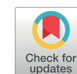
Journal of Steroid Biochemistry and Molecular Biology 202 (2020) 105701

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

Expression of nuclear progesterone receptors (nPRs), membrane progesterone receptors (mPRs) and progesterone receptor membrane components (PGRMCs) in the human endometrium after 6 months levonorgestrel low dose intrauterine therapy



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ARTICLE INFO

Keywords:
Endometrial hyperplasia
Levonorgestrel: intrauterine
Nuclear progesterone receptors
Membrane progesterone receptors
Progesterone receptor membrane components
Receptor regulation

ABSTRACT

The classical steroid receptors (nuclear receptors), including those for progesterone (nPRs), are thoroughly characterized. The knowledge about so-called non-genomic effects, which are mediated by extra-nuclear initiated signals, has increased immensely the last decades. In a previous clinical study of endometrial hyperplasia, we observed that the antiproliferative progestin effect persisted after 3 months treatment with levonorgestrel (LNG) intrauterine system (IUS) even with a complete downregulation of nPRs. This raised the question of what other mechanisms than signaling through nPRs could explain such an observation. In the present study, RT-qPCR was employed to characterize mRNA expression for nPRs, membrane progesterone receptors (mPRs) and progesterone receptor membrane components (PGRMCs) in women (n = 42) with endometrial hyperplasia that received intrauterine low dose LNG for 6 months. At the end of this period endometrial tissue showed that nPRs were virtually completely downregulated (≈ 10 % of baseline) whereas the levels of remaining mPRs, subtype-α, -β and -γ were 76 %, 59 % and 73 % of baseline, respectively. PGRMC1 was downregulated to 15 % of baseline, in contrast to PGRMC2, which was upregulated to about 30 % above baseline. We used human cancer cells from uterine cervix (C-4I cells) as control. Progesterone caused a concentration-dependent antiproliferative effect but in several and separate studies, we were unable to detect nPRs (immunocytochemistry) in the C-4I cells. The use of RT-qPCR showed that nPRs were undetectable in C-4I cells, in contrast to mPRs and PGRMCs with a distinct mRNA expression. The present study suggests that mPRs and/or PGRMCs preserve the antiproliferative effect of LNG in the human endometrium and are responsible for the concentration-dependent antiproliferative effect of progesterone in C-4I cells.

1. Introduction

Physiogical and pharmacological progesterone effects were known long before the search for molecular mechanisms was initiated. The identification of high affinity binding proteins (nPRs) in cytoplasm with [^{3}H]-progesterone was a breakthrough [1]. In the classical genomic signaling pathway progesterone and other progestins bind to nPRs located in the cytoplasm, which leads to a conformational change with dissociation of heat shock proteins, dimerization, translocation to the nucleus and binding to hormone responsive elements in target genes [2]. However, other mechanisms of progesterone action have also been reported, such as non-genomic effects due to the rapid onset [3]. The knowledge about these extranuclear-initiated mechanisms gradually emerged [4–7]. The discovery and characterization of membrane progesterone receptors (mPRs) [8,9]were important contribution to the understanding of non-genomic effects.

One of the earliest recognized effects of steroid hormone action was receptor down-regulation (including nPRs) in response to ligand

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https://doi.org/10.1016/j.jsbmb.2020.105701
Received 4 February 2020; Received in revised form 11 May 2020; Accepted 15 May 2020
Available online 29 May 2020
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binding [10]. However, limited information exists whether mPRs are subject to downregulation as response to various signal molecules, including hormones and drugs. In a clinical study of patients with levonorgestrel (LNG) intrauterine system (IUS) treatment of endometrial hyperplasia, we observed a persistent antiproliferative endometrial effect even if nPRs were completely downregulated [11]. In studies of the human cell line C-4I, derived from cervical cancer of the uterus [12], progesterone inhibited growth in a concentration-dependent manner [13,14]. Despite repeated analyses with immunocytochemistry, we were unable to detect nPRs in the C-4I cells. The inhibitory constant (IC_{50}-value) of progesterone was 5.9 μM (recalculation based on raw data) [13] and 2.1 μM [14]. These observations of progesterone effects without presence of nPRs, suggested that non-genomic and receptor-like mechanism were involved in the maintained antiproliferative effect in patients with endometrial hyperplasia and the concentration-dependent antiproliferative effects in the C-4I cells. In addition to mPRs, PGRMCs were potential candidates for the non-genomic effects in human endometrium and cells derived from a cancer of the uterine cervix. PGRM1C and PGRMC2 belongs to a family of membrane-associated progesterone receptors (MAPRs), also detected in the female reproductive tract in humans [15]. In the present work, we tested whether mPRs and /or PGRMCs were present in the endometrium after LNG-IUS therapy with downregulated nPRs [11], and if mPRs and /or PGRMCs were present in the C-4I cells with undetectable protein expression of nPRs [13,14]. Gene expression (mRNA of nPRs, mPRS and PCRMCs) was employed as surrogate markers for protein expression of receptors / membrane components.

