Disrupted balance between phase I and phase II estrogen-metabolising enzymes may contribute to the growth of the endometriotic tissue in ovarian endometriosis

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Aims

To examine the expression of the estrogen phase I and phase II metabolizing enzymes in ovarian endometriosis and normal endometrium at the mRNA level.

To study the protein expression of CYP1B1 and COMT in endometriotic in normal endometrial tissues.

Introduction

Endometriosis is a complex estrogen-dependent disease defined as the presence of endometrial tissue outside the uterine cavity. Estrogens stimulate cell proliferation via the estrogen receptor and undergo extensive oxidative metabolism at different positions, catalyzed by various cytochrome P450 isoforms. Formation of 2- and 4-hydroxy-estrogens (catechol estrogens, CEs) is catalysed mainly by CYP1A1/1A2 and CYP1B1, respectively. CEs can be oxidized to the corresponding estrogen ortho-quinones with concomitant formation of the reactive oxygen species (ROS). NAD(P)H:quinone oxidoreductase (NQO1/2) regulate the reduction of toxic estrogen quinones back to catechols. Furthermore, estrogens can undergo 16 α-hydroxylation, extrahepaticaly catalysed mainly by CYP3A5 and CYP3A7. CE and 16 α-hydroxy-estrogens are further metabolised by the conjugative enzymes: catechol-O-methyltransferase (COMT), sulfotransferases (SULTs), UDP glucuronosyltransferases (UGTs), and glutathione S-transferases (GSTs) and yields less harmful products (Figure 1).

Conclusion

Our data show imbalance between the phase I and phase II enzymes in ovarian endometriosis. Increased hydroxylation and decreased conjugation of estrogens caould lead to exessive ROS formation, which may activate cytokines that control the implantation and the growth of endometrial cells outside the uterus and thus contribute to the development of ovarian endometriosis.

Additionaly, we observed higher MB-/S-COMT ratio, suggesting that MB- and S-COMT play diferent roles in CE detoxification in endometriosis, where MB-COMT may be involved mainly in 2-CE methylation. However, the exact role of each COMT isoform should be further studied.

Results and discussion

Expression levels of genes encoding phase I (CYP1A1, CYP1A2, CYP1B1, CYP3A5, CYP3A7) and phase II (SULT1A1, SULT1E1, SULT2B1, COMT, UGT2B7, NQO1, NQO2, GSTP1) estrogen-metabolizing enzymes were studied by real-time PCR in 31 samples of ovarian endometriosis and 29 normal endometrium samples (Figure 1). We found significantly higher levels of CYP1A1 and CYP3A7 ($p < 0.0001$) suggesting increased 2- and 16 α-hydroxylation of estrogens. CYP1B1 and CYP3A5 expression was not altered, while CYP1A2 expression was not detected. The levels of CYP1A1, CYP3A5, and CYP3A7 were much lower than the levels of CYP1B1, however, levels of CYP1A1 and CYP3A7 were 4 and 2 fold higher, respectively, in endometriosis than in normal endometrium which suggest that in ovarian endometriosis 2-, 4-, and 16α-hydroxylations of estrogens are important.

Among conjugative enzymes, we found increased mRNA levels of COMT ($p < 0.0001$) and unchanged mRNA levels of SULT1A1 and NQO2, while all the other gene levels were significantly decreased: UGT2B7, NQO1, GSTP1, SULT2B1 (all $p < 0.0001$), and SULT1E1 ($p = 0.0311$). COMT and GSTP1 were the most abundant, which suggest that they represent the main detoxification force in these tissues. Increased levels of COMT imply enhanced methoxylation of CE in endometriosis. Lower levels of SULTs suggest decreased conjugation of hydroxy-estrogens by sulfatation, while lower NQO1 and GSTP1 levels might lead to insufficient quinone detoxification and ROS formation. Levels of UGT2B7 were negligible in endometriosis, while its expression was seen in all normal endometrium samples, suggesting decreased 4-CE detoxification.

Figure 1.

