

IL1 α and IL4 signalling in human ovarian surface epithelial cells

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Abstract

The human ovarian surface epithelium (hOSE) is a mesothelial layer that surrounds the ovary and undergoes injury and repair cycles after ovulation-associated inflammation. We previously showed that IL4 is a key regulator of progesterone bioavailability during post-ovulatory hOSE repair as it differentially up-regulated β -HSD1 and β -HSD2 mRNA transcripts and total β -hydroxysteroid dehydrogenase activity whereas it inhibited androgen receptor (AR) expression. We now show that the pro-inflammatory effect of IL1 α on β -HSD1 expression is mediated by nuclear factor- κ B (NF- κ B), whereas its anti-inflammatory action on β -HSD2 expression is exerted via p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and NF- κ B signalling pathways. The anti-inflammatory IL4 effects on β -HSD1 and β -HSD2 mRNA expression

are mediated through STAT6 and PI3K signalling networks. IL4 effects on AR and β -HSD2 expression involve the p38 MAPK pathway. We also document that IL4 up-regulates lysyl oxidase (LOX) mRNA transcripts, a key gene for extracellular matrix (ECM) deposition and inhibits IL1 α -induced expression of cyclooxygenase-2 (COX-2) mRNA, a gene involved in breakdown of ECM, showing a further role in post-ovulatory wound healing. We conclude that IL1 α and IL4 actions in the post-ovulatory wound healing of hOSE cells are mediated by different signalling transduction pathways. The p38 MAPK signalling pathway may have possible therapeutic benefit in inflammation-associated disorders of the ovary, including cancer.

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Introduction

The human ovarian surface epithelium (hOSE) is an epithelial layer of mesodermal origin that surrounds the ovary. This compartment of the ovary is considered the main source of epithelial ovarian cancer (EOC; Auersperg *et al.* 2001). Natural reproductive events such as ovulation, accompanied by inflammation and fuelled by pre- and post-ovulatory reproductive hormones (i.e. gonadotrophins, oestrogens, androgens and progesterone) are believed to protect from or promote the development of EOC (Fathalla 1971, Espey 1994, Risch 1998, Ness & Cottle 1999). Identifying the local anti-inflammatory steroidal mechanisms that normally protect OSE at ovulation could facilitate novel molecular markers for diagnosing or treating ovarian cancer (Rae & Hillier 2005). We previously showed expression of β -hydroxysteroid dehydrogenase (β -HSD) in hOSE, the enzyme responsible for the intracrine generation of apoptotic and anti-inflammatory progesterone and cytoproliferative androgens (Papacleovoulou *et al.* 2009a). Furthermore, we showed that local biosynthesis of progesterone and androgen as well as their downstream signalling via progesterone (PR) and androgen (AR) receptors are under inflammatory control

during post-ovulatory injury and repair cycles of hOSE (Papacleovoulou *et al.* 2009b). We demonstrated differential regulation of β -HSD1 and β -HSD2 mRNA by the pro-inflammatory IL1 α , thereby resulting in a balance of total β -HSD protein and activity. AR or PR mRNA levels were not affected by this treatment. Collectively, these data were suggestive of a balance in local steroid biosynthesis and steroid action during post-ovulatory wounding (Papacleovoulou *et al.* 2009b). Therefore, IL1 α displays, as elsewhere in the body, a pleiotropic role in ovulation-associated inflammatory responses of the human ovarian cell surface (Rae *et al.* 2004b, Papacleovoulou *et al.* 2009b). In human, binding of the IL1 ligand transactivates the IL1R1 receptor, resulting in the activation of inflammatory cascades including the classic inflammatory nuclear factor- κ B (NF- κ B) pathway (Mercurio *et al.* 1997) as well as the mitogen-activated protein kinase (MAPK) pathways, namely the stress/osmotic associated protein kinase/jun N-terminal kinase and p38 MAPK signalling pathways (Freshney *et al.* 1994). Extracellular signal-regulated kinases 1 and 2 (ERK1/2), albeit mostly activated by mitogenic factors, are also activated by IL1 α in selective cases (Bird *et al.* 1991, Waterfield *et al.* 2003).

Besides the role of IL1 α in post-ovulatory wounding, we also reported that IL4 substantially induced both 3 β -HSD1 and 3 β -HSD2 mRNA expression along with total 3 β -HSD protein and activity. Moreover, IL4 attenuated AR mRNA and AR protein levels without affecting expression of PR mRNA. These data established IL4 to have a role in the alleviation of inflammation and restoration of post-ovulatory stigma with potential therapeutic advantage in malignancy (Papacleovoulou *et al.* 2009b). IL4 is a T-lymphocyte-associated cytokine that is involved in several cell immune responses with a principal role in ameliorating inflammation-associated diseases such as cancer through inhibition of Th-1 responses (Nelms *et al.* 1999, Godfrey *et al.* 2000, Nagai & Toi 2000). IL4 action is mediated through its coupling to the IL4 receptor (IL4R) which is expressed in several immune cell types as well as in epithelial cells, including the ovarian cell surface (Burke *et al.* 1996). Transactivation of IL4R commonly results in activation of phosphatidylinositol 3-kinase (PI3K), ERK1/2 MAPK or signal transducers and activators of transcription protein 6 (STAT6) signalling pathways (Sun *et al.* 1993, Reichel *et al.* 1997).

We now elaborate the signalling transduction pathways that are involved in IL1 α and IL4 effects on hOSE cells. We also report that IL4 suppresses IL1 α -induced cyclooxygenase-2 (COX-2) mRNA transcripts and induces lysyl oxidase (LOX) mRNA levels, both key genes in extracellular matrix (ECM) degeneration and deposition, respectively, further supporting its fundamental role in the repair mechanisms of the ovarian cell surface.

Materials and Methods

Subjects

Primary hOSE cells were collected from 18 pre-menopausal women (mean age 36 years) who underwent surgery for non-malignant, benign gynaecological disorders, such as fibroids, heavy menstruation and pelvic pain. Written consent to obtain tissue was provided by all patients and the Lothian Research Ethics Committee (LREC) approved the project (project numbers 05/S1103/14 and 04/S1103/36). Clinical profile of each patient is listed in Table 1.

