**Background:** Interleukin 1 (IL1) plays an important role in the physiology of human endometrium and is recognized as a major and early embryonic signal. Tight control over the local endometrial action of this cytokine is critical for normal reproductive functions. The coordinated regulation of IL1 receptors types I and II (IL1R1 and IL1R2) and IL1 receptor antagonist (IL1RA) in endometrial cells may represent one of the principle mechanisms involved in the control of IL1 local effects. The objective of this study was to investigate the regulation of IL1Rs in human endometrial epithelial cells in response to IL1.

**Methods:** Cultures of KLE endometrial epithelial cell line and primary human endometrial epithelial cells, immunofluorescent staining, enzyme-linked immunosorbent assay, western blotting, nuclear transcription (run-on) and real-time PCR were used to investigate the expression of IL1R1, IL1R2 and IL1RA.

**Results:** Cells appeared to react to IL1 by up-regulating the expression of the signaling activating IL1R1 and to moderate in parallel IL1 effects by elevating the expression of the decoy inhibitory IL1R2 and the receptor antagonist IL1RA. Regulation of IL1R1 and IL1RA by IL1B involved gene transcription activation and that of IL1R2 involved mRNA stabilization.

**Conclusion:** Considering IL1’s immunomodulatory, proangiogenic and tissue remodeling properties, and its role as an embryonic signal, modulation of endometrial cell responsiveness to IL1 via the concomitant regulation of its own activating and inhibitory receptors and receptor antagonist may represent an important regulatory mechanism of IL1-induced physiological changes occurring in the human endometrium during the normal menstrual cycle and embryo development.

**Key words:** human endometrium / IL1 / receptors

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**Introduction**

It is now well accepted that the immune system is closely implicated in normal endometrial functions. Many of the physiological changes occurring in the human endometrium are similar to those taking place during inflammatory and reparative processes, and it is thus not surprising to find that cytokines known for their pro-inflammatory properties can be involved in many features of endometrial physiology. Cytokines and immune cells contribute to the defense mechanisms of the mucosal epithelium (Kelly et al., 2001), play a major role in endometrial tissue regeneration, growth, differentiation and shedding throughout the normal menstrual cycle and remodeling during embryonic implantation and growth (Kelly et al., 2001; Trundley and Moffett, 2004). Interleukin 1 (IL1), a major pro-inflammatory cytokine, is one of the principal cytokines that participate in endocrine and local regulation of many endometrial and reproductive functions. IL1 is known for its ability to regulate the expression of the matrix metalloproteinases (MMPs) following the withdrawal of progesterone (Kelly et al., 2001), which are implicated in the degradation of the extracellular matrix and the mechanisms underlying menstruation (Salamonsen and Woolley, 1996). IL1 was found at the feto-maternal interface and is considered to be an embryonic signal (Krussel et al., 2003; Fazleabas et al., 2004). The cytokine was shown to increase endometrial secretion of prostaglandin E2 and leukemia inhibitory factor, the expression of integrin β3 subunit (Tabibzadeh et al., 1990; Sawai et al., 1997; Simon et al., 1997) and to stimulate MMP9 activity in trophoblasts (Librach et al., 1994). Blockade of IL1R1 by IL1R antagonist (IL1RA), a natural antagonist of IL1 (Dripps et al., 1991), inhibited the implantation in mice (Simon et al., 1997),
which further stresses the important role of IL1 in implantation. IL1 has two known receptors, now designated as IL1R1 and IL1R2. Cell activation by IL1 appears to be mediated exclusively by IL1R1 and to require IL1 receptor accessory protein for cell signaling (Boraschi and Tagliabue, 2006; Jacques et al., 2006). In contrast, IL1R2 has no signaling properties, and appears to act as a ‘decoy receptor’ (Martin and Wesche, 2002; Dunne and O’Neill, 2003). The extracellular domain of the receptor can be shed from the cell surface as a soluble molecule, which is capable of capturing IL1, preventing its interaction with the functional receptor and playing an important role in the regulation of IL1 action in the inflammation sites (Colotta et al., 1993; Bossu et al., 1995; Symons et al., 1995; Boraschi and Tagliabue, 2006).