2. Materials and methods

2.1. Clinical study design

A group of 61 women were recruited to a prospective, multicenter pilot study to assess the efficacy of LNG-IUS 13.5 mg (Jaydess™, Bayer Pharmaceuticals, Berlin, Germany) for treatment of endometrial hyperplasia [16,17]. Only those women (n = 49) with a completed treatment period of six months were included in the presented study.

2.2. Study subjects

All women had consulted their gynecologists due to abnormal uterine bleeding. Prior to study inclusion they underwent a clinical examination. Endometrial biopsies were obtained for histopathological diagnosis, D-score [18] and RT-PCR analysis. In seven of the 49 women, endometrial biopsy material was insufficient for qPCR analysis. Table 1 shows the characteristics of the 42 women. Histopathological material from the endometrium was obtained prior to study inclusion (baseline biopsy) and after completing LNG-IUS therapy (post therapy biopsy). Therapy response in post therapy biopsy was defined as ordinary proliferative endometrium or endometrium with progestin effect (glandular atrophy and pseudo-decidualization of the stromal cells).

2.3. Handling of tissue and cells, and analytical details

Details about a) Endometrial biopsies, b) C4-I cell culture and cell sampling, and c)Tissue and cell preservation awaiting analysis, d) RNA isolation and cDNA synthesis, e)Analysis of reference gene stability, f) Selection of primers and reaction efficiency and g)Quantitative real-time PCR are presented in [19].

3. Results

3.1. Endometrial samples - nPRs, mPRs and PGRMCs

Maintained exposure of LNG to the endometrium caused a profound effect on the mRNA expression of classical progesterone receptors. After 6 months with low dosage LNG-IUS (Jaydess™ treatment the nPRB ($p < 0.001$) and nPRA + nPRB ($p < 0.001$) were almost completely downregulated with approximately 10 % receptor mRNA detectable compared to the baseline values (Table 2).

LNG administration did also cause downregulation of mPRs but markedly less compared with nPRs (Table 2). The remaining receptor subpopulations after LNG-administration were 76 %, 73 % and 59 % of baseline values for mPRα, mPRγ and mPRβ, respectively. Only the reduction of mPRβ expression was statistically different from baseline values ($p < 0.02$). The mRNA expression of PGRMCs was also influenced during the LNG treatment (Table 2). The expression of PGRMC1

Table 1
Demographic and histopathological characteristics of the women (n = 42) with endometrial hyperplasia included in the study. Menopausal status was defined according to s-estradiol (nmol/l) and s-FSH (IU/l).

Characteristics	Median (25; 75 percentile)	Range
Age (years)	49 (43.8; 53.3)	30 – 84
Weight (kg)	75 (68.8; 87.0)	56 – 120
Height (cm)	167 (161; 170)	149 – 176
BMI (kg/m^2)	28 (26.1; 30.9)	20.1–40.6
	n	%
Parity	7	16.7
0	14	33.3
1–2	21	50
3 – 4		
Menopausal status*	22	52.4
Premenopausal	5	11.9
(estradiol ≥ 0.12, FSH ≤ 30)	15	35.7
Perimenopausal	0	0
(estradiol ≥ 0.12. FSH > 30)	24	57.1
Postmenopausal	6	14.3
(estradiol < 0.12)FSH > 20)	12	28.6
Menstrual pattern		
Normal		
Metrorrhagia		
Menorrhagia		
Postmenopausal bleeding		
Histopathological subtype of endometrial	16	38.1
hyperplasia *	25	59.5
Simple hyperplasia	1	2.4
Complex hyperplasia		
Atypical hyperplasia		
D-score category**	24	41.5
> 1	17	58.5
0–1	0	0
< 0	42	100
Therapy response	0	0
Yes		
No		

* WHO classification, [44].
** D-score category was missing for one patient.