Collection and culture of primary hOSE cells

Ovarian surface epithelial cells were brushed from the ovarian surface and cultured as described previously (Rae *et al.* 2004a, Papacleovoulou *et al.* 2009b). 'Flakes' of hOSE cells from individual patients were then inoculated into donor calf serum pre-coated T75 flasks (Corning Inc. Glass Works, Corning, NY, USA) containing MCDB 105: M199 media (1:1 v/v), 15% (v/v) FBS, 2 mM L-glutamine, 50 μ g/ml streptomycin and 50 IU/ml penicillin (all obtained from Life Technologies Inc., Sigma Chemical Co. and Cambrex, Berkshire, UK) in a humidified tissue culture incubator gassed with 95% air, 5% CO₂ at 37 °C. Medium was refreshed every 7 days until cells reached confluence (2–4 weeks). A further advantage of 2- to 4-week cell culture in FBS-containing medium before experimentation was neutralisation of any disparity of different hormonal levels among individual patients that could be encountered as a result of cell collection at different stages of

Table 1 Basic clinical profile of patients

Patient no.	Age (years)	Surgery	Reason for surgery	Cycle day	Study	Gene tested
1	43	Oophorectomy	HMB	NS	IL1 α +IL4	3 β -HSDs, AR, PR, LOX, COX-2
2	47	TAHBSO	Fibroids	NS	IL1 α +IL4	3 β -HSDs, AR, PR, LOX, COX-2
3	43	TAH	Fibroids	NS	IL1 α +IL4	3 β -HSDs, AR, PR, LOX, COX-2
4	43	TAHBSO	Fibroids	NS	IL1 α +IL4	3 β -HSDs, AR, PR, LOX, COX-2
5	22	DiagLapar	Pelvic pain	NS	IL1 α +IL4	3 β -HSDs, AR, PR, LOX, COX-2
6	46	TAH	HMB	NS	PD98059	3 β -HSDs, AR, LOX
7	40	TAH	Fibroids	NS	PD98059	3 β -HSDs, AR, LOX
8	33	DiagLapar	Mid-cycle pain	(19) Luteal	PD98059	3 β -HSDs, AR, LOX
9	42	TAHBSO	HMB	NS	SB203580	3 β -HSDs, AR, LOX
10	44	TAHBSO	Prophylactic	(21) Luteal	SB203580	3 β -HSDs, AR, LOX
11	40	DiagLapar	Pelvic pain	(15) Follicular	SB203580	3 β -HSDs, AR, LOX
12	47	TAH	Prophylactic	(22) Luteal	SB203580	3 β -HSDs, AR, LOX
9	42	TAHBSO	HMB	NS	BAY117082	3 β -HSDs, AR, LOX
10	44	TAHBSO	Prophylactic	(21) Luteal	BAY117082	3 β -HSDs, AR, LOX
11	40	DiagLapar	Pelvic pain	(15) Follicular	BAY117082	3 β -HSDs, AR, LOX
13	28	DiagLapar	Pain	(9) Follicular	BAY117082	3 β -HSDs, AR, LOX
8	33	DiagLapar	Mid-cycle pain	(19) Luteal	LY294002	3 β -HSDs, AR, LOX
14	43	LapSter	Fibroids	(21) Luteal	LY294002	3 β -HSDs, AR, LOX
15	23	DiagLapar	Dysmenorrhoea	(4) Follicular	LY294002	3 β -HSDs, AR, LOX
16	24	DiagLapar	Dysmenorrhoea	(28) Luteal	Leflunomide	3 β -HSDs, AR, LOX
17	43	TAHBSO	Fibroids	(13) Luteal	Leflunomide	3 β -HSDs, AR, LOX
18	22	DiagLapar	Pelvic Pain	(9) Follicular	Leflunomide	3 β -HSDs, AR, LOX

TAH, total abdominal hysterectomy; TAHBSO, total abdominal hysterectomy and bilateral salpingo-oophorectomy; HMB, heavy menstruation bleeding; DiagLapar, diagnostic laparoscopy; NS, not specified due to irregular cycle, follicular/luteal phases for menstrual cycles ranging from 28 to 35 days.

the menstrual cycle or different pathological conditions (Yong *et al.* 2002, Rae *et al.* 2004a). This step appeared essential, since the use of multiple patients to produce sufficient replicates for each distinct experiment allowed us to overcome restrictions in cell numbers along with limitations in long-term culture of cells from the same subject. Moreover, all the conclusions we make are based on the reproducibility of data obtained from at least three separate patients and therefore are potentially reflective of a broader *in vivo* physiology. The purity of epithelial cultures was confirmed with phase-contrast microscopy and by staining with a mouse monoclonal antibody that immunoreacts with cytokeratins 5, 6, 8 and 17 (Papacleovoulou *et al.* 2009b).

Experimental treatments

After the establishment of confluent cellular monolayers, the culture medium was removed and cells from an individual patient were washed with Dulbecco's PBS (DPBS; Sigma) and enzymatically dispersed by incubation in 5 ml trypsin/EDTA (Sigma) in Hank's balance salt solution (0.05% w/v trypsin, 0.5 mM EDTA) at 37 °C for 5 min. The resultant cell suspension was washed with 10 ml DPBS and centrifuged at 800 g for 3 min. Cell pellets were then re-suspended in 1 ml pre-warmed FBS-containing culture medium and the cell number counted using a haemocytometer. Cell viability was assessed using trypan blue (Sigma) staining exclusion criteria and ranged between 85 and 95%. Cell suspensions were adjusted to 3.5×10^5 viable cells per well (35 mm) of six-well culture plates. After establishment of a cell monolayer (24 h), the culture medium was replaced with serum-free medium containing 0.01% (w/v) BSA instead of FBS for 24 h. Based on dose- and time-dependent data previously described (Rae *et al.* 2004a, Papacleovoulou *et al.* 2009a,b), cells were exposed to IL1 α , IL4 (0.5 ng/ml; R&D Systems, Abingdon Science Park, Abingdon, UK) or both in the absence or presence of signalling pathway inhibitors (Merck Biosciences) for 48 h. Cells receiving vehicle (DMSO) served as controls.

Culture and experimental treatments of the OSE-C2 cell line

Given the limited numbers of primary hOSE cells that can be obtained, OSE-C2 cells were used as a model for signal transduction studies (Papacleovoulou *et al.* 2009a). Cells were enzymatically dispersed and seeded into six-well culture plates. Cell attachment was allowed for 24 h before serum starvation for another 24 h in culture medium with 0.01% (w/v) BSA, followed by experimental treatments with serial concentrations of IL1 α and IL4 (0.5, 5.0 and 10 ng/ml) for 0, 15, 30, 60 min and 8 h to test activation of NF- κ B and p38 MAPK signalling pathways.

Cell harvest, RNA purification and quantity analysis

After experimental treatments, the cell monolayers (six-well 35 mm plates) were washed twice with 1 ml DPBS.

