigma; St. Louis, Missouri, USA) at 4°C. Subsequently, the proteins were detected by incubating the membranes with a goat anti-rabbit IgG-alkaline phosphatase antibody (1:20 000 for LPAR1, LPAR2, LPAR3, LPAR5, LPAR6, ATX, PLA2, ERα, and ERβ #A3687; Sigma), donkey anti-goat IgG-alkaline phosphatase antibody (1:20 000 for LPAR4, #A4187; Sigma) or goat anti-mouse IgG-alkaline phosphatase antibody

(1:20 000 for GAPDH, #A3562; Sigma) for 1.5 hours at 25.6°C. After washing again in TBS-T, the immune complexes were visualized using an alkaline phosphatase visualization procedure. The specific bands were quantified using the Kodak 1D software (Eastman Kodak; Rochester, New York, USA). The GAPDH was used as an internal control for protein loading.

Immunohistochemistry

Tissues were fixed overnight in 10% buffered formalin, dehydrated, and embedded in paraffin.

Five-micrometer serial sections of each sample were used in this study. Sections were cut, floated onto albumin-coated slides, dried at 56°C, deparaffinized in xylene, rehydrated, and washed in tap water for 10 minutes, followed by immersion in methanol with 3% (v/v) H_2O_2 for 30 minutes. Specimens were treated in a microwave oven in 0.1 mol/L citrate buffer (pH 6.0) for 30 minutes at 100°C, slowly cooled to room temperature, and washed with PBS for 5 minutes at room temperature. After quenching the endogenous peroxidase with 3% hydrogen peroxide in PBS for 10 minutes at room temperature, the sections were incubated with a blocking solution (protein block, VectaStain ABC HRP Kit, PK-4001 for Rabbit IgG and PK-4005 for Goat IgG; Vector Laboratories) for 20 minutes at room temperature. Then, the slides were incubated overnight at 4°C with rabbit polyclonal anti-LPAR2, ATX, and cPLA2 antibodies (1:100; #sc-25490, #sc-66813, and #sc-454, respectively; Santa Cruz Biotechnology), rabbit polyclonal anti-LPAR1 and LPAR3 antibodies (4 μg/mL or 1:200, #10005280 and #10004840, respectively; Cayman Chemicals), rabbit polyclonal anti-LPAR5 and LPAR6 antibodies (4 μg/mL or 1:300, #ab140837 and #ab113055, respectively; Abcam), or goat polyclonal anti-LPAR4 (1:100; #sc-46021; Santa Cruz Biotechnology). The optimal dilution of the monoclonal antibodies was determined to stain the normal endometrium during the proliferative phase. For the negative control, sections were incubated with normal rabbit or goat IgG (1:100; Santa Cruz Biotechnology; #sc-2027 and sc-2028, respectively). After several washes with PBS, the sections were incubated with a biotinylated goat anti-rabbit IgG (1:50 for LPAR1, LPAR2, LPAR3, LPAR5, LPAR6, ATX, PLA2, ERα, and ERβ # PK-4001; Vector Laboratories) or with biotinylated rabbit anti-goat IgG (1:50 for LPAR4, #PK-4005; Vector Laboratories) for 30 minutes, washed 3 times in PBS, and incubated for 30 minutes at room temperature with the rabbit or goat VectaStain avidin-biotin-peroxidase complex (Vector Laboratories; #PK-4001 and #PK-4005, respectively), according to the manufacturer's instructions. The color reaction was developed using the DAB peroxidase (HRP; Horseradish Peroxidase) Substrate Kit (SK-4100; Vector Laboratories).

Statistical Analysis

The mean (SD) and median were calculated. Student *t* test was used to compare normally distributed continuous variables, and the Mann-Whitney *U* test was used for abnormal distributions. Pearson correlation coefficients were estimated. Linear regression analysis was also used. The analyses were performed using the GraphPad Prism 6 software, and $P < .05$ was considered statistically significant.

Results

Lysophosphatidic acid level and the expression profile of ATX, PLA2, LPAR1, LPAR2, LPAR3, LPAR4, LPAR5, LPAR6, ERα, ERβ, PR-A, and PR-B in OEC and normal endometrium were analyzed.