Thus, the above reported literature suggests that regulation of IL1R expression in endometrial cells may represent one of the principle mechanisms involved in the control of IL1 local effects and the modulation of endometrial cell responsiveness to IL1. The present study showed that human endometrial epithelial cells respond to IL1 by up-regulating IL1R1, IL1R2 and IL1RA in a time- and dose-dependent manner. Cells appeared to react to IL1B by augmenting the expression of the signaling activating IL1R1 and deploying in parallel counter-regulatory mechanisms by increasing membrane-bound (mb) IL1R2 expression, and by the release of soluble (s) IL1R2 and IL1RA. For IL1R1 and IL1RA this appeared to involve gene transcriptional activation, and for IL1R2, this involved increased mRNA stabilization. In view of IL1’s immunomodulatory, proangiogenic and tissue remodeling properties and its role as embryonic signal, it is quite plausible that modulation and fine-tuning of endometrial cell receptivity to IL1 represents an important mechanism of IL1-induced endometrial changes during embryo implantation, growth and development.

Materials and Methods

Tissue collection and cell culture

Endometrial biopsies were taken from women aged between 33 and 40 years (mean age ± SD, 35.5 ± 3.2 years) requesting tubal ligation. All women had normal and regular menstrual cycle. Women had not taken anti-inflammatory or hormonal medications for at least 3 months before laparoscopy. Informed consent for donation of anonymous endometrial samples was obtained before collection. Tissue samples were placed in sterile Hank’s balanced salt solution containing 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 mg/ml amphotericin B (Invitrogen Life Technologies, Burlington, ON, Canada) at 4°C and transported immediately to the laboratory. Endometrial epithelial cells were isolated and cultured according to our previously described procedure (Akoum et al., 1995).

KLE endometrial carcinoma cells, which have previously been used in studies related to endometrial cell functions (Koustiti et al., 1997; Kumar et al., 1998; Shiozawa et al., 2001; Lembessis et al., 2004), were purchased from ATCC (Manassas, VA, USA).

Primary as well as KLE endometrial cells were seeded in 12-well culture plates and cultured in Dulbecco’s modified essential medium-F12 medium (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin (Invitrogen).

Cell treatment

The culture medium was changed every 2 days until confluence. Cells were then treated with fresh, serum-free DMEM-F12 containing different concentrations of IL1B (0–10 ng/ml). After different periods of time, the culture medium was collected and kept at −20°C for sIL1R1, sIL1R2 and IL1RA measurement, and cells were recovered for RNA and protein assays and stored at −20°C.

IL1R1, IL1R2 and IL1RA enzyme-linked immunosorbent assay

Concentrations of sIL1 and sIL1R2 and IL1RA in cell culture supernatants were measured using our previously reported procedures (Kharfi and Akoum, 2001; Bellehumeur et al., 2005; Akoum et al., 2007). Briefly, IL1R1 enzyme-linked immunosorbent assay (ELISA) is based on the use of a mouse monoclonal anti-human IL1R1 antibody for coating (R&D Systems, Inc., Minneapolis, MN, USA) (500 ng/well in phosphate-buffered saline (PBS)/0.5% bovine serum albumin (BSA)), a goat polyclonal anti-human IL1R1 antibody (1 μg/ml in PBS/0.5% BSA) for detection (R&D systems), peroxidase-conjugated rabbit anti-goat immunoglobulins (Zymed Laboratories, Inc., San Francisco, CA, USA) and TMB (3,3′,5,5′-tetramethylbenzidine) (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada) as substrate for peroxidase. IL1R ELISA is based on the use of a mouse monoclonal anti-human IL1R2 antibody for capture and a goat polyclonal anti-human IL1R2 antibody for detection (R&D systems). IL1RA concentrations were measured according to a similar procedure, but with the use of a mouse monoclonal anti-human IL1RA antibody for capture (R&D systems) and a rabbit polyclonal anti-human IL1RA antibody for detection (Genzyme, Cambridge, MA, USA). The optical density was determined at 450 nm, and IL1R1, IL1R2 and IL1RA concentrations were calculated by interpolation standard curve using recombinant human (rh) sIL1R1, sIL1R2 (R&D Systems) and IL1RA (GenWay Biotech Inc., San Diego, CA, USA).