Homogenisation of cells was achieved by lysis in 0.35 ml guanidine thiocyanate-containing buffer (lysis buffer, RLT; Qiagen) supplemented with 0.01% (v/v) β -mercaptoethanol (Sigma) to allow release of intracellular RNA. The resultant cell lysate was transferred to a 2 ml eppendorf tube. Lysates were then processed for RNA purification using the RNeasy Mini kit (Qiagen) as per manufacturer's guidelines. Extracted RNA was quantified using a Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies, Inc., Wilmington, DE, USA) as per supplier's instructions.

Quantitative (q) reverse transcription real-time PCR

Two-step Taqman quantitative PCR (qPCR) was performed to measure the transcription levels of human β 3-HSD1, β 3-HSD2, AR, PR, LOX and COX-2 mRNA. DNase-treated RNA (200 ng/25 μ l reaction) was reverse transcribed to cDNA using a first-strand cDNA synthesis kit (AB Applied Biosystems, Applied Biosystems, UK) with random hexamers as the priming system. cDNA (2 μ l) was used for qPCR, using commercial Applied Biosystems reagents. Each reaction was carried out in duplicate. Reaction setup followed by data analysis was performed as per manufacturer's instructions (ABI Applied Biosystems). The primers and probes for β 3-HSD1, β 3-HSD2 and LOX transcripts were purchased pre-validated (Assay-On-Demand; Applied Biosystems), with specified amplification efficiencies of 100 (\pm 10%) with the slope C_T method (-3.32). Primers for AR and PR transcripts were those described earlier (Papacleovoulou *et al.* 2009b), while the primer/probe set for COX-2 were: forward 5'-CC-TTCCTCCTGTGCCTGATG-3', reverse 5'-ACAATC-CATTTGAACAGGAAGCT-3' and probe 5'-FAM-T-GCCCGACTCCCTTGGGTGTCA-TAMRA/MGB-3' (NCBI accession number U_04636) as described previously (Rae *et al.* 2004a). These also produced consistent amplification efficiencies within the range of 100 \pm 10% in multiplex analyses with 18S. A ribosomal 18S primer/probe set that was also purchased pre-validated with the slope C_T method (-3.32 ; Assay-On-Demand; Applied Biosystems) was also included and served as an internal reference control (18S C_T values were subtracted from target gene C_T values; dC_T). In each experimental set, the mean dC_T of the control (sample with no treatments) was subtracted from the mean dC_T of each sample (ddC_T). Finally, the relative copy number between the control and each sample (fold change) was determined by the formula 2^{-ddC_T} . Mean values reflecting the PCR cycle when the target transcript started to be accumulated relative to 18S (mean dC_T in a 40 cycle PCR) for basal transcript levels were 16.4 ± 2 for β 3-HSD1, 22 ± 3 for β 3-HSD2, AR 17.4 ± 1 for AR, 21.8 ± 2 for PR, 9.1 ± 1 for LOX and 17.2 ± 2 for COX-2. Samples were evaluated in 96-well plates using an ABI Prism 7900 Sequence Detector (Applied Biosystems). The specificities of the various primer/probe sets were examined in mRNA samples from a variety of human reproductive and adrenal tissue samples and no aberrant outcomes were noted. In the case of the *HSD3B1*

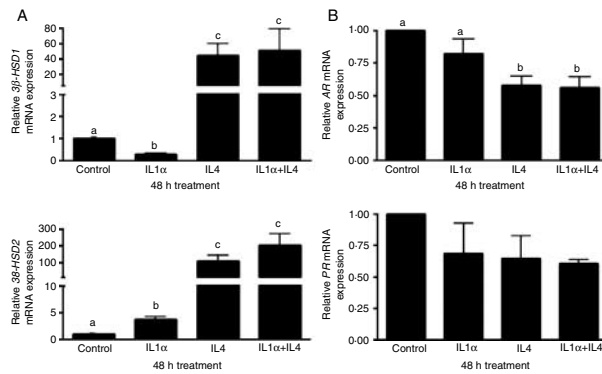


Figure 1 Effects of IL4 on steroid-related genes in IL1 α -treated hOSE cells. Primary hOSE cells were treated with IL4 (0.5 ng/ml) in the presence or absence of IL1 α (0.5 ng/ml) and quantitative real-time PCR was performed to measure 3 β -HSD1 and 3 β -HSD2 mRNA (A, b = $P < 0.05$ and c = $P < 0.001$) or AR and PR mRNA (B, b = $P < 0.05$). Combined data of cells from five patients. Data are presented as mean \pm s.e.m. Multiple measures of ANOVA were used to identify statistically significance among different treatment groups. Bars with different letter symbols denote statistically different datasets.

and *HSD3B2* sets, their specificities were assured after addition of their respective plasmid cDNAs to tissue cDNA preparations (not shown).

Western immunoblotting

OSE-C2 cell monolayers were washed with cold PBS and lysed in 150 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl pH 7.4, 1% NP40 and 10% glycerol (all from Sigma) containing a cocktail of proteinase inhibitors (Roche Diagnostics GmbH). Cell extracts were then prepared by sonication. Total protein (25 μ g) was size-fractionated by electrophoresis (12% SDS-PAGE) and transferred to PVDF membrane (Millipore, Bedford, MA, USA) followed by blocking for 2 h in 5% dried semi-skimmed milk diluted in PBS containing 0.05% Tween 20 (PBST; Sigma). After overnight incubation at 4 $^{\circ}$ C with anti-human rabbit phospho-p65, total p65, phospho-p38 and total p38 antibodies (1:1000; Santa Cruz Biotechnology, Heidelberg, Germany) diluted in 1% dried semi-skimmed milk/PBST, immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (Millipore).

Statistical analysis

All data from each experimental set were combined and presented as means \pm s.e.m. The numbers of replicate experiments are denoted by the number (n) of independent replicates (different passage numbers in the case of OSE-C2 cells and cells from separate patients in the case of primary hOSE cells) and are given in figure legends and text. Basic statistical analysis was performed by one-way ANOVA with

the GraphPad Prism 4.00 software (GraphPad Software Inc., San Diego, CA, USA). Repeated measures ANOVA and Newman-Keuls *post hoc* testing were run for multiple comparisons. To avoid any potential bias yielded by transformations through the equation 2^{-ddC_T} for the optimisation of the relative copy number of the target genes compared with the untreated control sample (that is always set at 1), all statistics were performed at the dC_T level (subtraction of the 18S C_T value from the C_T value of the target gene), thus avoiding a situation of no variability about the mean of control groups. A P value < 0.05 was taken as statistically significant in robust datasets. All P values given in the Results section reflect statistical difference relative to untreated control cells. Different superscript symbols between column bars represent significance between different treatments.

