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

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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.

Keywords: Endometriosis; progestin; inflammation; progesterone receptor; glucocorticoid receptor; mineralocorticoid receptor; membrane progesterone receptor; androgen receptor.

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 receptors 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 E. 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 hour 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 seconds and 60°C for 10 seconds.

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 250µl 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 minutes and protein concentration determined via the bicinchoninic acid assay (QuantiPro BCA; Sigma). Proteins were seperated by diluting approximately 40µg of total protein into LDS sample buffer (Invitrogen Life Technologies) and running the solution on a 8% Novex NuPAGE Bis/Tris gel (Invitrogen Life Technologies).

Proteins were transferred onto a 0.45 µM nitrocellulose membrane using the iBlot 2 dry transfer system (Invitrogen Life Science) for 7 minutes. The iBind™ Western System (Invitrogen Life Science) was used for protein-antibody conjugation with the following antibodys; rabbit anti-PR antibody (Cell Signaling Technology), rabbit anti-PGRMC1 (Cell Signaling Technology), rabbit anti-PGRMC2 antibody (Abcam, Cambridge, United Kingdon), rabbit anti-GR antibody (Cell Signaling Technology), rabbit anti-AR antibody (Cell Signaling Technology) and rabbit anti-MCR antibody (Abcam) all at a dilution of 1:200. The mouse anti-Actinβ antibody (Abcam) was used as a loading control at a dilution 1:1000. Anti-rabbit HRP linked antibody 1:1000 (Cell Signaling technology) and the anti-mouse HRP linked antibody 1:50000 (Sigma) were used as secondary antibodies. Immunodetection was performed with the Super-signal West femto Kit (Pierce; Thermo Scientific, Lausanne, Switzerland) on a Witec Fusion pulse series (Witec, Luzern, Switzerland). Semi-Quantitative analysis was performed by band densitometry using the ImageJ software

(Schneider, 2012). The proteins of interest were normalised to actinβ and the protein expression after the treatments were expressed as a percentage of control.

2.5 Statistical analysis

The statistical analysis was performed by GraphPad Prism 5 software (GraphPad Software, California, USA). Analysis of relative mRNA and protein expression was determined by non-parametric Friedman's one-way analysis of variance (ANOVA) test for repeated measures with a *post-hoc* Dunn's multiple comparison test to compare between both the treatment groups and the control. Significance was considered as $p < 0.05$.

3. Results

3.1 Patients characteristics

Eutopic endometrial tissue was collected from six different women, all of which had lesions subsequently confirmed as endometriosis by histological examination. Using the revised American Fertility Society (rAFS) score (Revised American Society for Reproductive Medicine classification of endometriosis, 2007), four women were considered stage IV, one woman stage III and the final woman stage I. The age range was 26-45 and the body mass index 21.3-28.7 Kg/m^2. All women were confirmed to be in the proliferative phase via measurement of peritoneal progesterone concentrations (**Table 1**).

3.2 Influence of inflammatory cytokines on PR expression

TNFα treatment signficantly ($p = 0.0017$) decreased PR mRNA transcription from control (100%). Although this did not reach signficance after 10ng/ml ($40.47 \pm 14.16\%$, $n = 6$; $p = 0.0867$) it did reach significance after 100ng/ml ($30.89 \pm 17.88\%$, $n = 6$; $p = 0.0030$) (**Figure 1A**). IL-1β treatment also signficantly ($p = 0.0055$) reduced PR mRNA transcription from control (100%) after both 1ng/ml ($48.29 \pm 22.60\%$, $n = 6$; $p = 0.419$) and 10ng/ml ($41.50 \pm 22.60\%$, $n = 6$; $p = 0.0078$) (**Figure 1B**).