Table 2
Expression (mRNA) of nuclear progesterone receptors, membrane progesterone receptors and progesterone receptor membrane components in the endometrium, before (baseline) and after 6 months LNG (levonorgestrel) intrauterine treatment. Statistical significance was evaluated by Wicoxon signed rank test with P < 0.05 as the significance limit (Sigmaplot™). Data presented as median and 25; 75 percentiles. The data are presented as CNRQ (Calibrated Normalized Relative Quantity).

Genes	Baseline biopsy	Post therapy biopsy	p-value
nPRB	3.255 (1.315;4.965)	0.290 (0.290; 0188)	< 0.001
nPRA + nPRB	3.45 (2.16;4.94)	0.39 (0.23;071)	< 0.001
mPRα	1.09 (0.72;1.78)	0.83 (0.59;1.71)	n.s.
mPRβ	1.24 (0.91;1.88)	0.73 (0.43;1.24)	< 0.02
mPRγ	1.25 (0.56;2.11)	0.91 (0.49;1.39)	n.s.
PGRMC1	2.59 (1.59;3.45)	0.40 (0.25;0.72)	< 0.001
PGRMC2	0.84 (0.71;1.17)	1.10 (0.72; 1.63)	n.s.

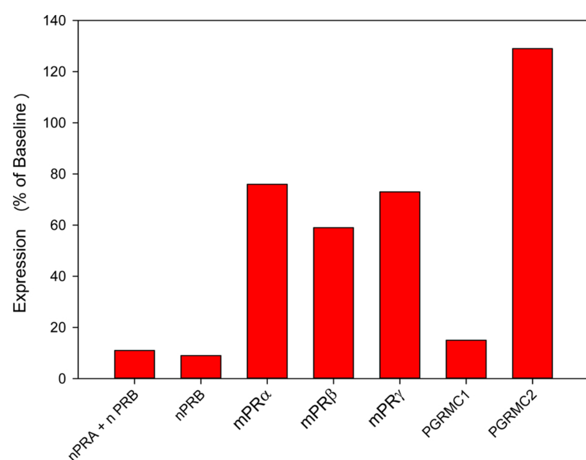

Fig. 1. Regulation of endometrial nPRs, mPRs and PGRMCs by levonorgestrel IUS. The gene expression (mRNA) after 6 months is presented as % of baseline values. The analytical equipment and experimental methods are described in [19].

was reduced significantly to 15 % of baseline levels, whereas PGRM2 increased with about 30 % above baseline levels. Fig. 1 shows the relative effect of continued LNG exposure on endometrial progesterone receptors and membrane components.

3.2. C-4I cells - nPRs, mPRs and PGRMCs

The human cervical cancer cell line (C-4I) was employed in the present study as a control. The absence of nPR expression was confirmed with RT- qPCR technology. Table 3 shows that mRNA for the mPRs and PGRMCs was expressed during logarithmic growth.

4. Discussion

Progesterone modulates gene expression via classical nPRs and causes rapid effects via mPRs and the less characterized PGRMCs. In receptor pharmacology desensitization is a well-known phenomenon wherein continued exposure to an agonist (hormone, neurotransmitter or drug) results in reduced tissue response. At least two mechanisms may cause this process: Receptor downregulation and uncoupling between receptor and effector component(s). One of the earliest recognized effects of steroid hormone action was receptor downregulation in response to ligand binding [10]. In a previous study, we found that endometrial nPRs were completely downregulated after 3 months treatment with LNG-IUS (Mirena™) releasing ≈ 20 μg/day, in women diagnosed with endometrial hyperplasia [11]. The mechanism behind this ligand-dependent down-regulation involves phosphorylation of nPRs by p42yp44 MAPKs at serine-294, thus targeting nPRs for ubiquitination and destruction by the 26S proteasome [20]. In two more recent studies the treatment period was extended to six months

Table 3
Expression (mRNA) of nuclear progesterone receptors, membrane progesterone receptors and progesterone receptor membrane components in human cancer cells of the uterine cervix (C-4I cells, ATCC® CRL-1594™). The data are presented as CNRQ (Calibrated Normalized Relative Quantity).