Results

Effects of IL4 on steroid-related gene regulation in IL1 α -treated hOSE cells

Effects of pro-inflammatory IL1 α and anti-inflammatory IL4 on 3 β -HSD1, 3 β -HSD2, AR and PR transcriptional regulation were tested in treated and untreated hOSE cells (Fig. 1A and B). As expected, IL4 substantially increased both 3 β -HSD1 and 3 β -HSD2 mRNA levels, whereas IL1 α attenuated 3 β -HSD1 and increased 3 β -HSD2 mRNA transcripts. However, in IL4 plus IL1 α (0.5 ng/ml) co-treated cells for 48 h, IL4 appeared to override the IL1 α response of 3 β -HSD1 and 3 β -HSD2 mRNA (Fig. 1A, upper and lower panels, respectively; $n = 5$, b = $P < 0.05$ and c = $P < 0.001$). Likewise, co-treatment did not alter IL4-attenuated AR mRNA (Fig. 1B, upper panel; $n = 5$ and b = $P < 0.05$). No effect on PR mRNA was observed with

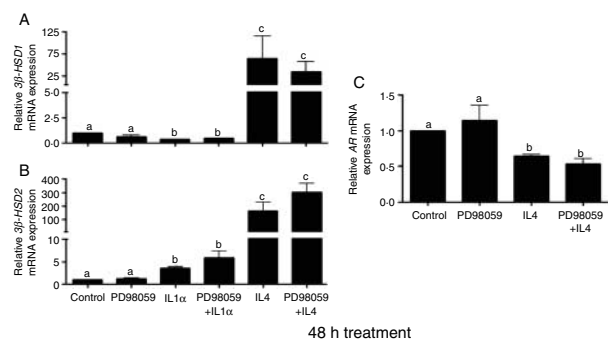


Figure 2 Effects of PD98059 on IL1 α and IL4 actions. Primary hOSE cells were treated with IL4 or IL1 α (0.5 ng/ml) in the presence or absence of 50 μ M PD98059 and quantitative real-time PCR was performed to measure 3 β -HSD1 (A), 3 β -HSD2 (B) and AR (C) mRNA. Combined data of cells from three separate patients. Data are presented as mean \pm s.e.m. Multiple measures of ANOVA were used to identify statistical significance among different treatment groups. Bars with different letter symbols denote statistically different datasets (b = $P < 0.05$ and c = $P < 0.001$).

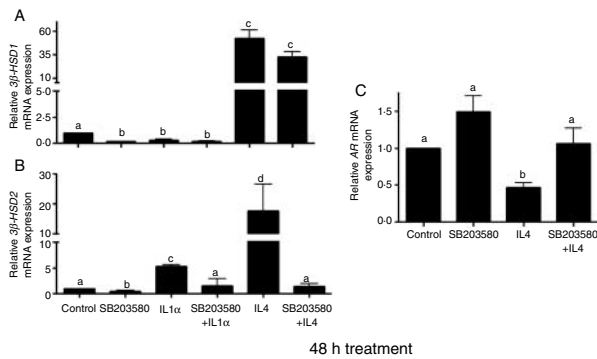


Figure 3 Effects of SB203580 on IL1 α and IL4 actions. Primary hOSE cells were treated with IL4 or IL1 α (0.5 ng/ml) in the presence or absence of 10 μ M SB203580 and quantitative real-time PCR was performed to measure 3 β -HSD1 (A), 3 β -HSD2 (B) and AR (C) mRNA (b = $P < 0.05$ and c = $P < 0.001$). Combined data of cells from three separate patients. Data are presented as mean \pm S.E.M. Multiple measures of ANOVA were used to identify statistical significance among different treatment groups. Bars with different letter symbols denote statistically different datasets.

any of the cytokines either alone or in combination (Fig. 1B, lower panel).

Effects of MAPK signalling pathways in IL1 α and IL4 mediation of 3 β -HSD and AR mRNA expression

To examine if IL1 α and IL4 exert their effects on 3 β -HSD1, 3 β -HSD2 and AR mRNA transcripts through activation of an ERK1/2 and/or a p38 MAPK-related pathways, hOSE cells were treated *in vitro* with 50 μ M of PD98059, a selective inhibitor for ERK1/2 or 10 μ M SB203580 that specifically inhibits the p38 MAPK pathway in the presence or absence of 0.5 ng/ml of IL1 α or IL4 for 48 h (Bazuine *et al.* 2005, Moon *et al.* 2007).

Suppression of the ERK1/2 pathway did not affect IL1 α -mediated (b = $P < 0.05$) or IL4-elevated 3 β -HSD1 and 3 β -HSD2 mRNA levels (c = $P < 0.001$; Fig. 2A and B respectively). The effects of the cytokines on 3 β -HSD1, 3 β -HSD2 and AR mRNA levels did not appear to involve ERK1/2 signalling pathways. Moreover, the same inhibitor did not affect the IL4-attenuated AR mRNA (Fig. 2C; b = $P < 0.05$). Intriguingly, addition of the p38 MAPK inhibitor to hOSE cells did not further inhibit IL1 α -decreased 3 β -HSD1 mRNA levels and it did not affect the IL4-increased levels of 3 β -HSD1 mRNA (Fig. 3A; n = 4, b = $P < 0.05$ and c = $P < 0.001$). Addition of the SB203580 p38 MAPK inhibitor significantly suppressed 3 β -HSD1 and 3 β -HSD2 mRNA. When added in combination with IL1 α and IL4, induction of 3 β -HSD2 mRNA levels (0.5 ng/ml) for 48 h were completely blocked (Fig. 3B; n = 4, c = $P < 0.05$ and d = $P < 0.001$), suggesting that p38 MAPK is indispensable for 3 β -HSD2 but not 3 β -HSD1 transcriptional regulation. IL4-suppression of AR mRNA was reversed when SB203580 was added in IL4-treated hOSE cells (Fig. 3C; n = 4 and b = $P < 0.05$).

The role of the NF- κ B signalling pathway in IL1 α and IL4 mediation of 3 β -HSD and AR mRNA expression

Considering that IL1 α responses are commonly mediated by NF- κ B-associated inflammatory pathways and given that IL1 α plus IL4 (0.5 ng/ml) did not have an additive effect on the transcriptional regulation of 3 β -HSD1, 3 β -HSD2 and AR mRNA levels (Fig. 1), we tested the role of NF- κ B pathway in IL1 α and IL4 responses in hOSE cells. Effects of IL1 α on 3 β -HSD1 and 3 β -HSD2 were blocked in the presence of 1 μ M BAY117082 (Skurk *et al.* 2004), whilst IL4-induced 3 β -HSD1 and 3 β -HSD2 mRNA levels were not affected (Fig. 4A and B; n = 4, b = $P < 0.05$ and c = $P < 0.001$). The same inhibitor slightly but not significantly reversed IL4-attenuated AR mRNA (Fig. 4C; b = $P < 0.05$).