Western blot analysis after both TNFα and IL-1β treatment separated the PRA and PRB isoforms based on their size and actinβ was used as a loading control (**Figure 1C**). Quantitation of the different isoforms of PR indicated that 10ng/ml TNFα stimulated a signficant reduction in the expression of PRA ($54.20 \pm 8.50\%$, $n = 6$; $p = 0.0419$), but not PRB ($72.10 \pm 21.87\%$, $n = 6$; $p = 0.4965$). Treatment with 100ng/ml TNFα however signficantly reduced both PRA ($35.28 \pm 9.59\%$, $n = 6$; $p = 0.0078$) and PRB ($59.13 \pm 20.45\%$, $n = 6$; $p = 0.0419$) (**Figure 1D**). Similarly, no signficant reduction from control was observed after 1ng/ml IL-1β treatment for either PRA ($78.15 \pm 11.09\%$, $n = 6$; $p = 0.4965$), or PRB ($78.89 \pm 11.22\%$, $n = 6$; $p = 0.7730$) however both PRA

(49.93 ± 10.30%, n = 6; p = 0.0419) and PRB (50.49 ± 12.51%, n = 6; p = 0.0188) were signficantly reduced with 10ng/ml IL-1β (**Figure 1E**).

3.3 Influence of inflammatory cytokines on membrane PR expression

The influence of TNFα on PGRMC1 mRNA did not reach significance (p = 0.0521). A *post-hoc* analysis indicated the decrease after 10ng/ml TNFα (66.20 ± 22.59%, n = 6; p = 0.0826) was not significant, in contrast to the decrease after 100ng/ml of TNFα (63.71 ± 15.01%, n = 6; p = 0.0419) (**Figure 2A**). IL-1β treatment did not significantly (p = 0.1804) decrease PGRMC1 mRNA transcription at 1ng/ml (61.79 ± 19.81%, n = 6; p = 0.0826), however it did significantly decrease it after 10ng/ml (59.26 ± 11.67%, n = 6; p = 0.0419) (**Figure 2B**). Western blot analysis found PGRMC1 protein in all samples (**Figure 2C**). Semi-quantitative analysis however did not identify a signficant influence of either TNFα (p = 0.4306) (**Figure 2D**) or IL-1β (p = 0.4306) (**Figure 2E**).

TNFα treatment did not significantly influence (p = 0.1840) PGRMC2 mRNA at 10ng/ml TNFα (94.89 ± 39.92%, n = 6) or 100ng/ml TNFα (70.89 ± 31.19%, n = 6) (**Figure 3A**). IL-1β also did not significantly influence PGRMC2 mRNA transcription (p = 0.1840) and a post-hoc analysis confirmed this was not significant at 1ng/ml (60.95 ± 28.00%, n = 6) or 10ng/ml (64.17 ± 28.88%, n = 6) (**Figure 3B**). Western blot confirmed PGRMC2 protein expression in ESC (**Figure 3C**). TNFα did not have a strong influence on PGRMC2 protein expression (p = 0.4306) (**Figure 3D**). However IL-1β reduced PGRMC2 protein at 10ng/ml IL-1β (81.02 ± 6.52%, n = 4; p = 0.0267) (**Figure 3E**).

3.4 Influence of inflammatory cytokines on AR expression

TNFα treatment did not signficantly (p = 0.7402) decrease AR mRNA transcription at 10ng/ml (72.12 ± 38.13%, n = 6), or 100 ng/ml (72.98 ± 28.15%, n = 6) (**Figure 4A**). IL-1β treatment also had no influence (p = 0.2522) on AR mRNA at 1ng/ml (104.57±11.20%, n = 6), or 10ng/ml (104.43 ± 31.74%, n = 6) (**Figure 4B**). Western blot confirmed AR protein expression in ESC (**Figure 4C**), although no significant influence on AR protein expression was observed after 10ng/ml (112.2 ± 14.83%, n = 6; p > 0.9999), or 100ng/ml TNFα (126.1 ± 14.09%, n = 6; p = 0.2980) (**Figure 4D**),

or 1ng/ml (109.1 ± 24.29%, n = 6; p > 0.9999), or 10ng/ml IL-1β (79.04 ± 15.26%, n = 6; p = 0.4965) (**Figure 4E**).