Receptor type	Detected	Mean ± SD	Minimum	Maximum
nPRB	No			
PRA + /nPRB	No			
mPRα	Yes	1.000 ± 0.290	0.790	1.495
mPRβ	Yes	1.016 ± 0.218	0.782	1.301
mPRγ	Yes	1.008 ± 0.034	0.962	1.033
PGRMC1	Yes	1.016 ± 0.200	0.778	1.252
PGRMC2	Yes	1.008 ± 0.144	0.864	1.199

and the dose was reduced with LNG-IUS (Jaydess™), releasing ≈ 10 μg/day [16,17]. Endometrial biopsies were obtained prior to and after LNG-IUS therapy.

The biopsy material of the present study was analyzed with qPCR technology to determine the mRNA expression of the genes encoding for nPRs, mPRs and PGRMCs. However, the observed changes in mRNA can, but may not, reflect expression of the respective proteins. Occurrence of translational and posttranslational modification may exist. Examples of this are modified protein folding and glycosylation patterns. This demands caution in the phenotypic interpretation of changes in mRNA levels.

An increased ratio between nPRB and nPRA stimulates of growth in endometrial cancers [21,22]. In the present study, the endometrial expression of nPRs was reduced to 10 % or less of baseline values. It is possible that the mechanism behind the clinical effect of LNG-IUS therapy (antiproliferative effect), is the removal of functional nPRB receptors in the endometrium. Since some categories of endometrial hyperplasia may represent preliminary stages of endometrial cancer [23], the present study implies that maintained progestin therapy may prevent the development of malignancy.

The relative binding affinities of LNG and progesterone are 100 % and 50 %, respectively, above the reference steroid for nPR, promegestone (R5020) [24]. The high LNG affinity for nPRs, in addition to persistent high local LNG concentration in the endometrium observed with IUS *in situ* [25], suggest that the downregulation of nuclear receptors was initiated and maintained by LNG.

However, LNG-IUS may also modify mPRs activity indirectly by reducing the serum progesterone levels due to suppression of ovulation. In a study that comprised 27 fertile regularly menstruating women, serum samples were obtained in the mid-luteal phase without and after 3 months with LNG-IUS *in situ*. Progesterone levels showed a significant fall from 32.8 to 8.4 nanomol/L in serum [26].

The discovery of mPRs can account for at least some of the extranuclear-initiated (non-genomic) effects. They were described as proteins with seven transmembrane domains, G-protein-coupled with inhibition of adenylate cyclase activity [9]. Subsequent studies indicated that the mPRs did not belong to the GPCR superfamily but to the PAQR (progesterone and adipoQ receptors) subfamily [27,28]. Furthermore, neither the cellular localization(s) nor the effector mechanism(s) have been settled; for review see: [29].

In human endometrium the post-ovulatory rise in progesterone coincided with a significant induction of mPRα and a gradual downregulation of mPRγ [27] and represents physiological receptor regulation. The present study shows a pharmacological effect on the mPR mRNA expression. Downregulation was observed for the three investigated mPRS sdubtypes, but with a dissimilar magnitude. The mechanisms behind the downregulation of mPRs are not clarified but evidence for a receptor endocytosis via a clathrin-mediated pathway have been presented [30]. While LNG has high affinity for nPRs [24], norgestrel (the racemic mixture of LNG and the inactive dextroisomer) has no or very low affinity for human mPRα [28,31]. This is a strong argument against a direct role of LNG in the downregulation of mPRs.

It appears that mPRs have significant roles in premalignant and malignant diseases of the female genital tract. These receptors have been detected in the cervical cancer cell lines HeLa [32] and C4-I (present report), in diverse epithelial human ovarian cancer biopsies [33] and in commonly used ovarian cancer cell lines [34]. Endometrial cancer showed decreased expression of mPRα and mPRβ, but unaltered expression of mPRγ in endometrial cancer compared to control tissue [35].