The role of the PI3K signalling pathway in IL1 α and IL4 mediation of 3 β -HSD and AR mRNA expression

To test if IL1 α and/or IL4 action involved the PI3K signalling transduction pathway to exert their effects on the transcriptional regulation of 3 β -HSD1, 3 β -HSD2 and AR mRNA expression, we suppressed PI3K signalling pathway activity with the selective inhibitor, LY294002 (10 μ M; Fig. 5; Vlahos *et al.* 1994). Stimulatory effects of IL4 (0.5 ng/ml) on 3 β -HSD1 and 3 β -HSD2 mRNA levels were partially but significantly attenuated (Fig. 5A and B, respectively; n = 3, c = $P < 0.001$ and d = $P < 0.01$). Moreover, *in vitro* addition of LY294002 completely blocked IL1 α -increased 3 β -HSD2 mRNA with no effect on IL4-attenuated AR mRNA (Fig. 5B and C, respectively; n = 3 and b = $P < 0.05$).

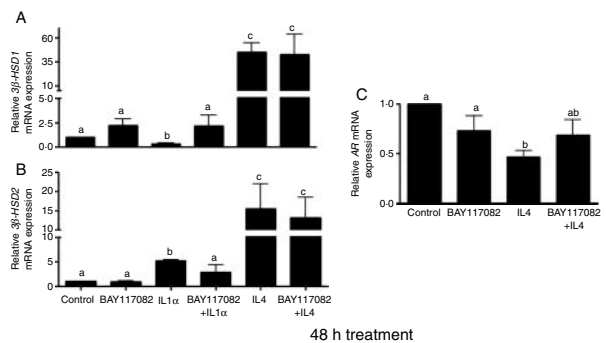


Figure 4 Effects of BAY117082 on IL1 α and IL4 actions. Primary hOSE cells were treated with IL4 or IL1 α (0.5 ng/ml) in the presence or absence of 1 μ M BAY117082 and quantitative real-time PCR was performed to measure 3 β -HSD1 (A), 3 β -HSD2 (B) and AR (C) mRNA (b = $P < 0.05$ and c = $P < 0.001$). Combined data of cells from four separate patients. Data are presented as mean \pm S.E.M. Multiple measures of ANOVA were used to identify statistical significance among different treatment groups. Bars with different letter symbols denote statistically different datasets.

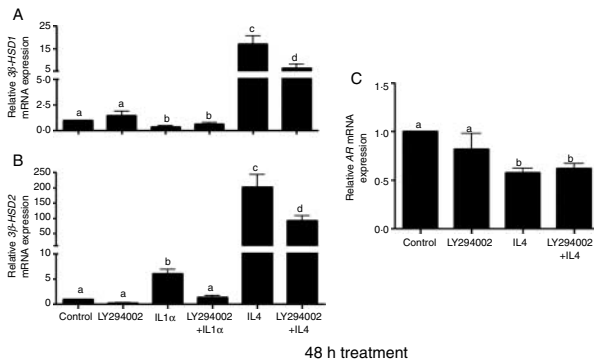


Figure 5 Effects of LY294002 on IL1 α and IL4 actions. Primary hOSE cells were treated with IL4 or IL1 α (0.5 ng/ml) in the presence or absence of 10 μ M LY294002 and quantitative real-time PCR was performed to measure *3 β -HSD1* (A), *3 β -HSD2* (B) and *AR* (C) mRNA ($b = P < 0.05$, $c = P < 0.001$ and $d = P < 0.01$). Combined data of cells from three separate patients. Data are presented as mean \pm s.e.m. Multiple measures of ANOVA were used to identify statistical significance among different treatment groups. Bars with different letter symbols denote statistically different datasets.

The role of the STAT6 signalling pathway in IL1 α and IL4 mediation of *3 β -HSD* and *AR* mRNA expression

The STAT6 pathway is ubiquitously activated by IL4 and is the major component of transcriptional regulation of IL4-responsive genes (Reichel *et al.* 1997). Therefore, we suppressed the STAT6 pathway *in vitro* by treatment of hOSE cells with the STAT6 inhibitor, leflunomide (100 μ M), in the presence or absence of IL1 α or IL4 (0.5 ng/ml) for 48 h (Akiho *et al.* 2005). Stimulatory effects of IL4 on *3 β -HSD1* and *3 β -HSD2* mRNA levels were significantly reduced (Fig. 6A and B, respectively; $n = 3$, $b = P < 0.05$, $c = P < 0.001$ and $d = P < 0.01$). On the other hand, IL4-reduction of *AR* mRNA (6C), along with IL1 α -mediated *3 β -HSD* transcripts, was not affected.

Activation of NF- κ B and p38 MAPK pathways in the OSE-C2 cell line

We previously reported that the OSE-C2 cell line responds similarly to primary hOSE cell cultures (Papacleovoulou *et al.* 2009a). Therefore, we tested potential activation of the inflammatory NF- κ B pathway in response to IL1 α and IL4 treatment on this cell line. Serial concentrations of IL1 α at several time points resulted in activation of the p65 (NF- κ B subunit) and p38 MAPK signalling pathways (Fig. 7). Phospho-p65 was activated at all IL1 α concentrations tested. Activation appeared to fade after 60 min of treatment. The p38 MAPK appeared to be activated within 15 min of IL1 α treatment but started to fade out after 60 min. On the other hand, IL4 did not affect the NF- κ B pathway, but activated the p38 MAPK signalling pathway when examined at the 15 and 30 min time points (Fig. 7).