Western blot analysis

Proteins were extracted from cultured cells in a buffer containing 0.5% Triton-X100, 10 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM ethylene glycol tetraacetic acid, 2 mM ethylene diamine tetraacetic acid, 0.05% NaN3 and a mixture of anti-proteases composed of 5 μg/ml aprotinin, 63 μg/ml leupeptin and 3 mM phenylmethylsulfonylfluoride. Cell homogenate was then incubated at 4°C for 45 min under gentle shaking and centrifuged at 11 000 × g for 30 min to recover the soluble extract. Total protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad laboratories). Protein extracts (10 μg) and culture supernatants (40 μl) were then separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto 0.45 μm nitrocellulose membranes and analyzed by western blotting as described previously (Akoum et al., 2007). Briefly, IL1R1 and IL1R2 were detected using specific goat polyclonal antibodies (1:100 dilution in PBS containing 5% skim milk and 0.1% Tween 20) (blocking solution) (R&D systems), while IL1RA was detected using a specific rabbit polyclonal antibody (Genzyme). Fc-specific peroxidase-labeled rabbit anti-goat antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) (1:10 000 in blocking solution), were then used for IL1R1 and IL1R2, whereas a Fc-specific peroxidase-labeled goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) (1:15 000 in blocking solution), was used for IL1RA. ECL reagent (GE healthcare, Chalfont St Giles, UK) and exposure to Super RX films (Fuji, Tokyo, Japan) for 5–30 min for optimal detection (all bands visible but not overexposed). Membranes were stripped and rebotted with a monoclonal antibody specific to tubulin (Sigma Chemical Co., St. Louis, MO, USA) (1:5 000 dilution in PBS-0.01% Tween-20) used as internal control for protein loading and transfer. Quantification of bands was achieved by computer assisted densitometry (BioImage, Visage 110s, Genomic Solutions Inc., Ann Arbor, MI, USA).
Immunofluorescence

The same immunofluorescence procedure was applied for IL1R1, IL1R2 and IL1RA. Cells were seeded on 8-well sterile culture slides (5 × 103 cells/well) (BD Biosciences, Mississauga, ON, Canada) and cultured until confluence. Cells were then rinsed with the culture medium devoid of FBS and treated for 24 h with 0.1 ng/ml IL1B. At the end of the incubation period, cells were washed once with PBS and fixed for 15 min at room temperature with PBS/3.7% formaldehyde. After washing with PBS, cells were incubated with PBS containing 1% Triton X-100 for 15 min at room temperature, washed in PBS and incubated with a monoclonal mouse anti-human IL1R1 (R&D Systems) (10 μg/ml in PBS containing 0.2% BSA and 0.01% Tween 20 (PBS/BSA/Tween 20)), a monoclonal mouse anti-human IL1R2 (R&D Systems) (10 μg/ml in PBS/BSA/Tween 20) or a monoclonal mouse anti-human IL1R2 (R&D Systems) (20 μg/ml in PBS/BSA/Tween 20) for 1 h at room temperature in humid chamber. For controls, cells were incubated with PBS/BSA/Tween 20 only or with an equivalent concentration of mouse IgGs. After washing with PBS containing 0.1% Tween 20 and subsequently with PBS alone, coverslips were incubated for 1 h at room temperature with a biotin-conjugated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) (1:100 dilution in PBS/BSA/Tween 20). Subsequently, culture slides were washed with PBS and incubated with 1% streptavidin-fluorescein isothiocyanate in PBS/BSA/Tween 20 for 1 h at room temperature in humid chamber. After a final wash in PBS, samples were mounted in Mowiol containing 10% para-phenylenediamine (Sigma-Aldrich), an anti-fading agent, and observed under a Leica microscope (Leica Mikroskopie und Systeme, Wetzlar, Germany) equipped for fluorescence with a 100 watt UV lamp and connected to an image analysis system (ISIS; Metasystems, Altusheim, Germany).

Immunoprecipitation and SDS-page analysis

Confluent cell cultures were washed twice with cysteine-free RPMI 1640 medium and then incubated for 24 h at 37°C, 5% CO2 with 500 μl of cysteine-free RPMI 1640 medium containing different concentrations of IL1B (0–10 ng/ml) supplemented with 100 μCi of 35S-cysteine. Culture supernatants were recovered and precleared with protein G-Sepharose and goat anti-IL1R1, goat anti-IL1R2 or rabbit anti-IL1RA antibody. These antibodies do not cross-react with several cytokines that are closely related to IL1R1, IL1R2 or IL1RA. Beads were washed three times in 25 mM/l Tris–HCl buffer (pH 8.0, 150 mM/l sodium chloride, 0.05% sodium azide and 0.1% Nonidet P40) (Sigma Chemical Co). Immunoprecipitated proteins were the boiled for 5 min in 40 μl of sample buffer, electrophoresed in 15% acrylamide separating gel and exposed to BioMax MR Films (Eastman Kodak, Rochester, NY, USA) for 2 weeks.