The LPA level did not differ between the OEC and normal endometrium. All studied endometriotic cysts and normal endometria expressed all analyzed LPARs, ATX, PLA2 ERα, ERβ, PR-A, and PR-B at the mRNA and protein levels. LPAR2, LPAR4, LPAR5, and the enzymes responsible for LPA synthesis showed significantly higher mRNA and protein levels in the ectopic tissues than in the eutopic tissues ($P < .05$). LPAR3 showed lower expression in the OEC than in the healthy endometrium but only at the mRNA level. LPAR6 expression did not differ at the mRNA or protein level in either sample type. Additionally, our results showed that the mRNA and protein levels of PR-A and PR-B were significantly lower and that the levels of ERα and ERβ were significantly higher in OECs than in the normal endometria ($P < .05$). We found significantly higher ATX and PLA2 transcript and protein expression levels in OEC (mean: 0.04462 [0.0542] for the ATX mRNA, mean: 1.576 [1.729] for the ATX protein, mean 0.008631 (0.006146) for the PLA2 mRNA, and mean 2.030 (2.234) for PLA2 protein) compared to the normal endometria (mean: 0.007064 [0.0005231] for the ATX mRNA, 0.002481 (0.001459) for the PLA2 mRNA, mean: 0.2714 [0.1268] for the ATX protein, and mean: 0.4285 [0.07631] for the PLA2 protein; $P < .05$, Table 2, Figure 1A-D). The OEC samples showed the highest LPAR2 transcript and protein expression levels (ranging from 0.01171-0.08082, mean: 0.03275 [0.02532] for the LPAR2 mRNA and from 0.1525-4.932, mean: 1.968 [1.621] for the LPAR2 protein) in OECs compared to the normal endometria (ranging from 0.003485-0.01068, mean: 0.007077 [0.02261] for LPAR2 mRNA and from 0.1561-0.2114, mean: 0.1825 [0.02261] for the LPAR2 protein; $P < .05$, Table 2 and Figure 2C and D). We also found significantly higher LPAR4 and LPAR5 transcript and protein expression levels in OECs (mean: 0.02733 [0.002343] for the LPAR4 mRNA, mean: 1.656 [0.7704] for the LPAR4 protein, mean: 0.001046 [0.0008132] for the LPAR5 mRNA, and mean: 1.572 [0.8543] for the LPAR5 protein) compared to the normal endometria (mean: 0.0003563 [0.0001208] for the LPAR4 mRNA, mean: 0.6098 [0.3287] for the LPAR4 protein, mean: 0.0002464 [0.000127] for the PLAR5 mRNA, and mean: 0.661 [0.2071] for the LPAR5 protein; $P < .05$, Table 2 and Figure 3G-J). We found significantly lower LPAR3 transcript expression levels in the ectopic tissue (mean: 0.00118 [0.002289] for the LPAR3 mRNA) compared to the normal endometria (mean: 0.008916 [0.006857] for the LPAR3 mRNA; $P < .05$, Table 2 and Figure 3E). We did not find any difference in the LPAR3 protein levels between the ectopic and normal tissues ($P > .05$; Table 2 and Figure 3F). We also did not find any difference in the LPAR1 and LPAR6 transcript and protein expression levels

Table 2. Comparison of mRNA and Protein Levels of LPARs, ATX, PLA2, ERα, ERβ, PR-A, and PR-AB Between Studied Endometriotic Cysts (EC) and Normal Endometrium Tissues (Control) Using Q-RT-PCR and Western Blot Studies.

			Q-RT-PCR Study			Western Blot Study		
Gene/Protein Names	Group	Number	Mean	SD	*P* Value	Mean	SD	*P* Value
LPAR1	Control	10	0.011	0.008	NS	0.5	0.06	NS
	EC	25	0.022	0.012		1.4	1.0	
LPAR2	Control	10	0.007	0.0036	.002	0.18	0.02	.06
	EC	25	0.033	0.025		1.9	1.,6	
LPAR3	Control	10	0.009	0.007	.008	0.09	0.02	NS
	EC	25	0.001	0.002		0.06	0.03	
LPAR4	Control	10	0.0003	0.0001	.008	0.6	0.3	.008
	EC	25	0.0028	0.002		1.7	0.8	
LPAR5	Control	10	0.0002	0.0001	.0045	0.7	0.2	.004
	EC	25	0.001	0.0008		1.6	0.9	
LPAR6	Control	10	0.052	0.03	NS	1.1	0.3	NS
	EC	25	0.054	0.034		1.4	0.7	
ATX	Control	10	0.007	0.005	.002	0.3	0.1	.008
	EC	25	0.045	0.054		1.6	1.7	
PLA2	Control	10	0.0025	0.0015	.035	0.4	0.08	.04
	EC	25	0.009	0.006		2.0	2.2	
ERα	Control	10	0.004	0.0024	.001	0.02	0.004	.015
	EC	25	0.081	0.13		0.2	0.05	
ERβ	Control	10	0.0002	0.0002	.002	0.1	0.04	.003
	EC	25	0.012	0.017		0.3	0.1	
PB-A	Control	10	0.73	0.33	.0045	3.9	0.8	<.0001
	EC	25	0.12	0.12		1.2	0.6	
PB-B	Control	10	0.15	0.06	.007	3.5	1.0	.0005
	EC	25	0.05	0.05		1.3	0.7	

Abbreviations: ATX, autotaxin; LPAR, LPA receptor; NS, nonsignificant; Q-RT-PCR, quantitative reverse transcription PCR; SD, standard deviation.

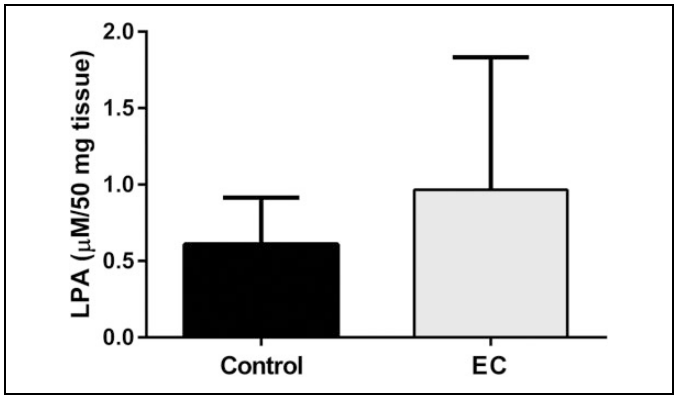

Figure 1. Concentration of LPA (μM/50 mg tissue) in ovarian EC (gray bars) and normal proliferative endometrium (control, black bars). LPA indicates lyspohosphatidic acid; OEC, ovarian endometriotic cysts.