3.5 Influence of inflammatory cytokines on GR expression

TNFα stimulated a signficant (p = 0.0289) increase in GR mRNA transcription at 10ng/ml (203.28 ± 85.85%, n = 6; p = 0.188), but not 100ng/ml (175.13 ± 49.16%, n = 6; p = 0.1667) (**Figure 5A**). IL-1β treatment resulted in a statistically (p = 0.0055) significant increase after both 1ng/ml (203.73 ± 145.43%, n = 6; p = 0.419) and 10ng/ml (230.84 ± 168.92%, n = 6; p = 0.0078) **(Figure 5B)**. Western blot analysis confirmed GR protein expression in ESC **(Figure 5C).** Although a non-significant change was observed after 10ng/ml (113.7 ± 7.65%, n = 6, p = 0.7730) and 100ng/ml (102.5 ± 12.48%, n = 6; p = 0.7730) TNFα (**Figure 5D**). In contrast IL-1β treatment significantly increased GR expression at 1ng/ml (147.2 ± 17.71%, n = 6; p = 0.0419) and 10ng/ml (189.3 ± 18.30%, n = 6; p = 0.0078) (**Figure 5E**).

3.6 Influence of inflammatory cytokines on MCR expression

TNFα significantly (p = 0.0081) decreased MCR mRNA at 10ng/ml (28.30 ± 28.88%, n = 6; p = 0.0188) and 100ng/ml (20.66 ± 9.46%, n = 6; p = 0.0188) (**Figure 6A**). There was a borderline non-significant (p = 0.0521) decrease in MCR mRNA transcription after IL-1β at 1ng/ml (46.16 ± 83.44%, n = 6; p = 0.0867) and statistically significantly decrease at 10ng/ml (22.28 ± 9.13%, n = 6; p = 0.0418) (**Figure 6B**). Western blot analysis confirmed MCR protein in ESC (**Figure 6C**). Semi-quantitative analysis indicated TNFα treatments increased MCR protein expression, although not significantly at both 10ng/ml (108.4 ± 17.11%, n = 6; p > 0.9999) and 100ng/ml (129.8 ± 20.34%, n = 6; p = 0.2980) (**Figure 6D**). IL-1β also stimulated an increase in MCR protein that was significant at 1ng/ml (158.5 ± 20.64%, n = 6; p = 0.0419) and 10ng/ml (196.3 ± 38.00%, n = 6; p = 0.0078) (**Figure 6E**).

4. Discussion

In the present study we determined the expression of nuclear and membrane targets for progestins in ESC isolated from women with endometriosis and analysed their change in expression in response to inflammatory cytokine exposure. TNF-α and IL-1β induced mRNA and protein expression changes of most targets and in most cases with a common trend for the mRNA and protein. Most significantly, these results show a significant reduction in PR and an increase in GR expression. Additionally, the mRNA expression of the PGRMC1 was significantly reduced only by the strongest concentrations of TNFα, although this was not translated to the protein expression. Little change was observed for AR, and MCR was the only target that that did not show a consistent trend. These results therefore show a potentially significant contribution of the inflammatory microenvironment to the local activity of progestins in endometriosis treatment.

Endometriosis is an inflammatory condition that is widely believed to start in the eutopic endometrium (Burney and Giudice, 2012). Through retrograde menstruation these endometrial cells enter the peritoneal cavity, a phenomenon that occurs in approximately 90% of women. However in women with endometriosis numerous biochemical and molecular alterations in the endometrial cells allow the cells to implant and grow in the ectopic environment. The presence of these cells stimulates the infiltration of immune cells and a chronic inflammatory response. In this study we used endometrial stromal cells isolated from women with endometriosis and exposed them to inflammatory cytokines, TNFα and IL-1β. By using eutopic tissue we were able to start with cells not yet exposed to the inflammatory milieu of the peritoneal cavity, in order to better determine the influence of this environment on these potentially endometriotic cells. Furthermore in this study we focussed specifically on cells from women with endometriosis. It would be interesting to determine if this effect also occurred in women without endometriosis, but was beyond the scope of this study.