Roles of IL4 in ECM deposition

As expected, IL1 α induced *COX-2* mRNA, whereas IL4 did not affect it (Fig. 8A, upper panel; $n = 5$ and $b = P < 0.001$). However, IL4 attenuated IL1 α -induced *COX-2* mRNA expression levels approximately twofold (Fig. 8A, upper panel, b vs c $P < 0.05$). The treatment of primary hOSE cells with IL4 also resulted in the induction of *LOX* mRNA expression (Fig. 8A, lower panel; $n = 5$ and $c = P < 0.01$). Moreover, the *LOX* mRNA increase appeared to be triggered by IL1 α treatment as well (Fig. 8A, lower panel; $n = 5$ and $b = P < 0.05$), confirming previous studies (Rae *et al.* 2004b). Nevertheless, IL4-induced *LOX* mRNA expression was more profound than that occurring with IL1 α treatment. Importantly, p38 MAPK signalling appeared to mediate *LOX* mRNA transcriptional levels by IL4 and IL1 α (Fig. 8B, upper panel; $n = 4$, $b = P < 0.05$ and $c = P < 0.01$), whereas NF- κ B signalling pathway was not involved in IL1 α -stimulated *LOX* mRNA expression (data not shown). Besides, the p38 MAPK signalling pathway, IL4-induced *LOX* mRNA was shown to be PI3K dependent (Fig. 8B, lower panel; $n = 3$, b , $c = P < 0.05$ and $d = P < 0.01$).

Discussion

Herein, we show a panel of signalling transduction pathways that are potentially involved in the effects of IL1 α and IL4 on *3 β -HSD1* and *3 β -HSD2* transcripts. We also show that besides the role of IL4 in sustaining progesterone bioavailability and downstream action in hOSE cells (Papacleovoulou *et al.* 2009b), IL4 is also essential for *COX-2* and *LOX* mRNA regulation, genes that are essential for ECM breakdown and deposition.

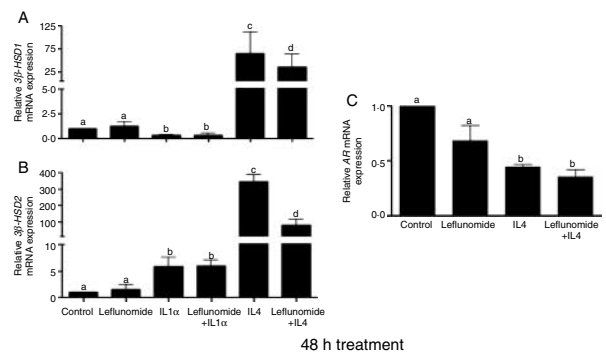


Figure 6 Effects of leflunomide on IL1 α and IL4 actions. Primary hOSE cells were treated with IL4 or IL1 α (0.5 ng/ml) in the presence or absence of 100 μ M leflunomide and quantitative real-time PCR was performed to measure *3 β -HSD1* (A), *3 β -HSD2* (B) and *AR* (C) mRNA ($b = P < 0.05$, $c = P < 0.001$ and $d = P < 0.01$). Combined data of cells from three separate patients. Data are presented as mean \pm s.e.m. Multiple measures of ANOVA were used to identify statistical significance among different treatment groups. Bars with different letter symbols denote statistically different datasets.

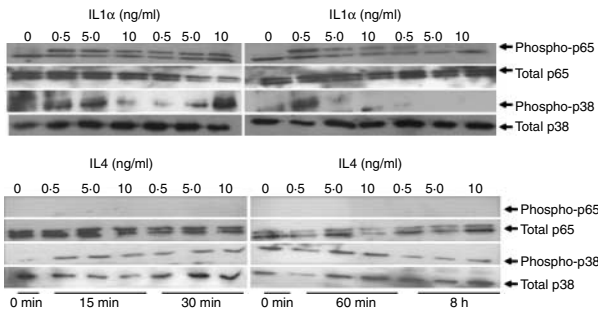


Figure 7 Activation of phospho-p65 and phospho-p38 MAPK pathways in the OSE-C2 cell line. Representative immunoblotting of dose- and time-dependent activation of the NF- κ B and p38 MAPK pathway in response to IL1 α (0.5, 1 and 10 ng/ml) (upper panel) and IL4 (0.5, 1 and 10 ng/ml) (lower panel) in OSE-C2 cells. Protein was harvested by stimulated cell monolayers and was immunoblotted for the aforementioned proteins to assess activation of the pathways in response to cytokines.

Involvement of the p38 MAPK signalling pathway in the transcriptional regulation of 3 β -HSD by IL1 α and IL4 is a novel finding in the documented record of 3 β -HSD regulation in a variety of tissues (Rainey *et al.* 1994, Leers-Sucheta *et al.* 1997, Gingras *et al.* 2000, Peng *et al.* 2004). However, this effect is consistent with p38 MAPK involvement in cell death and cell apoptosis. Our data are suggestive that, at least in hOSE cells, IL1 α -induced p38 MAPK triggers apoptotic effects through induction of transcriptional activity of apoptotic-associated genes such as 3 β -HSD1 and 3 β -HSD2 mRNA. Physiologically, follicular rupture is followed by sloughing of the ovarian cell surface, a process that is considered to be mediated by apoptosis (Murdoch 1995). Accordingly, most of the IL1-regulated genes that involve p38 MAPK are proteolytic (e.g. t-PA), ECM-related (e.g. gelatinase; Funakoshi *et al.* 2001) or pro-inflammatory (e.g. COX-2; Ogata *et al.* 2007). Intriguingly, mRNA expression of these genes is altered in post-ovulatory inflammation and tissue remodelling of hOSE (Murdoch 1999, Rae *et al.* 2004a, Gubbay *et al.* 2005). Besides, the pro-inflammatory-related genes mediated by IL1 α -induced p38 MAPK, herein we show that transcriptional regulation of anti-inflammatory and apoptotic genes such as 3 β -HSDs is also possible, potentially reflecting a negative feedback loop mechanism of IL1 α action through which hOSE recovers from tissue damage. Further support of this concept is the fact that IL1 α massively up-regulates 11 β -HSD1 mRNA and activity in hOSE, thereby sustaining local anti-inflammatory glucocorticoid regeneration to counteract hOSE post-ovulatory damage (Yong *et al.* 2002). Remarkably, IL1 α -induction of 11 β -HSD1 mRNA is also reversed by the SB203580 inhibitor (Rae MT, unpublished observations). Moreover, the p38 MAPK pathway appeared to be indispensable for the anti-inflammatory responses of IL4 in 3 β -HSD2 and AR transcriptional activity but not in 3 β -HSD1 mRNA expression, indicative that the same signal can result in differential regulation of target genes. This

concordance with the studies on IL1 α -induced 3 β -HSD2 mRNA indicates that transcriptional regulation of this gene involves p38 MAPK action. Interestingly, p38 MAPK is not notable as a universal signalling pathway for IL4 action but is cell type-dependent (Hunt *et al.* 2002).

The effects of the cytokines on 3 β -HSD1, 3 β -HSD2 and AR mRNA did not appear to involve ERK1/2 signalling pathways consistent with the mitogenic role of this group of MAPKs along with the proposed anti-inflammatory and pro-apoptotic roles of 3 β -HSD1 and 3 β -HSD2 in hOSE cells. On the other hand, ERK1/2 activation by FSH and HGF to trigger cytoproliferation of hOSE has been described (Choi *et al.* 2002, Gubbay *et al.* 2004).