in the endometriotic tissue (mean: 0.02215 [0.01191] for the LPAR1 mRNA, mean: 0.05414 [0.03393] for the LPAR6 mRNA, 1.398 [1.039] for the LPAR1 protein, and 1.369 [0.7138] for LPAR6 protein) compared to the normal endometria (mean: 0.01062 [0.007627] for the LPAR1 mRNA, mean: 0.0518 [0.03029] for the LPAR6 mRNA, 0.4948 [0.0641] for the LPAR1 protein, and 1.119 [0.298] for the LPAR6 protein; $P > .05$, Table 2 and Figure 3A, B, K and L). Additionally, we found significantly higher ERα and ERβ transcript and protein expression levels in the endometriotic tissue (mean: 0.08066 [0.1308] for the ERα mRNA, mean: 0.2058 [0.05128] for the ERα protein, mean: 0.01673 [0.01637] for the ERβ mRNA, and mean: 0.02974 [0.09832] for the ERβ protein) compared to the normal endometria (mean: 0.003622 [0.002413] for the ERα mRNA, mean: 0.01588 [0.003691] for the ERα protein, mean: 0.00022 [0.000177] for the ERβ mRNA, and mean: 0.1365 [0.03934] for the ERβ protein; $P > .05$; Table 2) and significantly lower PR-A and PR-B transcript and protein expression levels in the ectopic tissue (mean: 0.1192 [0.1192] for the PR-A mRNA, mean: 1.205 [0.5521] for the PR-A protein, mean: 0.04818 [0.02409] for the PR-B mRNA, and mean: 1.297 [0.7243] for the PR-B protein) compared to the normal endometria (mean: 0.7296 [0.3283] for the PR-A mRNA, mean 3.838 [0.8417] for the PR-A protein, mean: 0.1525 [0.05823] for the PR-B mRNA, and mean: 3.512 [1.038] for the PR-B protein; $P > .05$, Table 2).

Correlations Between the LPAR, ATX, and PLA2 Expression Levels and the ERα, ERβ, PR-A, and PR-B Expression Levels in OECs

ERα expression correlated positively with ATX expression at both the mRNA and protein levels ($P < .0001$, $r^2 = .7882$; $P < .0002$, $r^2 = .5369$, respectively; Figure 4A). There was also a statistically significant positive correlation between ERα

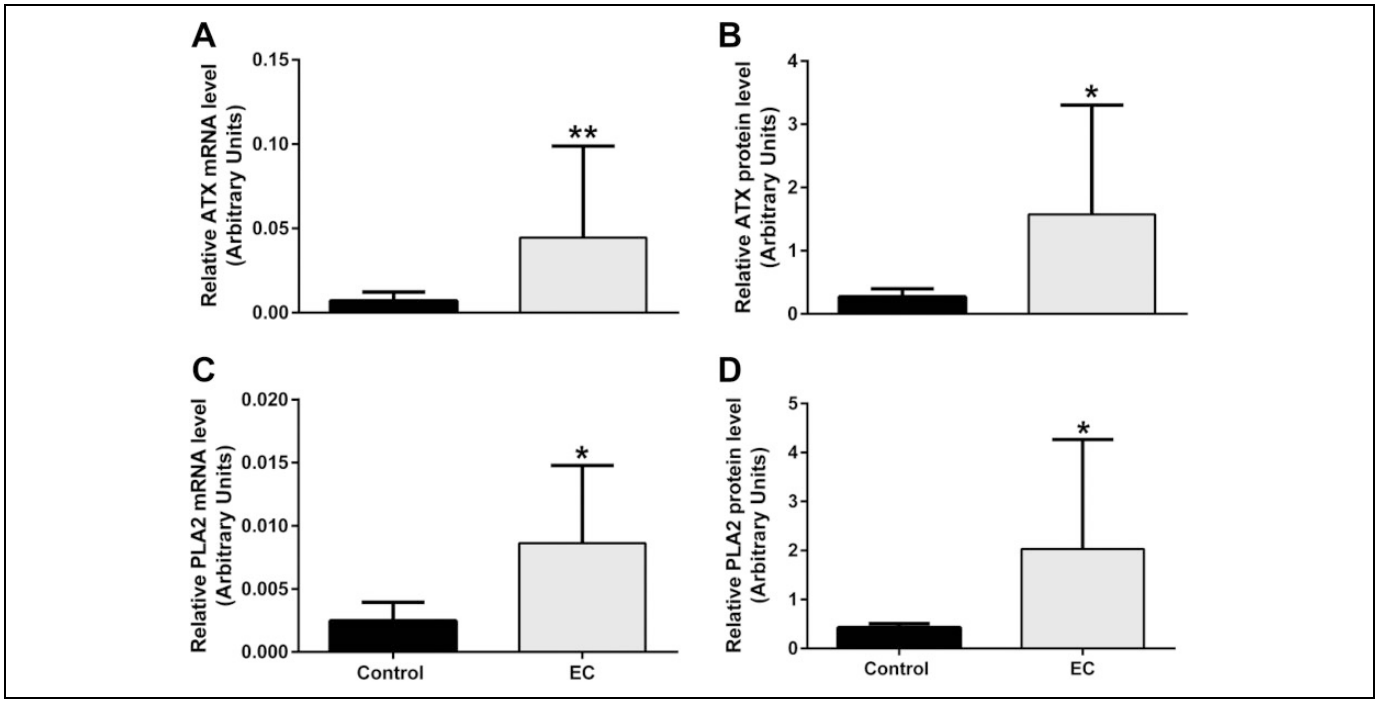

Figure 2. The expression of mRNAs (A and C) and proteins (B and D) for ATX and PLA2, respectively in OEC (gray bars) and normal proliferative endometrium (control, black bars). All values are expressed as the mean ± SEM of ATX and PLA2 expression. Different asterisks indicate significant statistical differences ($P < .05$) as determined by Student *t* test for independent pairs. ATX indicates autotaxin; LPAR, lysophosphatidic acid receptor; PLA2, phospholipase A2; OEC, ovarian endometriotic cysts.