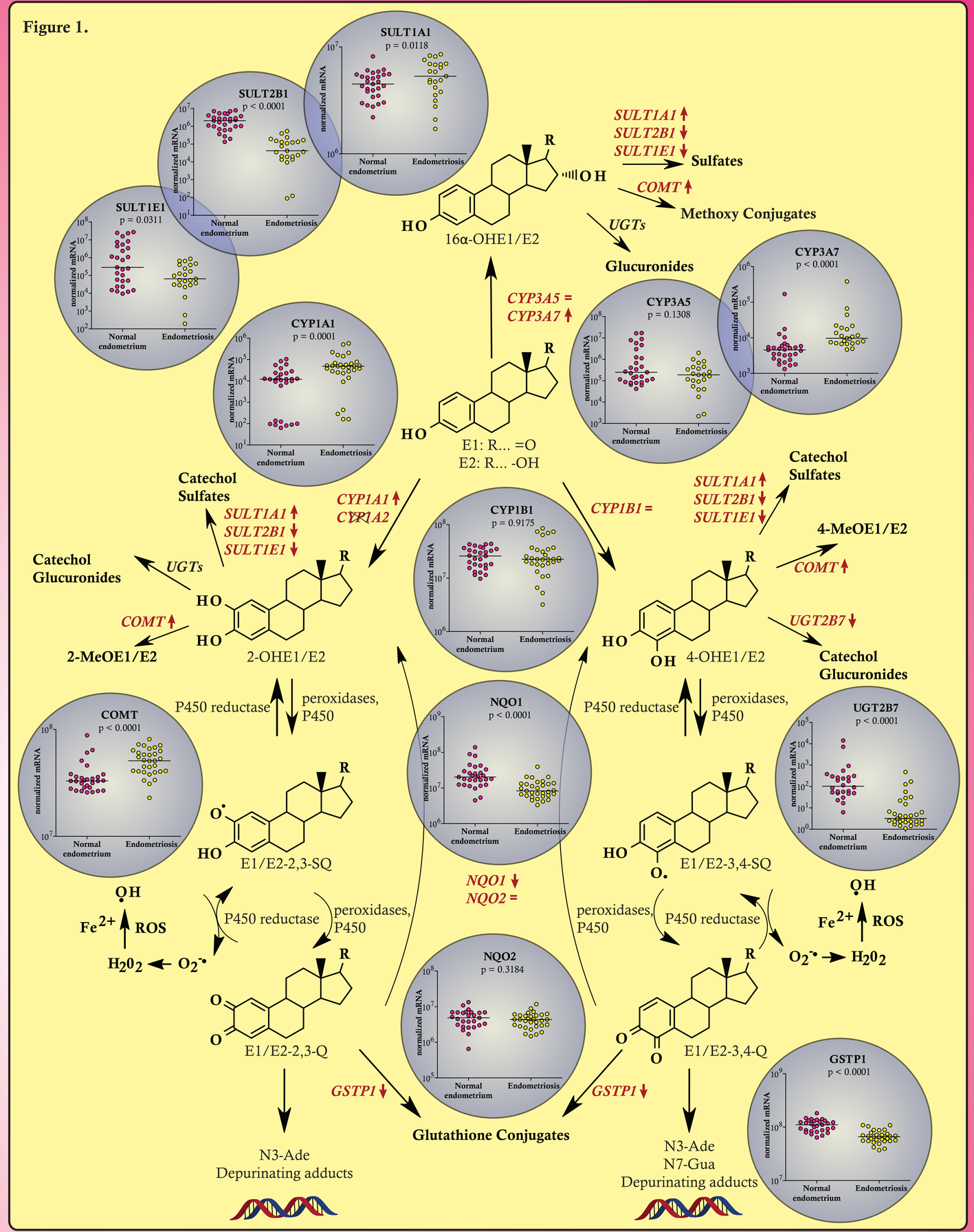
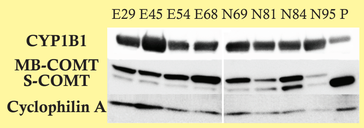


Figure 1. Phase I and Phase II metabolism of estrogens. Enzymes of our interest are shown in red. ↓ down regulated genes, ↑ up regulated genes and = statistically insignificant change in expression levels . The scatter plot graph show relative expression levels of the *CYP1A1, CYP1B1, CYP3A5, CYP3A7, COMT, SULT1A1, SULT1B1, SULT1E1, UGT2B7, NQO1, NQO2,* and *GSTP1* genes in normal endometrium and endometriosis. The levels of gene expression were multiplied by 10^{14} and are presented on a logarithmic scale. Bars denote the median expression levels.

Figure 2.



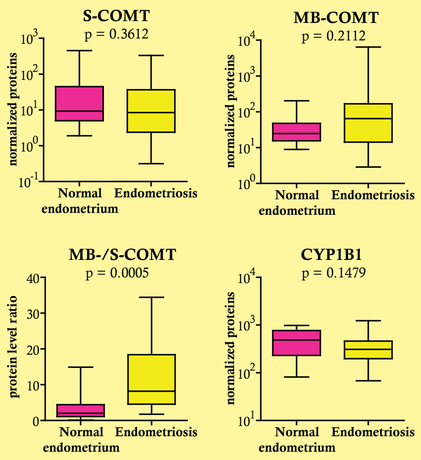


Figure 2. Western blot analysis of protein fractions of normal endometrium (N) and endometriotic tissue (E). Representative samples are shown, and cyclophilin A was used as normalisation control. Box and whiskers graph show S-COMT, MB-COMT, and CYP1B1 protein expression, as well as MB-/S-COMT ratio.