We are the first to show that a NF- κ B pathway is involved in transduction of 3 β -HSD1 and 3 β -HSD2 mRNA by cytokines in hOSE cells treated with BAY117082. The involvement of this pathway in pro-inflammatory IL1 α effects on 3 β -HSD1 is consistent with the nature of NF- κ B responses. However, this pathway also appears to be involved in the stimulation of 3 β -HSD2 mRNA by IL1 α . IL1 α activated NF- κ B to induce 3 β -HSD2 and thus progesterone formation and action through PR could in turn abolish pro-inflammatory NF- κ B responses. In support of this, it has been proposed previously that NF- κ B and PR mutually suppress each other's activity (van der Burg & der Saag 1996).

IL4-induced 3 β -HSD1 and 3 β -HSD2 mRNA levels were not affected by BAY117082, implying that IL4 exerts its anti-inflammatory effects through antagonising IL1 α -induced

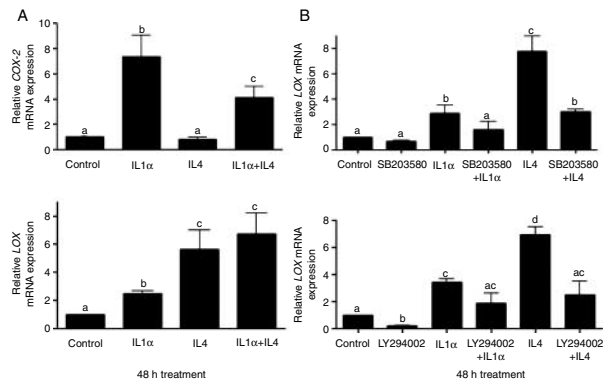


Figure 8 Effects of IL4 on IL1 α -induced COX-2 and LOX mRNA levels in primary hOSE cells. (A) Primary hOSE cells were treated with IL4 or IL1 α (0.5 ng/ml) and quantitative real-time PCR was performed to measure COX-2 (upper panel) or LOX (lower panel) mRNA (b = $P < 0.05$, c = $P < 0.001$ and d = $P < 0.01$). Combined data of hOSE cells from five patients. Data are presented as mean + s.e.m. Bars with different letter symbols denote statistically different datasets. (B) Primary hOSE cells were treated with IL4 or IL1 α (0.5 ng/ml) in the presence or absence of 10 μ M SB203580 (upper panel) or LY294002 (lower panel) and quantitative real-time PCR was performed to measure LOX mRNA (b, c = $P < 0.05$ and d = $P < 0.01$). Combined data of hOSE cells from three patients. Data are presented as mean + s.e.m. Multiple measures of ANOVA were used to identify statistical significance among different treatment groups. Bars with different letter symbols denote statistically different datasets.

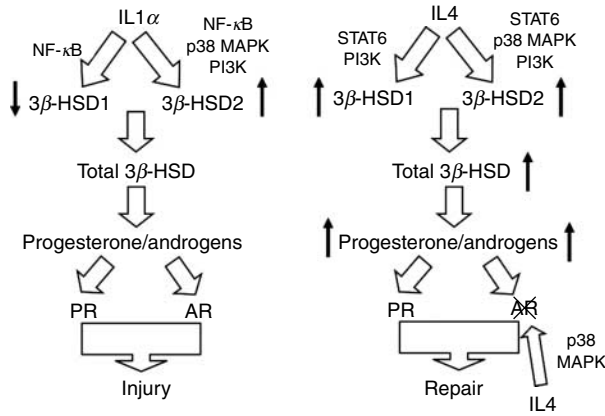


Figure 9 Regulation of steroid signalling by IL1 α and IL4 in the human ovarian surface epithelium. Schematic illustration of the proposed model and mechanisms regarding the role of IL1 α and IL4 in steroid biosynthesis and downstream signalling through the cognate receptors during post-ovulatory injury and repair cycles of hOSE. The p38 MAPK signalling pathway appears essential for anti-inflammatory actions of IL1 α on 3 β -HSD2 and IL4 on 3 β -HSD2 and AR.

pro-inflammatory effects. IL4 anti-inflammatory activities appear to be achieved through inhibition of the pro-inflammatory NF- κ B responses and activation of pro-apoptotic p38 MAPK pathway. This is consistent with our findings in OSE-C2 cells, a cell line that was previously shown to behave similarly to primary hOSE cells (Papacleovoulou *et al.* 2009a), where IL4 treatment could induce phospho-p38 MAPK but not the phospho-p65 signalling pathway.

IL1 α -induced 3 β -HSD2 mRNA levels were also found to be a result of activation of the PI3K signalling pathway. Although the PI3K pathway is not the most common target of IL1R1 transactivation, recent studies in rat Sertoli cells suggest involvement of this pathway in IL1 signalling cascades (Riera *et al.* 2007). In addition, the PI3K pathway appeared to be a component in the effects of IL4 on 3 β -HSD1 and 3 β -HSD2 mRNA. This pathway is mainly involved in mitogenic activities of IL4, since it usually activates the *AKT* proto-oncogene that sustains cell proliferation and survival and blocks apoptosis (Franke *et al.* 1997). In support of this, IL4 is mainly secreted during the luteal phase of the menstrual cycle when ovarian tissue remodelling and repair of the stigma take place (Papacleovoulou *et al.* 2009b). Physiologically, IL4-stimulated 3 β -HSDs through PI3K activation to induce local generation of progesterone in hOSE might be a mechanism through which IL4 monitors controlled proliferation of only integral epithelial cells. As such, genetically damaged cells undergo progesterone-associated apoptosis during post-ovulatory repair. Also, in breast cancer cell lines, PI3K signalling pathways were demonstrated to be an intermediate component of IL4-induced 3 β -HSD activity (Gingras *et al.* 2000). On the other hand, impaired PI3K signalling at post-ovulatory repair might lead to dysfunction of transcription of PI3K-related genes. This could have profound implications in

the aetiology of ovarian cancer as links between amplification of PI3K components and EOC have been described (Mills *et al.* 2001, Wong *et al.* 2001).

The STAT6 pathway is ubiquitously activated by IL4 and is the major component of transcriptional regulation of IL4-responsive genes (Reichel *et al.* 1997). It is not clear from the present data if the multiple signalling pathways that participate in IL4 responses are parallel or complementary. Thus, STAT6 transactivation might involve PI3K activity as seen in the case of 3 β -HSD1 or even p38 MAPK as seen in the case of 3 β -HSD2 mRNA.