expression and PLA2 expression in the endometriotic cysts ($P < .0001$, $r^2 = .05907$ for the transcriptional level and $P < .0005$, $r^2 = .5646$ for the protein level; Figure 4B). Additionally, we observed positive correlations between ERα and LPAR6 expression in the endometriotic cysts at both the mRNA and protein levels ($P < .0001$, $r^2 = .5711$; $P < .0002$, $r^2 = .7175$, respectively; Figure 4F). A statistically positive correlation was found between ERα and LPAR3 expression ($P < .0001$, $r^2 = .6378$) but only at the mRNA level (Figure 4D). A negative correlation was found between PR-B and LPAR2 expression ($P < .0001$, $r^2 = .7931$) at the protein level only (Figure 4C). A positive correlation was also observed between ERβ and LPAR4 expression at the transcriptional and protein levels in the ectopic tissue ($P < .0001$, $r^2 = .7259$; $P < .0001$, $r^2 = .9185$, respectively; Figure 4E). No other correlation in the endometriotic cysts between ERα, ERβ, PR-A, and PR-B and the expression levels of the studied genes were observed.

Immunohistochemical Staining of ATX, PLA2, and LPARs in the OEC and Normal Endometrium

Intense positive immunostaining for ATX and cPLA2 was observed in both stromal and epithelial cells and in the hemosiderin-containing macrophages (Figure 5A and B) of the OECs. A moderate signal was observed for ATX in the epithelial cells and stroma of the normal endometrium (Figure 5C). Pale immunostaining was observed for cPLA2 in the epithelial cells of the normal endometrium (Figure 5D). No positive staining was observed in the sections that were stained with IgG (negative control; small windows in the left top corners of Figure 5A-D).

The immunohistochemical analyses of all examined LPARs (LPARs 1-6) showed the localizations of all examined proteins to both the stromal and epithelial cells (Figure 6A-F) and the presence of LPARs 2-6 in the hemosiderin-containing macrophages (Figure 6B-F), with an intense staining in the OECs. Immunohistochemical staining of the control endometrial sections confirmed pale antibody reactions in the stromal cells for LPAR3 and LPAR6 (Figure 6I and L), epithelial cells for LPAR2, LPAR4, and LPAR6 (Figure 6H, J, and K) and hemosiderin-containing macrophages for LPAR2 (Figure 6H). Negative control samples exhibited no staining (small windows in the top left corners).

Discussion

Several medical therapies exist for the treatment of endometriosis. Nevertheless, the disease is not entirely susceptible to surgical or pharmacological treatment, and the risk of recurrence, side effects, high cost, and contraceptive outcomes for women who desire pregnancy are still high. Although endometriosis remains an enigmatic disease, with symptoms that include chronic pelvic pain, inflammation, dysmenorrhea, dyspareunia, and infertility, which degrade the quality of life for women significantly, there is still an urgent need to develop efficient prognostic markers and individual, targeted therapies for OECs.