One of the factors believed to be inherent to endometrial cells of women with endometriosis and that leads to lesion growth is progesterone resistance (Cheng et al, 2007; Burney et al, 2007; Attia et al, 2000). Although contradictory results have also been reported (Shao et al, 2014), as some studies

have shown PRA and B are decreased, or absent in endometriomas (Attia et al, 2000, Bergqvist and Ferno 1993), whereas other studies failed to show a consistent expression in extra-ovarian endometriosis (Bukulmez et al, 2008; Jones et al, 1995; Prentice et al, 1992). In this study we found low, but consistent PR mRNA expression and PRA and PRB protein expression in all replicates, although with a significant degree of variation within each cellular preparation. Furthermore exposure to both TNFα and IL-1β significantly reduced the PR mRNA and protein expression suggesting the inflammatory environment contributes to the progesterone resistance.

It is also possible that progesterone resistance is reinforced by inflammation-stimulated changes to the membrane proteins, PGRMC 1 and 2. We found a slight reduction in PGRMC1 mRNA transcription at the highest TNFα treatments and no reduction in PGRMC2. At the protein level no reduction was observed for either PGRMC1 or 2. The slight mRNA reduction, but consistent protein expression may be due to the short exposure time used. Longer exposure times for the evaluation of membrane receptor expression are needed in future investigations. A downregulation of PGRMC 1 and 2 has recently been demonstrated in eutopic endometrial stromal cells of women with endometriosis (Bunch et al, 2014).

In addition to the influence on PR expression we found a significant influence on the GR expression. GR are strongly expressed throughout the menstrual cycle in the stromal compartment of the endometrium (Bamberger et al, 2001). A recent study, consistent with our data, demonstrated that TNFα stimulates cortisol synthesis and activity in endometriotic lesions, with an upregulation of GR expression (Monsivais et al, 2012). Glucocorticoids control inflammatory processes by negatively regulating the expression of pro-inflammatory gene products allowing them to induce both apoptosis, or pro-survival signalling cascades (Stringer-Reasor et al, 2015). In many other chronic inflammatory diseases IL-1β and TNFα also influence the local glucocorticoid synthesis by increasing 11β-hydroxysteroid dehydrogenase type 1 (HSD11β2) transcription (Chapman et al, 2009).

MCR was the only progestin target we examined with a discrepancy between the mRNA and protein levels. One previous paper showed an increase in MCR mRNA expression after TNFα in endometriotic stromal cells (Monsivais et al, 2012). The reasons for the discrepancies in this study are not clear, although the simplest explanation is that low expression influenced quantitation accuracy. It has also recently been recognised however that numerous post-translational modifications of MCR alter their functional activity (Faresse et al, 2014). The effect of mineralo-corticoids on uterine stromal cells and hence endometriotic lesions is unknown, deserving further investigation.

Lastly we confirmed AR presence in endometrial stromal cells, although inflammatory cytokines had very little effect on their expression. The physiological role of AR in the uterus is not clear, although it can be detected in both epithelial and stromal cells, with higher concentrations in the latter. The highest stromal cell concentrations occur during the proliferative phase with a decrease during the secretory phase. When activated, AR has an anti-proliferative effect and acts antagonistically to estradiol on cell replication. Estradiol and androgens can increase the uterine AR expression (Moutsatsou et al, 2003).

The biological effects of progestins at the cellular level are mediated by the interaction with membrane and nuclear PR, AR, GR and MCR and thus their biological effects and therapeutic potential can be influenced by the expression of these receptors (Schindler et al, 2008). Furthermore the anti-proliferative, anti-inflammatory and anti-angiogenic effects of progestins on endometriotic lesions may also be mediated at a local level and will depend on the expression of their targets. Current theories suggest that endometriotic lesions are derived from eutopic endometrial cells that exist in an ectopic inflammatory environment. We have been able to show that both the RNA and protein expression of progestin targets in endometrial stromal cells from women with endometriosis can be significantly influenced by inflammation. From this data, it could be suggested the any steroidal hormonal treatment aimed at reducing endometriotic lesions should have a strong

progestational activity, while exploiting favourable GR activity to enhance its effect in an inflammatory environment.