RT–PCR

Total RNA was extracted by Trizol according to the manufacturer’s instructions. cDNA was synthesized using 100 ng of RNA and 2.5 μM random hexamers in 20 μl of a solution containing 50 mM KCl, 10 mM Tris–HCl, 5 mM MgCl2, 1 mM of each dNTP, 20 U of RNase inhibitor and 50 U of reverse transcriptase using Gene Amp PCR Core Kit (Perkin-Elmer, Foster City, CA, USA). The reaction was incubated at 25°C for 15 min, 42°C for 30 min and 99°C for 5 min. Quantitative real-time PCR was carried out using an ABI 7000 thermal cycler (Applied Biosystems, Foster city, CA, USA). Each standard PCR reaction contained 2.5 μl of RT product, 0.5 μl of each primer (final concentration, 5 pmol/l), 12.5 μl SYBR Green PCR Master Mix consisting of Taq DNA polymerase reaction buffer, dNTP mix, SYBR Green I, MgCl2 and Taq DNA polymerase. Following a 10 min denaturation at 95°C, the reactions were cycled 40 times with 15 s denaturation at 95°C and 60 s annealing at 60°C. IL1R1 primers (forward, 5′-AGAGGGAAAAACCCACAAAGG-3′; reverse, 5′-CTGG CCGGTAGCATTACAGAT-3′; amplimer size 106 bp), IL1R2 primers (forward, 5′-TGCGACCTAGTCGTCACTACT-3′; reverse, 5′-TTG CGGATGAGTAGAACG-3′; amplimer size 112 bp) and GAPDH primer (forward, 5′-CAGGGGTCTGTGTTTAACTCTGG-3′; reverse, 5′-TGGGA GGATATTGGGAACA; amplimer size 102 bp), IL1R1 primers (forward, 5′-AACAGAAGGAGACAAGGC-3′; reverse, 5′-CCTCG TCAGGATATTGTG-3′; amplimer size 149 bp) and GAPDH (forward, 5′-CAGGGGTCTGTGTTTAACTCTGG-3′; reverse, 5′-TGGGTGAA CATATTGGGAACA; amplimer size 102 bp) were designed with Primer expressTM, version 2.0 (Applied Biosystems), spanned intron-exon boundaries and were tested in duplicate, each run including a no-reverse transcription (RT) control.

RNA stability

Cells were treated with IL1B (0 or 1 ng/ml) for 12 h in DMEM:F12 without phenol-red medium. Transcription was then stopped with actinomycin D (10 μg/ml), and cells were harvested after different times of incubation with actinomycin D for RNA extraction, RT and real-time PCR.

Nuclear run-on assay

KLE cells or primary endometrial epithelial cells were treated with IL1B (0 and 1 ng/ml) for 6 or 12 h in DMEM:F12 without phenol-red medium. Isolation of nuclei, nuclear transcription and extraction of radio-labeled RNA were performed as described previously (Akoum et al., 2000). Briefly, cells were scraped in a lysis buffer containing 0.25 mol/l sucrose, 10 mM/l HEPES (pH 8.0), 10 mM/l MgCl2, 2 mM/l dithiothreitol (DTT), and 0.1% Triton X-100, and homogenized in a Dounce homogenizer (Kontes Co., Vineland, NJ, USA) on ice. Nuclei were isolated by centrifugation at 6000 × g for 5 min at 4°C and in vitro transcription was carried out at 26°C for 45 min in 200 μl reaction buffer containing 20 mM/l HEPES (pH 8.0), 5 mM/l MgCl2, 90 mM/l NaH4Cl, 0.5 mM/l MnCl2, 16% (vol/vol) glycerol, 0.04 mM/l EDTA, 2 mM/l DTT, 0.4 mM/l each of ATP, CTP, GTP and 0.25 mCi [32P]UTP (3000 Ci/mM). RNA was extracted with phenol/chloroform, precipitated with ethanol and dissolved in 850 μl hybridization buffer containing 50 mM/l PIPES (pH 7.0), 0.5 mol/l NaCl, 2 mM/l EDTA, 0.4% (wt/vol) SDS, and 33% (vol/vol) formamide. The radioactive RNA was used to probe 5 μg alkali denatured plasmid IL1R1 (gift from Dr R. Kastelein, DNAx, Palo Alto, CA, USA), IL1R2 (gift from Paola Bossù, Research Center Dompé Sa, L’Aquila, Italy), IL1RA (Origene, Rockville, MD, USA) and 28S DNAs (ATCC) immobilized on nylon membranes using a slot blot apparatus (Hoeffer, San Francisco, CA, USA). Empty plasmid vector was used as negative control. Hybridization was carried out for 3 day at 42°C using 5–10 million cpm/ml of hybridization buffer. Membranes were washed four times with 2 × SSC, 0.1% SDS at 65°C for 30 min, incubated with 10 μg/ml RNase A and 100 μg/ml proteinase K for 30 min at 37°C, respectively, washed twice again with 2 × SSC, 0.1% SDS at 65°C for 30 min and finally exposed to X-ray films (BioMax, Eastman Kodak) at –80°C.
Statistical analysis