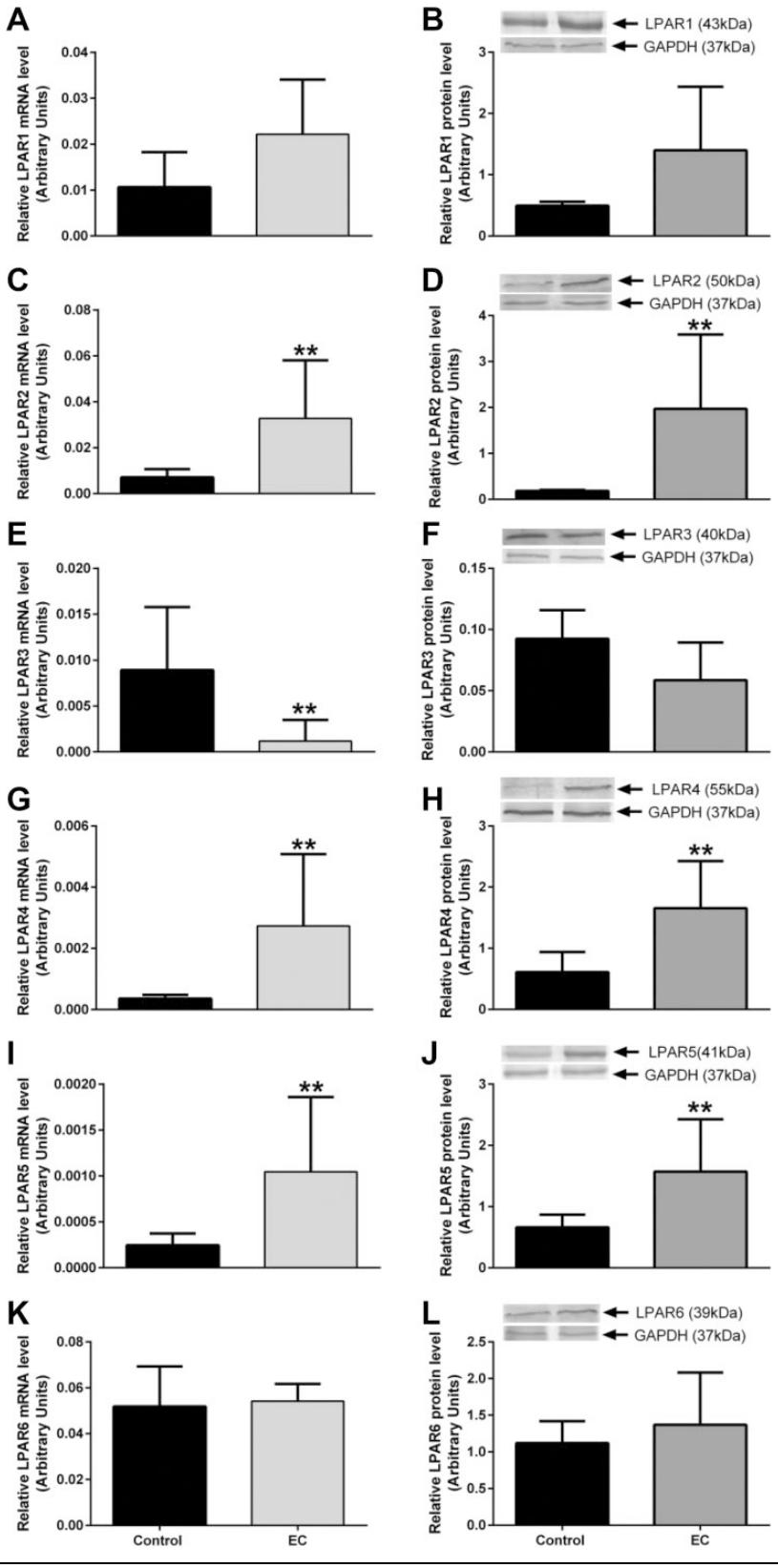

Figure 3. The expression of mRNAs (A, C, E, G, I, and K) and proteins (B, D, F, H, J, and L) for LPAR1, LPAR2, LPAR3, LPAR4, LPAR5, and LPAR6, respectively, in OEC (gray bars) and normal proliferative endometrium (control, black bars). All values are expressed as the mean $\pm$ SEM of LPAR1, LPAR2, LPAR3, LPAR4, LPAR5, and LPAR6 expression. Different asterisks indicate significant statistical differences ($P < .05$) as determined by Student *t* test for independent pairs. LPAR indicates lysophosphatidic acid receptor; OEC, ovarian endometriotic cysts.

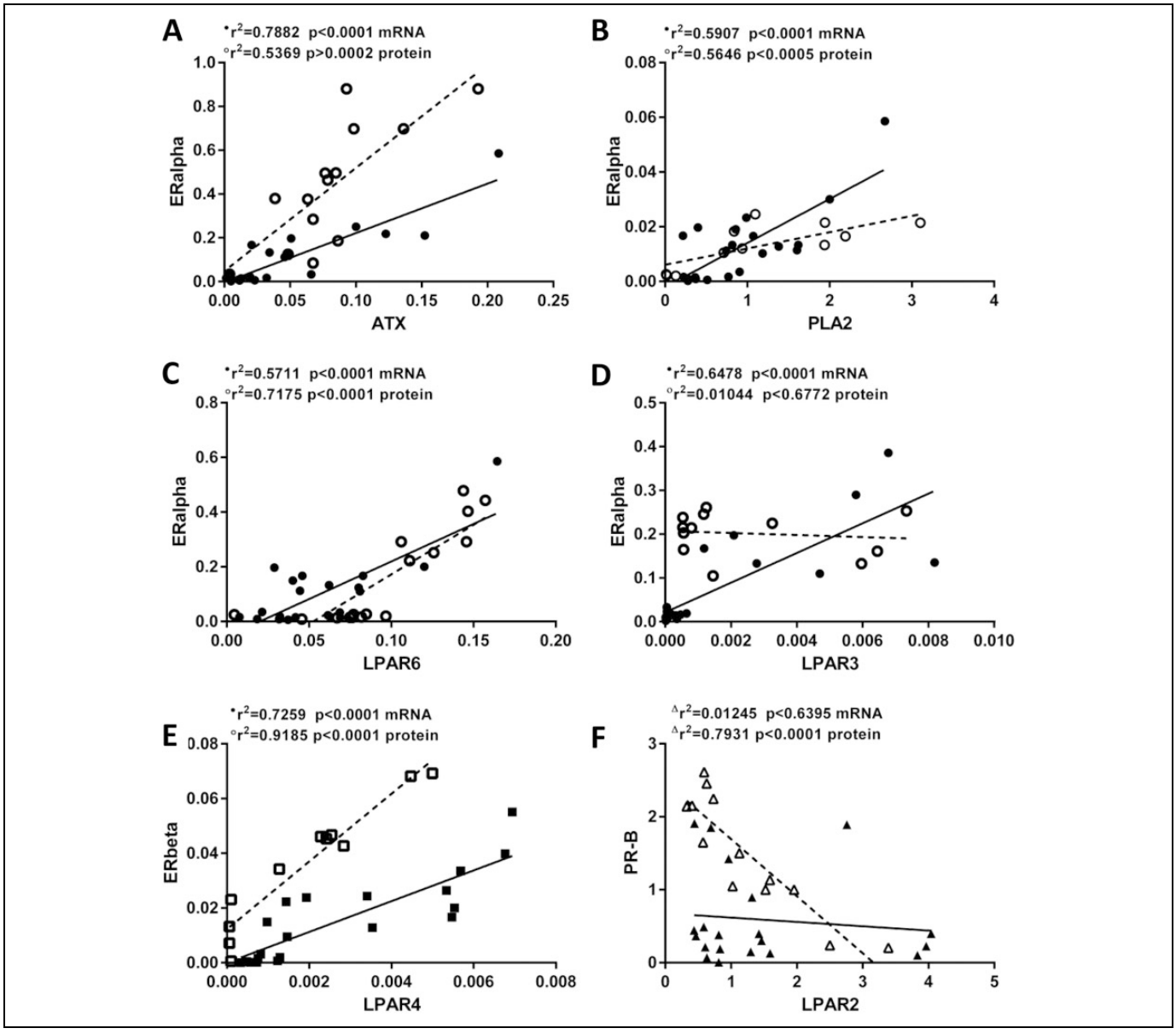

Figure 4. Pearson correlation analysis among ERα and ATX and (A), PLA2 (B), LPAR3 (D), LPAR6 (C); ERβ and LPAR4 (E); PR-B and LPAR2 (F) on mRNA transcript and protein levels in OEC. ATX indicates autotaxin; LPAR, lysophosphatidic acid receptor; PLA2, phospholipase A2; OEC, ovarian endometriotic cysts.

