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Inflammation influences steroid hormone receptors targeted by progestins in endometrial stromal cells from women with endometriosis

Giovanni Grandi[a], Michael D. Mueller[b,c], Andrea Papadia[b], Vida Kocbek[c], Nick A. Bersinger[b,c], Felice Petraglia[d], Angelo Cagnacci[a], Brett McKinnon[b,c,*]

[a] Azienda Ospedaliero-Universitaria Policlinico, University of Modena and Reggio Emilia, Via del Pozzo 71, 41124 Modena, Italy
[b] Department of Obstetrics and Gynaecology, Inselspital, Berne University Hospital, Effingerstrasse 102, Berne CH-3010, Switzerland
[c] Department of Clinical Research, University of Berne, Murtenstrasse 35, Berne CH-3010, Switzerland
[d] Policlinico "Santa Maria delle Scotte", Department of Molecular and Development Medicine, University of Siena, Viale Bracci, 53100 Siena, Italy

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ABSTRACT

Endometriosis is an estrogen-dependent disease characterised by the growth of endometrial epithelial and stromal cells outside the uterus creating a chronic inflammatory environment that further contributes to disease progression. The first choice treatment for endometriosis is currently progestin mediated hormone modulation. In addition to their progestogenic activity however, progestins also have the potential to bind to other nuclear receptors influencing their local activity on endometriotic cells. This local activity will be dependent on the steroid hormone receptor expression that occurs in endometrial cells in a chronic inflammatory environment. We therefore aimed to quantify receptors targeted by progestins in endometrial stromal cells after exposure to inflammation. Using primary endometrial stromal cells isolated from women with endometriosis we examined the mRNA and protein expression of the progesterone receptors A and B, membrane progesterone receptors 1 and 2, androgen receptors, mineralocorticoid receptors and glucocorticoid receptors after exposure to the inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β). The results indicate that both cytokines reduced the expression of progesterone receptors and increased the expression of the glucocorticoid receptors in the endometrial stromal cells. The change in expression of progestin targets in endometrial stromal cells in an inflammatory environment could contribute to the progesterone resistance observed in endometriotic cells and ultimately influence the design of hormonal therapies aimed at treating this disease.

1. Introduction

Endometriosis is an estrogen-dependent disease defined by the presence of endometrial glands and stroma outside the uterine cavity, affecting up to 15% of women during reproductive age (Giudice and Kao, 2004) and linked to pelvic pain and subfertility (McKinnon et al., 2015; Schliep et al., 2015; de Ziegler et al., 2010). The growth of ectopic lesions stimulates an infiltration of immune cells (Halme et al., 1983; Hornung et al., 2001) and the subsequent cytokine production creates a positive feedback loop that further stimulates inflammation from endometriotic cells (Bersinger et al., 2008, 2011). This positive feed-back loop creating a chronic inflammatory environment that contributes to both disease progression and symptomology (McKinnon et al., 2015). Current medical therapies for women with endometriosis who do not wish to conceive are aimed at hormonal modulation (Dunselman et al., 2014; Practice Committee of American Society for Reproductive Medicine, 2008) by either progestins alone, or in combination with estrogen (Dunselman et al., 2014; Practice Committee of American Society for Reproductive Medicine, 2008; Vercellini et al., 2003).

Progestins are synthetic compounds that produce a progestogenic effect through binding to progesterone receptors (PR) (Schindler et al., 2008). The beneficial activity of progestins in endometriosis treatment stems from the activation of pituitary PR suppressing ovulation and creating amenorrhea and a hypoestrogenic environment (Vercellini et al., 2003). The progestins affinity for PR however varies significantly between the different compounds, as does their cross-reactivity to other nuclear recep-

* Corresponding author at: Frauenheilkunde, Inselspital, Murtenstrasse 35, Berne CH-3010, Switzerland.
E-mail address: brett.mckinnon@dkf.unibe.ch (B. McKinnon).

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tors including androgen receptors (AR) (Schindler et al., 2008), glucocorticoid receptors (GR) (Schindler et al., 2008) and mineralocorticoid receptors (MCR) (Schindler et al., 2008). Non-nuclear effects are also possible through the interaction with progesterone-binding membrane proteins (PGRMC) 1 and 2 (Kowalik et al., 2013). Ultimately therefore, the biological influence of progestins is the combination of their relative affinities to multiple targets.

Whether progestins also exert a local effect on the endometriotic lesions is not clear. A recent systematic review concluded that there was sufficient evidence to indicate a local effect on immortalized endometrial epithelial cell lines and primary stromal cells *in vitro* by the progestin dienogest, the mechanism by which this was mediated however could not be resolved (Grandi et al., 2016). Endometriotic stromal cells are considered progesterone resistant due a down regulation of PR, especially the active subtype B (PRB), although this remains controversial (Shao et al., 2014**)**. Membrane PGRMC1 and PGRMC2 mRNA are also significantly lower in the eutopic endometrium of women with endometriosis compared to those without (Bunch et al., 2014). AR is present in both eutopic endometrium and peritoneal endometriosis (Carneiro et al., 2008) and GR is significantly higher in endometriotic lesions compared to eutopic endometrial tissue (Monsivais et al., 2012). Furthermore, the expression of these receptors is influenced by the inflammatory microenvironment. PR has an intricate reciprocal relationship with the nuclear transcription factor (NF)κB, responsible for controlling the inflammatory response (Guo, 2007) and the mRNA expression of GR is down regulated in endometriotic stromal cells after tumor necrosis factor α (TNFα) treatment (Monsivais et al., 2012).