In conclusion, this study demonstrates that the inflammatory cytokines TNFα and IL-1β alter the expression of progestin targets in endometrial stromal cells of women with endometriosis. This effect may contribute to the progesterone resistance and a suboptimal local response to progestin therapy, while compounds with strong GR activity may influence the local response. A better understanding of the influence of the inflammatory environment on progestin targets may help improve progestin-like drugs that mediate desirable local effects.

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Table 1: Clinical characteristics of subjects included in the study.

Study Number	rAFS stage	Age	BMI (Kg/m^2)	Peritoneal Fluid Progesterone Concentration nmol/L
1	I	45	23.2	9.98
2	IV	34	21.4	1.37
3	IV	26	21.3	4.17
4	IV	27	28.7	4.04
5	III	38	24.7	0.95
6	IV	34	23.0	5.53

Legends for Figures:

Figure 1: The influence of TNFα and IL-1β on PR mRNA and protein expression. (A) TNFα treatment of ESC significantly reduced PR mRNA expression. **(B)** IL-1β treatment significantly reduced PR mRNA expression. **(C)** Western blot confirmed the expression of PRA (α) and PRB (β) isoforms in ESC and semi-quantitative analysis on band densitometry normalized to actinβ and the control lane confirmed that both **(D)** TNFα and **(E)** IL-1β mediated a decrease in the expression of both PRA and PRB isoforms. * $p < 0.05$, ** $p < 0.01$,

Figure 2: The influence of TNFα and IL-1β on PGRMC1 mRNA and protein expression. (A) TNFα treatment of ESC did not significantly reduce PGRMC1 mRNA expression, as did **(B)** IL-1β. **(C)** Western blot analysis confirmed expression of PGRMC1 in ESC and semi-quantitative analysis after normalization to actinβ and a loading control found that there was not a significant decrease in the protein concentration after either **(D)** TNFα treatment, or **(E)** IL-1β treatment. * < 0.05.

Figure 3: The influence of TNFα and IL-1β on PGRMC2 mRNA and protein expression. (A) TNFα treatment did not significantly downregulate PGRMC2 mRNA expression. **(B)** nor did IL-1β. **(C)** Western blot analysis confirmed expression of PGRMC2 in ESC and a semi-quantitative analysis found no significant variation in protein expression after **(D)** TNFα treatment, however **(E)** IL-1β lead to a significant down regulation in protein expression after 10ng/ml. * $p < 0.05$.

Figure 4: The influence of TNFα and IL-1β on AR mRNA and protein expression. (A) TNFα treatment of ESC did not significantly influence the mRNA expression of AR. **(B)** IL-1β treatment also had no influence on AR mRNA expression. **(C)** Western blot analysis confirmed the protein expression of AR in ESC, however semi-quantitative analysis indicated that neither **(D)** TNFα, nor **(E)** IL-1β produced a significant effect on the protein expression.

Figure 5: The influence of TNFα and IL-1β on GR mRNA and protein expression. (A) TNFα treatment lead to a significant increase in the expression of GR mRNA. **(B)** IL-1β also mediated an increase in GR expression, however this did not reach statistical significance. **(C)** Western blot analysis confirmed the protein expression of GR in ESC and a semi-quantitative analysis found that there was no significant increase in GR protein expression after **(D)** TNFα, although **(E)** IL-1β mediated a significant increase at both concentrations. * $p < 0.05$, ** $p < 0.01$.

Figure 6: The influence of TNFα and IL-1β on MCR mRNA and protein expression. (A) TNFα treatment mediated a significant decrease in MCR mRNA expression, as did **(B)** IL-1β at 10ng/ml. **(C)** Western blot confirm a low, but consistent MCR protein expression in all samples and a semi-quantitative analysis found that there was a non-significant increase in protein expression after **(D)** TNFα treatment. **(E)** IL-1β treatment however did result in a significant increase in MCR protein expression. * < 0.05, ** $p < 0.01$.