Previous data have suggested that upregulated LPA may play a significant role in ovarian pathology.[12] Elevated concentrations of LPA have been found in the plasma and ascites of patients with ovarian cancer.[15] Additionally, in vitro studies have shown that blocking LPA synthesis can lead to the inhibition of ovarian tumor cell activity.[16,17] Moreover, serum AX/lysoPLD activity in patients receiving ovarian stimulation is higher than in women with natural cycles,[15] and LPA is induced in incubated human follicular fluid by AX/lysoPLD,[15] suggesting that ovarian stimulation in women may increase LPA levels. These findings imply the importance of LPA in human ovarian disorders. In our study, although the LPA level did not statistically differ between the endometriotic cyst wall and the normal endometrium, we found that both enzymes responsible for LPA synthesis showed significantly higher mRNA and protein expression levels in the endometriotic cysts than in the control tissue. The hallmark of an endometriotic cyst wall is the presence of hemorrhagic lesions and abundant hemosiderin-laden macrophages in the endometrial stroma, which is the possible source of LPA in the examined tissue. Additionally, we observed positive correlations between the ERα mRNA and protein expression levels and the ATX and PLA2 mRNA and protein levels in the OEC wall. Estrogen receptor subtypes α and β are proteins with high affinities for E2 that are encoded by separate genes. A previous study showed higher levels of ERβ and lower levels of ERα in human endometriotic tissues and primary stromal cells compared with eutopic endometrial tissues and cells.[18,19] Nevertheless,

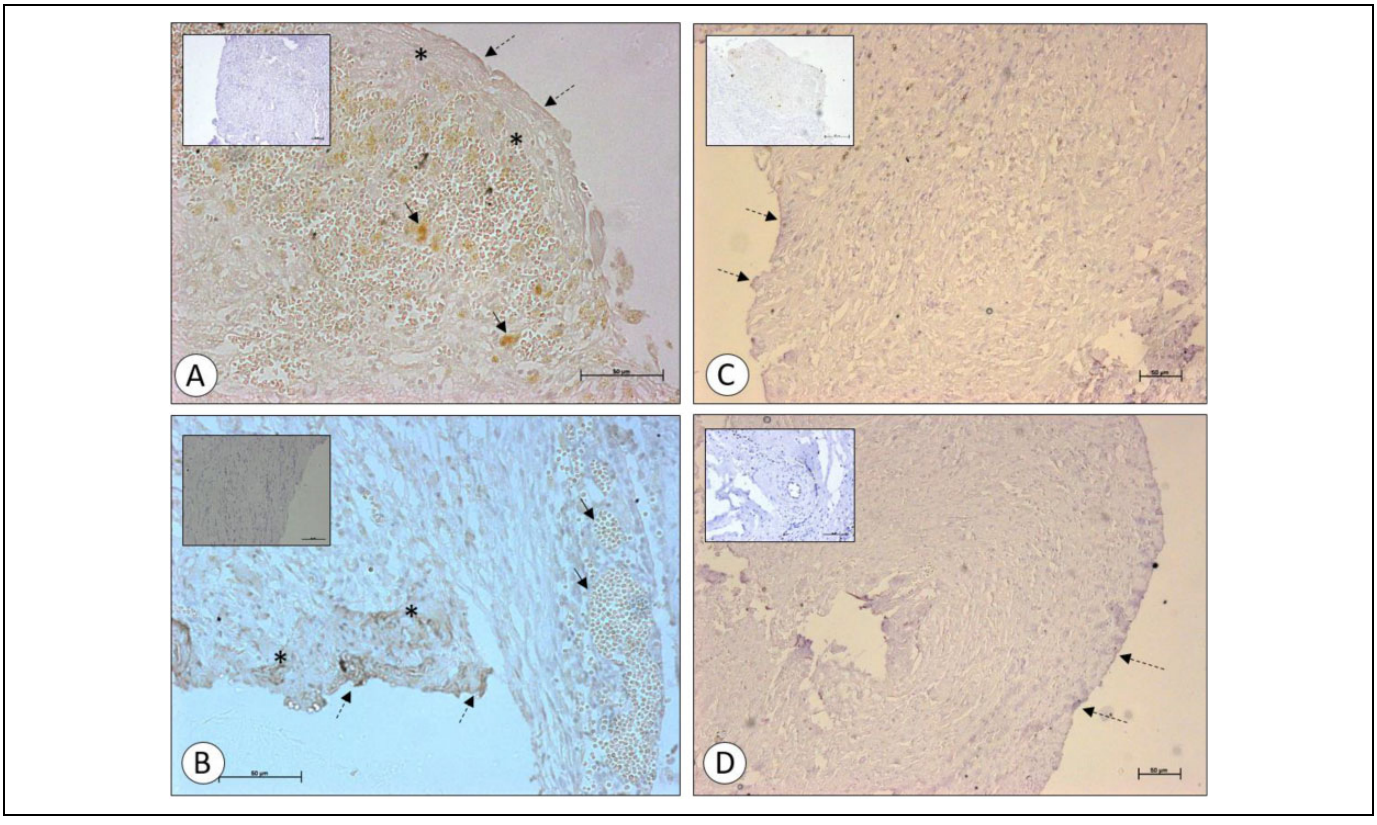

Figure 5. ATX and cPLA2 immunostaining in ovarian endometriotic cysts (A, B) and in normal endometrium (C, D) and negative control (small windows in the left top corners) were revealed. Positivity is shown by brown staining. Dashed arrows donate epithelial cells; asterisks denote stroma cells; black arrows denote macrophages containing hemosiderin. ATX indicates autotaxin.