Protein expression levels of CYP1B1 and COMT were examined in 19 samples of ovarian endometriosis and 20 samples of normal endometrium (Figure 2). The results were assessed by Western blotting and showed unchanged protein levels of CYP1B1 in endometriosis ($p = 0.1479$). MB-COMT protein levels were increased and S-COMT levels decreased, yet, not statistically significantly. However, the ratio between MB- and S-COMT was significantly increased in endometriotic tissue (4 fold, $p = 0.0005$). This data, together with increased CYP1A1 mRNA levels, suggest that MB-COMT is more important for 2-methoxy estrogen (MeOE), while S-COMT may be preferentially involved in 4-MeOE formation. The total COMT levels were 2.8 fold elevated, but it was not statistically significant.

Methods

Specimens. A total of 60 specimens were collected by biopsies during laparoscopy: 31 of ovarian endometriomas (mean age, 33 ± 6) and 29 of control normal endometrium, from women undergoing sterilisation (mean age, 41 ± 3) (Tables 2 and 3). The study was approved by the National Medical Ethics Committee of the Republic of Slovenia and tissue samples were obtained after hysterectomies or during sterilization procedure with the full and informed consent of the patients. The specimens were immediately placed into RNA Later (Qiagene) RNA stabilization solution, and kept at –20 °C until RNA extraction. Total RNA was extracted with Tri Reagent (Sigma) according to the manufacturer's instructions and transcribed into cDNA using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen).

Selection of normalization gene candidates. A cohort of 15 reference genes (*GAPDH, CCND1, YWHAZ, ACTB, HPRT1, RPS26, B2M, SDHA, TBP, PPIA, TFRC, UBC, MMP3, RPLP0, TUBA1*, and *PGK1*) was tested on samples of ectopic endometrium and control endometrium. The TaqMan® Gene Signature Array Configuration (384) (Applied Biosystems, Foster City, CA, USA) was used. The most stable reference genes: *PPIA, GAPDH* and *RPLP0* were selected using GeNorm algorithm.

Real-time PCR. The expression of 13 genes of interest and three selected reference genes was determined with the exon-spanning hydrolysis probes (FAM or VIC dye labeled), commercially available as 'Assay on Demand' (Applied Biosystems, Foster City, CA, USA). Quantification was accomplished with the LightCycler® 480 Real-Time PCR System (Roche) using TaqMan® Universal PCR Master Mix and universal thermocycling parameters recommended by Applied Biosystems. RT-PCR samples were run in triplicates using 0.25 µL of cDNA. The reactions were performed on 384 well-plates (Roche) with the reaction volume of 5 µL. The gene expression normalization factor for each sample was calculated based on the geometric mean of all three selected reference genes. MIQE guidelines were considered in the performance and interpretation of the qPCRs. Statistical calculations and tests were performed using the SPSS Software (SPSS Inc., USA). The Mann-Whitney test for unrelated samples was used. All of the statistical tests were two-tailed, and differences in p values of less than 0.05 were considered to be significant.

Western blot analysis. Proteins were isolated from the same tissues as RNA. Protein aliquots were separated by SDS PAGE. Proteins were transfered from gels to membranes and blocked with 5% non-fat milk for 2 hours. Afterwards the membranes were incubated over night with primary antibodies rabbit anti-CYP1B1 (Abcam, ab32649, 1:20000) and rabbit anti-COMT (Millipore, ab5873, 1:7500) in TTBS with 1 % non-fat milk powder. The next day the secondary antibodies (anti-rabbit peroxidase conjugated) were incubated with membranes for 2 hours. RapidStep ECL Reagent (Calbiochem) was used for the detection of bound antibodies according to manufacturer's instructions. Cyclophilin A (Abcam, ab58144, 1:1000) was used as normalisation control. Quantification of Western Blots was done with Multi Gauge (Fujifilm software, Fujifilm, Japan).

Table 1. Patients details for endometriosis and control group.

	Patients with Endometriosis ovarii	Patients undergoing sterilisation
Total number	31	29
Mean age	33 ± 6	41 ± 3
Proliferative phase	14	21
Late proliferative/early secretory	9	8
Secretory phase	5	0
Phase not determined	3	0