Figures

Figure 1

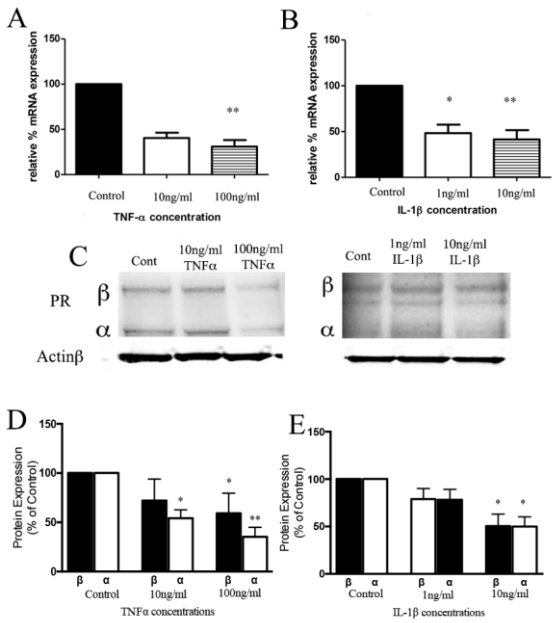
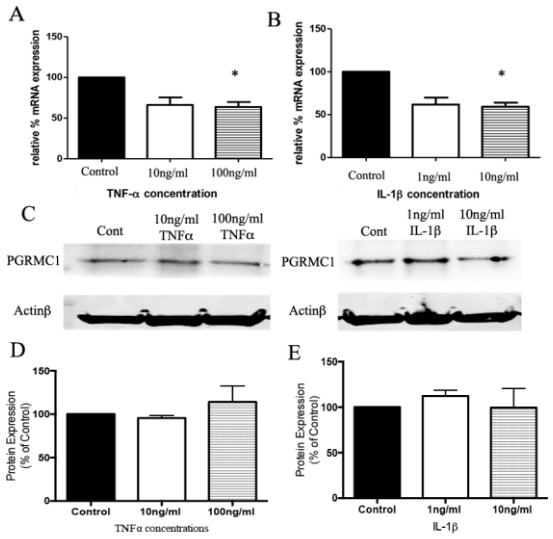


Figure 2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TNF-α concentration
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relative % mRNA expression
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10ng/ml
IL-1β concentration
C
Cont
10ng/ml TNFα
100ng/ml TNFα
PGRMC1
Actinβ
Cont
1ng/ml IL-1β
10ng/ml IL-1β
PGRMC1
Actinβ
D
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100
50
0
Control
10ng/ml
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50
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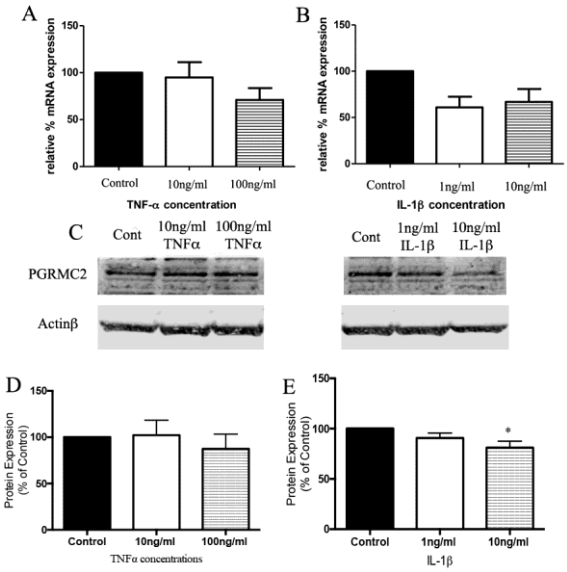
10ng/ml
IL-1β