Fujimoto et al[18] reported that the relative mRNA levels and immunohistochemical staining of ERβ were much lower than for ERα in ovarian endometriomas and normal uterine endometria. The authors assumed that typical endometriosis becomes established after transformation to an endometriotic lesion together with an alteration in the manner of ER isoform expression. Additionally, the characteristic stable ERβ-to-ERα ratio in each target organ has been suggested as necessary for intact estrogen action via ER isoform cascades.[18] When these observations are taken into consideration together with the positive correlation between the enzymes responsible for LPA synthesis and ERα expression, we can presume that LPA may influence the ERβ-to-ERα ratio in the endometriotic cyst wall, resulting in the LPA contribution to the establishment of ovarian endometriosis in humans.

Lysophosphatidic acid can induce proliferation, survival, and invasion of ovarian cancer cells. The outcomes of LPA signaling are determined by the constellation of LPARs expressed on cell surfaces. Lysophosphatidic acid binds and activates surface G protein-coupled receptors (GPCRs) of the EDG receptor family (LPAR1-3) and purinergic receptor family, including LPAR4 (GPR23), LPAR5 (GPR92), and LPAR6 (P2Y5).[20,21] In the present study, we have shown that LPAR2, but not LPAR1, is upregulated at the mRNA and protein level in the endometriotic cyst wall compared to the normal endometrium, which is partially consistent with data from Sutphen et al[20] and Xu et al,[22] who showed that LPAR2 and LPAR3, but not LPAR1, are upregulated in ovarian cancer tissues. Although recent data suggested that LPAR1 is the major mediator of LPA-induced ovarian cancer invasion and metastasis,[23] these results were established with ovarian cancer cell lines, which do not completely simulate clinical disease or the endometriotic cyst wall. Additionally, LPAR1 has been suggested as a negative growth regulator when overexpressed in ovarian cancer cell lines and T lymphocytes that functions by counterbalancing the effects of other LPARs that contribute to cell proliferation.[24] This observation is similar to studies of other GPCR families, where both positive and negative receptors exist and suggests that selective agonists for LPAR1 may decrease the growth of ovarian cancer cells and potential tumor cells from other lineages.[24] Furthermore, Murph et al[25] demonstrated that LPA acting via LPAR1 plays a tumor suppressive role in ovarian cells, which suggests that LPA1 may exert negative effects on the growth and survival of ovarian cancer cells. Together with the data presented in the literature and our observation that LPAR1 expression was not significantly higher in the endometriotic cyst wall than in the normal endometrium, we can presume that this receptor is not active in ovarian endometriosis. In the experiments that followed, we observed a negative correlation between LPAR2 and PR-B in the endometriotic cyst wall on the protein level. The molecular basis of P4 resistance in endometriosis has been associated with