In addition to mPRs we considered PGRM1C and PGRMC2 as potential candidates responsible for the non-genomic gestagen-induced antiprolifrative effect in the endometrium and in the C-4I cells. These two small proteins belong to a family with four members of membrane-associated progesterone receptors (MAPRs). The cloning of PGRMC1 (designated HPR6) and PGRMC2 (designated Dg6) was published in

1998 [36]. Ten years thereafter, a review summed up the accumulated knowledge on PGRMC1 biology [37]. PGRMC1 is a transmembrane protein, predominantly located in intracellular membranes, but has also been reported to be present at the plasma membrane ectoside in some cell types. The protein is not a progesterone receptor but a component of a multi-functional complex with partners dependent on the cell type. PGRMC1 is involved in regulation of cytochrome P450, steroidogenesis and vesicle trafficking, binding of steroids and other hydrophobic molecules, cell cycle regulation and many other processes, for review see [38]. In this context, the idea that PGRMC1 is responsible for the cell surface localization of mPRα is exciting [39].

PGRMC1 plays an important role in the antiapoptotic action of progesterone [40]. The significant reduction in endometrial mRNA expression observed in the present study may antagonize PGRMC1′s antiapoptotic effect. This is compatible with the normalization of the hyperplastic endometrium. The possibility exists that PGRMC1 may be a useful biomarker for successful LNG therapy with reversal of malignant development. This idea is in agreement with the reported upregulation of PGRMC1 in a number of cancer types including those of the female genital tract [41].

PGRMC2 has many similarities with PRGMC1. Both are composed of a single amino acid chain, have a ubiquitous expression with mainly intracellular localization, expressed in human female reproductive tissues and upregulated by progesterone [15]. However, the functional role of PGRMC2 is less characterized than that of PGRMC1 [42], but is probably a signal adapter protein with some functions that makes it distinguishable from PGRMC1 such as binding to CYP21A2 and CYP3A4 [43]. In SKOV-3 ovarian cancer cells PGRMC2, but not PGRMC1, inhibited cell migration whereas no differences were evident with regard to cell viability or response to cisplatin and progesterone [43]. It is suggested that PGRMC2 plays a role in tumor suppression and not, as PGRMC1, in tumor promotion [42]. The present study showed a striking difference in mRNA expression with an opposite modulation of the proteins. PGRMC1was significantly downregulated whereas PGRMC2 was moderately upregulated. Thus, it is tempting to suggest that PGRMC2 has an essential role in the suppression of the abnormal growth typical for endometrial hyperplasia.

The cell line C-4I with undetectable nPRs (immunohistochemistry), was included into this study as a systemic control. The present results without detectable mRNA expression of nPRs are in agreement with our previous observations. However, the C-4I cells showed distinct mRNA expression for mPRs and PGMRCs during logarithmic growth. We believe that progesterone signaling through mPRs and/or PGRMCs explains the concentration-dependent antiproliferative effect of progesterone in C-4I cells [13,14]. Based on the knowledge that PGRMC2 is involved in tumor suppression, it is possible that PGRMC2 coordinates the signals responsible for the antiproliferative effect of progesterone in C-4I cells. Under the given conditions in the present study, absence of nPRn was a common feature for the endometrium and the C-4I cells. However, the respective mechanism was entirely different with LNG-induced downregulation for the endometrium and the genetic makeup for C-4I cells. PGRMC2 might be the common link in the antiproliferative effect of gestagens, observed in the present study.

Financial support

We thank the Regional Research Board of Northern Norway (Helse Nord) and UiT - The Arctic University of Norway for funding.

CRediT authorship contribution statement

Elise Thoresen Sletten: Investigation, Formal analysis, Resources, Writing - review & editing. **Natalia Smaglyukova:** Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing. **Anne Ørbo:** Writing - review & editing, Funding acquisition, Supervision. **Georg Sager:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

Gynecological outpatient clinics in northern Norway (Tromsø, Harstad, Stokmarknes, Kirkenes, Finnsnes, Alta, Hammerfest) for their contribution in sampling of endometrial biopsy material. Lena M. M. Lyså and Bjørn T.G. Moe for skilled image analyses (D-score) on endometrial tissue specimens.

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