figure 3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TNF-α concentration
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relative % mRNA expression
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1ng/ml
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IL-1β concentration
C
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10ng/ml TNFα
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PGRMC2
Actinβ
Cont
1ng/ml IL-1β
10ng/ml IL-1β
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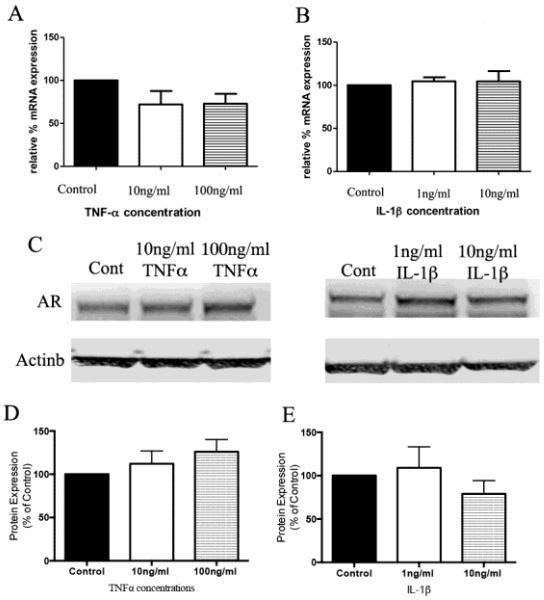
Figure 4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IL-1β concentration
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1ng/ml IL-1β
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10ng/ml
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TNFα concentrations
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Protein Expression (% of Control)
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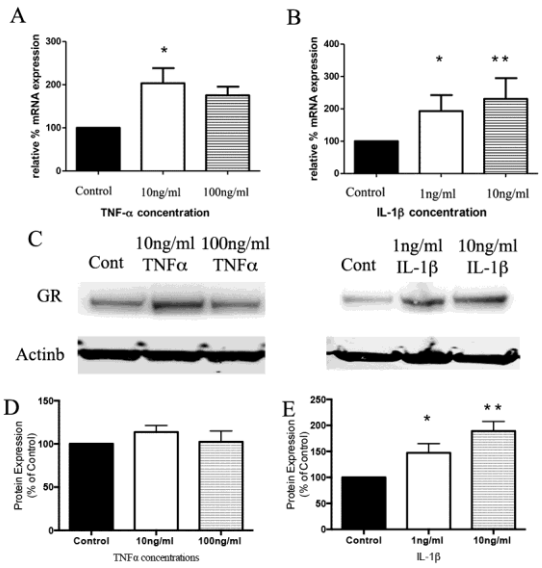
IL-1β

Figure 5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relative % mRNA expression
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200
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Control
1ng/ml
10ng/ml
IL-1β concentration
C
Cont
10ng/ml TNFα
100ng/ml TNFα
GR
Actinb
Cont
1ng/ml IL-1β
10ng/ml IL-1β
D
Protein Expression (% of Control)
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50
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Control
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TNFα concentrations
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Control
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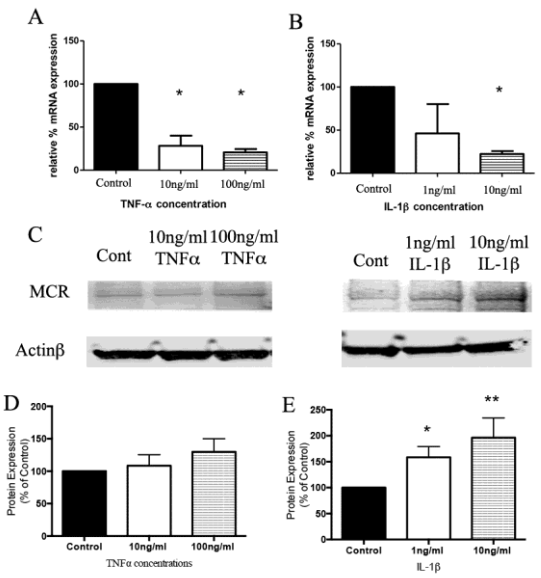
10ng/ml
IL-1β

Figure 6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100ng/ml TNFα
MCR
Actinβ
Cont
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10ng/ml IL-1β
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Protein Expression (% of Control)
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TNFα concentrations
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Protein Expression (% of Control)
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IL-1β