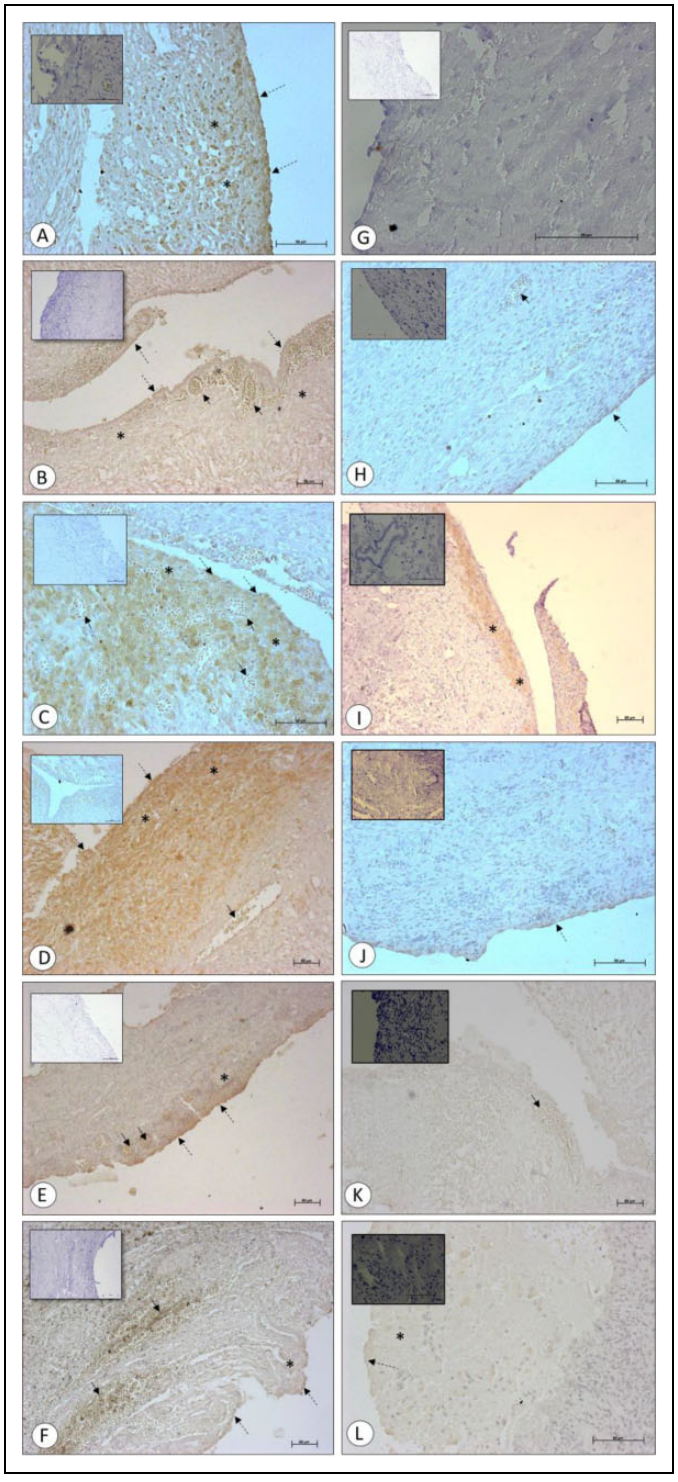

Figure 6. Lysophosphatidic acid receptor (LPAR1-6) immunostaining in ovarian endometriotic cysts (A-F) and in normal endometrium (G-L) and negative control (small windows in the left top corners) were revealed. Positivity is shown by brown staining. Dashed arrows denote epithelial cells; asterisks denote stroma cells; black arrows denote macrophages containing hemosiderin.

a reduction or absence in PRs, especially PR-B. Attia et al[6] showed that in patients with endometriosis, the expression of both the A and B isoform of the P4 receptor was detectable in the eutopic endometrium, whereas in the endometriotic implants, only the A transcript was found. On the other hand, Xue et al[26] demonstrated that the total PR and PR-B mRNA levels in endometriosis were significantly lower compared with the eutopic endometrium. The presence of the inhibitory isoform A and the absence of the stimulatory isoform B provide a possible explanation for the P4 resistance of endometriotic implants.[6] Our study demonstrated high LPAR2 expression in the OEC wall compared to the normal endometrium and showed a negative correlation between LPAR2 and isoform B of the P4 receptor in the OECs, which are observations that may indicate a contribution by LPAR2 to endometriosis that occurs through its impact on decreasing PR-B expression in ovarian endometriosis. On the other hand, we observed lower LPAR3 expression in the endometriotic cyst wall compared to the normal endometrium but only on the mRNA level. Despite the upregulation of LPAR3 in the ovarian cancer tissues,[27] the decreased LPAR3 expression that has been found in the endometria of woman with endometriosis may indicate impaired endometrial receptivity in patients with endometriosis.[28] We also observed a positive correlation between type 3 receptor for LPA and ERα at the mRNA level in the endometriotic cyst wall. In contrast to LPAR1 and LPAR2, which are activated by LPA with either saturated or unsaturated fatty acyl chains, LPA3 is preferentially activated by LPA with unsaturated fatty acyl chains.[29] Although LPAR1 to LPAR3 are members of the endothelial cell differentiation gene (Edg) family, LPAR4 to LPAR6 have been identified as non-Edg LPARs that belong to the purinergic receptor family.[21] Although the impact of LPAR1, LPAR2, and LPAR3 has been widely described in ovarian pathology, the expression of other known LPARs has not been described to date. To our knowledge, this is the first report to show the expression of LPAR4, LPAR5, and LPAR6 in ovarian endometriosis. We found that LPAR4 and LPAR5 are elevated in the endometriotic cyst wall compared to the normal endometrium at the mRNA and protein level. Additionally, positive correlations between LPAR4 and ERβ and between LPAR6 and ERα were found. Because the disease is most prevalent in women of reproductive age and regresses after menopause, the importance of steroid hormones in endometriosis is unquestionable. As previously suggested, the characteristic stable ratio of ERβ to ERα in each target organ has been suggested as a requirement for an intact estrogen-signaling axis via ER isoform cascades.[18] In the endometriotic cyst wall, ERβ might fail to act with ERα toward normal estrogen dependency, which might be damaged.[18] The observations in our study suggest that LPA may influence the ERβ-to-ERα ratio, especially via LPAR4 and LPAR6 in OECs.

In conclusion, our results demonstrated the presence of enzymes involved in LPA synthesis in OECs. Additionally, when we compared endometriotic cysts to the normal endometria, we were able to show overexpression of 3 of 6 examined LPARs and both enzymes that are responsible for LPA synthesis in the endometriotic cyst wall. Specifically, the high LPAR2 and LPAR4 transcript and protein expression levels in the endometriotic cyst wall together with the positive correlations of LPAR2

and LPAR4 with PR-B and ERβ, respectively, suggest that LPAR2 and LPAR4 are the main receptors responsible for LPA actions in ovarian endometriosis and that LPAR2 and LPAR4 are the most promising prognostic markers of ovarian endometriosis. Additionally, the selected receptors can represent novel molecular treatment options for endometriotic cysts.

The reason for the limited application of the expression of LPARs as potential biomarkers in the diagnostics of early ovarian endometriosis is the unavailability of the blood tests. Therefore, we suspect that the level of LPARs expression might facilitate pharmaceutic industry as well as streamline future prognosis prediction of ovarian endometriosis. Although the above data have indicated some relationship between the overexpression of LPARs and the occurrence of endometriotic cysts, further studies are required to establish the role of LPA signaling in the endometriosis. Furthermore, there is still demand to use the large population-based cohort or case-control studies to test the importance of LPARs in etiopathogenesis of endometriosis as well as implement their antagonists as novel medicaments in the therapy for OECs.

Authors' Note

All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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