For each result, a minimum of three experiments was carried out and all measures were performed in duplicate. Data followed a parametric distribution and were therefore expressed as means ± SEM. An unpaired t-test was used for comparing the means of two groups, and one-way analysis of variance (ANOVA) followed by the Dunnett test was used for multiple comparisons. All analyses were performed with GraphPad software, Prism 3.0 (GraphPad Software, USA). Differences were considered as statistically significant for \( P < 0.05 \).

Results

Increased expression of IL1R1 and IL1R2 protein in KLE cells following IL1B treatment

Western blot analysis of IL1R1 showed a 90 kDa band whose apparent molecular weight (MW) corresponds to the mb form of IL1R1 (mbIL1R1) as we previously reported (Bigonnesse et al., 2001), a 55 kDa band corresponding to sIL1R1 and a 45 kDa band which may correspond to degraded IL1R1 protein (Fig. 1). Western blot analysis of IL1R2 showed four major bands of 68, 63, 55 and 45 kDa. The 68 and the 45 kDa bands correspond to the reported MW of mbIL1R2 (Boraschi and Tagliabue, 2006; Akoum et al., 2007) and sIL1R2 (Akoum et al., 2007), respectively, although the 63 and the 55 kDa bands may correspond to degraded IL1R2 protein (Fig. 1). Western blot analysis of IL1RA showed one band whose apparent MW is equivalent to that of rhIL1RA (17 kDa). Naturally occurring sIL1R’s are released following natural proteolytic cleavage and shedding from the extracellular domain of mbIL1R’s (Arend et al., 1994; Fernandez-Botran et al., 1996; Orlando et al., 1997; Cui et al., 2003; Bellehumeur et al., 2005; Mantovani et al., 2007). Protein extraction from endometrial cells was performed in the presence of protease inhibitors, and the appearance of lower MW bands may result from the receptors’ cleavage and/or degradation during cell lysis and protein extraction. Pre-absorption of IL1R1, IL1R2 and IL1RA antibodies with an excess of rhIL1R1, rhIL1R2 and rhIL1RA, respectively, before incubation with blotted proteins markedly reduced the intensity of the detected