Progestins are a first-line treatment for endometriosis via the systematic modulation of estrogen concentrations. Whether progestins also have local effects on the endometriotic lesions is not clear, but will be dependent on the interaction between their cellular targets and the inflammatory environment. Therefore to better understand the local progestin effects in endometriosis, we analysed the expression of the cellular targets of progestins in eutopic endometrial stromal cells after exposure to TNFα and interleukin 1β (IL-1β), hierarchal cytokines that are increased in the ectopic environment of endometriosis.

2. Materials and methods

2.1. Sample collection

Ethical approval was obtained from the relevant institution review board and written informed consent was collected from all patients prior to surgery. Inclusion criteria were planned laparoscopy for suspected endometriosis or idiopathic infertility, regular menstrual cycles, ages between 18 and 45 years and no hormonal therapies within the 3 months prior to surgery. Exclusion criteria included prior or current pelvic inflammatory disease or liver dysfunction. During laparoscopy, all of which were performed during the proliferative phase both peritoneal fluid and endometrial biopsies were collected. Peritoneal fluid was collected from the cul-de-sac and eutopic endometrium obtained via an endometrial Pipelle® (Pipelle deCornier, Laboratorie CCD, Paris, France), as previously described (McKinnon et al., 2012). Peritoneal fluid was used for progesterone measurement to confirm cycle phase and eutopic endometrium was used for the isolation of primary eutopic endometrial stromal cells (ESC).

2.2. Isolation and culture of endometrial stromal cells

Primary ESC were isolated from the endometrial biopsies using methods described previously (Bersinger et al., 2011; McKinnon et al., 2013). Separation was performed via collagenase digestion (Collagenase from Clostridium Histolyticum, Sigma Life Sciences, Missouri, USA) and size exclusion membranes (100 μm and 40 μm mesh filters, BD Bioscience, New Jersey, USA). Isolated ESC were maintained in Iscoves's modified Eagle medium (IMEM) (Invitrogen Life Technologies, New York, USA) supplemented with 10% fetal calf serum (FCS) and 1% antibiotic/antimycotic (Invitrogen Life Technologies) at 37 °C in a humidified atmosphere with 5% CO_2. Culture medium was changed every 3 days. Cells were used for experiments until passage 7.

For experiments cells were seeded onto a 6-well plate and grown until approximately 80% confluent. Prior to treatments the media was changed to 0.5% FCS, 1% antibiotic/antimycotic. Treatments were performed either with 0.5% FCS media only (control), 0.5% media with TNFα (10 ng/ml or 100 ng/ml) (Recombinant Human TNF-alpha Protein, from *Escherichia coli*, R&D Systems Inc, Minneapolis, USA), or 0.5% media with IL-1β (1 ng/ml or 10 ng/ml) (Recombinant Human IL-1 beta, from *E. coli*, R&D Systems Inc). After a 6 h incubation both control and treatment cells were collected in either RNA cell lysis buffer for subsequent genetic analysis (Qiazol® Lysis Reagent, Qiagen, Maryland, USA) or in Radioimmunoprecipitation assay (RIPA) buffer containing Phosphatase and Protease inhibitor cocktail (Cell signalling technology, Massachusettes, USA), Ethylene glycol tetraacetic acid (EGTA), Ethylenediaminetetraacetic acid solution (EDTA) and Triton® X-100 (Sigma Life Sciences, Missouri, USA) for protein anaylsis.

2.3. Determination of gene expression

RNA extraction was performed with the RNAeasy® Plus Micro extraction Kit (Qiagen) following the manufacturer's instructions. One microgram of RNA was reverse transcribed in a final volume of 25 μl with the Moloney Murine leukemia virus Reverse transcriptase enzyme and random primers (Promega, Madison, USA) and the cDNA diluted 1:20. Genomic DNA absence was confirmed via a no RT control.

RNA expression was determined via a Real-time quantitative Polymerase chain reaction (qPCR) using Rotor-gene Taqman Fast advanced Master Mix (Qiagen) and the following TaqMan® gene expression arrays for both genes of interest PR (Hs01556702_m1), PGMRC1 (Hs00998344_m1), PGMRC2 (Hs01128672_m1), AR (Hs00171172_m1), GR (Hs00353740_m1), MCR (Hs01031809_m1) and reference genes hypoxanthine phosphoribosyltransferase-1 (HPRT1) (Hs01003267_m1), beta-actin (Hs01060665_g1), ubiquitin C (Hs00824723_m1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs00266705_g1) and ribosomal protein L13A (RPL13A) (Hs04194366_g1). The qPCR was performed in a Rotor-Gene RG 2000 (Corbett Research, New South Wales, Australia), under the following condition: 95° for 5 min, followed by 40 cycles of 95° for 5 s and 60 °C for 10 s.

Multiple reference genes were selected based on their stability across samples, as determined by the qBASE software suite (Biogazelle, Ghent University, Belgium). The change in mRNA expression for each gene of interested was also calculated via the qBASE software, based on the geometric mean of the multiple reference genes and the ΔΔCt method. The efficiency of each reaction, as determined via linear regression (Ruijter et al., 2009) was also incorporated into the equation. All RNA quantities are expressed as a percentage of control.

2.4. Determination of protein expression

Whole cell lysates were prepared from ESC cultured in the 6-well plates by adding 250 μl of RIPA buffer to each well and scraping the cells from the plate. Cell lysate was collected and cell debris removed via centrifugation at 12,000 rpm at 4 °C for 30 min and protein concentration determined via the bicinchoninic acid