Surprisingly, the STAT6 and PI3K signalling pathways that are commonly activated by IL4 did not appear to be involved in IL4-attenuated AR mRNA expression. Despite the fact that IL4 can influence the regulation of AR not only in hOSE cells, but also in other cell systems, there is no evidence of STAT6 involvement in these effects and there are also no reports of STAT6 recognition sites in the promoter of AR gene (Lee *et al.* 2003). A summary of the effects of the various inhibitors is depicted in Fig. 9.

In the presence of IL1 α , IL4 attenuated IL1 α -induced COX-2 mRNA expression levels approximately twofold, suggesting that IL4 inhibited prostaglandin synthesis during post-ovulatory repair, alleviating the degradation of connective tissue. At the same time, IL4 massively induced 3 β -HSD mRNA and 3 β -HSD protein and activity and thus capacity of local progesterone biosynthesis (Papacleovoulou *et al.* 2009b). Progesterone has also been documented to impede IL1 α -stimulation of COX-2 mRNA levels (Rae *et al.* 2004a). It seems that IL4 directly impacts upon IL1 α -stimulated COX-2 mRNA levels, but at the same time it promotes intracrine

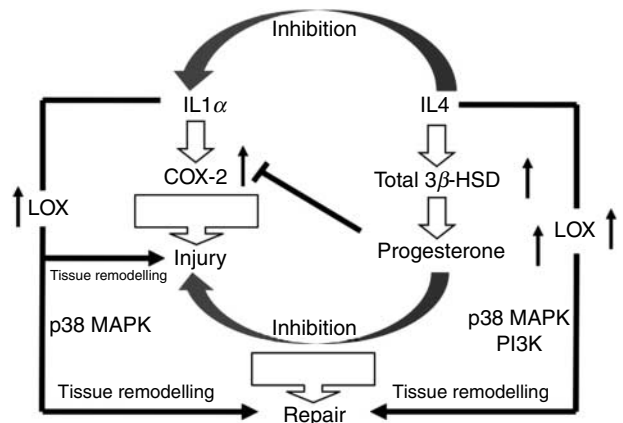


Figure 10 Role of IL4 in tissue remodelling of hOSE. This figure illustrates schematically the effects of IL4 on COX-2 and LOX levels in the presence or absence of IL1 α . Similarly to progesterone, IL4 attenuates IL1 α -induced COX-2. Concomitant IL4-stimulated progesterone biosynthesis might further enhance this inhibitory effect, suggestive of a positive feedback loop mechanism. IL4-stimulated LOX further supports its fundamental role in connective tissue deposition during post-ovulatory repair of hOSE. IL1 α and IL4-mediated LOX appears to be mediated by p38 MAPK and in the case of IL4 a crosstalk with PI3K was also shown.

generation of progesterone (through induction of 3 β -HSD) that in turn suppresses IL1 α -increased COX-2 mRNA expression (Rae *et al.* 2004a), indicative of a potential positive feedback loop mechanism.

The treatment of primary hOSE cells with IL4 also resulted in the induction of LOX mRNA expression. Proper transcriptional and translational regulation of LOX is fundamental for the deposition of ECM and the integral construction of the hOSE cell layer. Moreover, the LOX mRNA increase appeared to be triggered by IL1 α treatment as well, confirming previous studies (Rae *et al.* 2004b). Nevertheless, IL4-induced LOX expression was more profound than that occurring with IL1 α treatment, suggesting that LOX is more essential during post-ovulatory repair of the stigma. Importantly, combined effects of both cytokines were not additive, confirming our hypothesis of IL4-mediated inhibition of IL1 α pro-inflammatory responses. In physiology, LOX mRNA expression in response to IL1 α at the apex of ovulation is probably essential for minimisation of the injury of the inflamed tissue; however, a profound elevation during post-ovulatory repair secures regeneration of the ovarian cell surface on a fully structured connective tissue. This is further supported by the finding that IL4 abolished IL1 α -elevated COX-2 mRNA expression levels that potentially reflects an attenuated synthesis of prostaglandins that participate in degradation of the ECM. In addition, we have shown that TGF- β 1 up-regulated LOX mRNA in OSE-C2 cells (Papacleovoulou G, Critchley HOD, Hillier SG, Mason JI, unpublished observations, 2007), consistent with the anti-proliferative effects of TGF- β 1 in hOSE cells (Choi *et al.* 2001). Accordingly, LOX has been demonstrated to be elevated after ovulation in perch (Langenau, *et al.* 1999), as well as in final follicle maturation in the rat ovary (Harlow, *et al.* 2003), indicating its dynamic role in post-ovulatory ovarian tissue remodelling. Collectively, all these responses further support the potential function of LOX in the early stages of wound healing processes and protection from malignant transformation/invasion. Actions of cytokines in COX-2 and LOX mRNA are summarised in Fig. 10.

Importantly, p38 MAPK signalling appeared to mediate LOX mRNA transcriptional levels by IL4 and IL1 α , once again attesting to the anti-inflammatory nature of this signalling pathway at the ovarian cell surface. Furthermore, NF- κ B signalling pathways were not involved in IL1 α -stimulated LOX mRNA expression (data not shown). This is consistent with previous studies proposing that the tumour suppressive effects of LOX are mediated through inhibition of the NF- κ B pathway and associated pro-inflammatory responses (Wu *et al.* 2007). Moreover, this agrees with our suggestion that IL4 antagonises IL1 α pro-inflammatory responses through inhibition of NF- κ B and activation of the p38 MAPK pathway. Besides the p38 MAPK signalling pathway, mediation of IL4-induced LOX mRNA by the PI3K pathway is consistent with its involvement in IL4-stimulated 3 β -HSD1 and 3 β -HSD2 mRNA expression in primary hOSE cells (Fig. 10).

In conclusion, we have demonstrated that IL1 α and IL4 utilise different panels of signalling molecules to mediate 3 β -HSD1 and 3 β -HSD2 mRNA transcription, probably explaining their differential effects on these genes. The p38 MAPK appears essential for IL4 actions on 3 β -HSD2, AR and LOX mRNA, establishing this signalling pathway as a fundamental regulator of post-ovulatory wound healing with prospective use to target inflammation-associated disorders of the ovary, including cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

G P was the primary person responsible for conducting the experiments and writing the manuscript. H O D C was the primary person who organised the collection and documentation of the clinical specimens used for the experiments. S G H obtained grant support to complete this project. J I M directed the study, obtained grant support and contributed to the writing of the manuscript.

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