Figure 1  Effect of IL1B on mbIL1R, mbIL1R2 and IL1RA expression in KLE cells. Confluent KLE cell cultures were treated with 0.1 ng/ml IL1B for varying periods of time (0–24 h) (A) or with different concentrations of IL1B (0, 0.1, 1 and 10 ng/ml) for 12 h (B). Cells were recovered to evaluate IL1R1, IL1R2 and IL1RA protein expression in total cell protein extracts by western blot. α-Tubulin was also probed on the same membranes to ensure equal protein loading. The intensity of mbIL1R, IL1RA and corresponding α-tubulin bands was evaluated by densitometric analysis (C and D). Values were normalized to α-tubulin band intensity and expressed as % of control (ratio of normalized mbIL1R1, mbIL1R2 or IL1RA band intensity detected following treatment with IL1B to that detected following incubation with the control culture medium alone for an equivalent period of time). * \( P < 0.05 \) and ** \( P < 0.01 \) as compared with control. Data are from three different experiments. MM, minimal medium; mb, membrane-bound; s, soluble.
above-described bands, thereby demonstrating specific binding (data not shown). mbIL1R1, mbIL1R2 and IL1RA expression in KLE cells cultured without stimuli gradually decreased throughout the incubation time until 12 h and increased after 24 h (Fig. 1A). In the presence of IL1B (1 ng/ml), mbIL1R1, mbIL1R2 and IL1RA expression showed a noticeable time-dependent increase after 12 h of treatment (Fig. 1A). Culture stimulation for 12 h with various concentrations of IL1B (0–10 ng/ml) further showed a dose-dependent up-regulation of mbIL1R1, mbIL1R2 and IL1RA expression (Fig. 1B). We then performed a densitometric analysis of mbIL1Rs (90 and 68 kDa) and IL1RA. Data illustrated in Fig. 1C and D are consistent with the general pattern of the receptors’ expression that could be qualitatively seen in Fig. 1A and B. Statistical analysis showed a significant increase in the intensity of mbIL1R1 in cells treated with 1 ng/ml IL1B for 6 h ($P < 0.05$) and that of mbIL1R1, mbIL1R2 and IL1RA in cells treated with 1 ng/ml IL1B for 12 h ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively), as compared with cells incubated with the culture medium alone for an equivalent time (Fig. 1C). MbIL1R1, mbIL1R2 and IL1RA bands were also significantly more intense in cells exposed to 1 ng/ml IL1B for 12 h ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively), as compared with cells incubated with the control medium (Fig. 1D). Soluble IL1R1 was not detectable in the culture medium by ELISA (data not shown). Soluble IL1R2 concentrations in the culture supernatants increased in response to IL1B, and this increase was statistically significant after 12 and 24 h stimulation with 1 ng/ml IL1B ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 2B) and in cells exposed for 12 h to 0.1 and 1 ng/ml IL1B ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 2D). IL1R1, IL1R2 and IL1RA were not detectable by ELISA in cellular protein extracts (data not shown).

Immunocytofluorescence analysis corroborates the above described data and showed a noticeable increase in IL1R1, IL1R2 and IL1RA immunofluorescent signal in endometrial KLE cells in response to IL1B (Fig. 3).

Increased expression of IL1R1 and IL1R2 mRNA in KLE cells following IL1B treatment

The effect of IL1B on IL1R1 and IL1R2 mRNA expression in KLE cells was evaluated by quantitative real-time PCR. IL1R1 mRNA levels showed a time-dependent increase in cells exposed to IL1B, which was statistically significant after 6 ($P < 0.01$), 12 ($P < 0.001$) and 24 ($P < 0.05$) h of treatment as compared with control (cultures incubated for the same time period with the basal culture medium devoid of stimuli) (Fig. 4A). A statistically significant dose-dependent increase in IL1R1 mRNA was also seen in cultures treated with 1 ($P < 0.01$) and 10 ($P < 0.01$) ng/ml IL1B for 12 h (Fig. 4D). Also, IL1B (1 ng/ml) increased IL1R2 mRNA expression in a time-dependent manner. A significant increase in IL1R2 mRNA was detectable after 2 ($P < 0.01$), 6 ($P < 0.001$), 12 ($P < 0.001$) and 24 ($P < 0.001$) h of treatment with IL1B (Fig. 4B). IL1R2 mRNA increased as well in a dose-dependent manner in cells exposed to IL1B for

**Figure 2** Effect of IL1B on sIL1R2 release and IL1RA secretion by KLE cells.
Confluent KLE cell cultures were treated with 0.1 ng/ml IL1B for varying periods of time (0–24 h) (**A** and **B**) or with different concentrations of IL1B (0, 0.1, 1 and 10 ng/ml) for 12 h (**C** and **D**). The cultures supernatants were recovered to evaluate sIL1R2 release (**A** and **C**) and IL1RA secretion (**B** and **D**) by ELISA. Data were expressed as % of control (ratio of sIL1R2 or IL1RA concentrations detected in the presence of IL1 to those detected in the control basal culture medium for an equivalent period of time). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as compared with control. Data are from three different experiments.
12 h, reaching statistically significant levels at 1 (P ≤ 0.01) and 10 (P ≤ 0.05) ng/ml (Fig. 4E). Assessment of IL1RA mRNA levels showed a significant increase in cells treated with 1 ng/ml IL1B for 12 (P ≤ 0.01) and 24 (P ≤ 0.05) h (Fig. 4C). A dose-dependent response was also observed, as IL1RA mRNA levels were significantly increased in cells exposed for 12 h to 1 ng/ml IL1B, although only a trend for a significant increase was observed in cells exposed to 0.1 and 10 ng/ml IL1B (P = 0.06 and P = 0.09, respectively) (Fig. 4F).

**Effect of IL1B on IL1R2 and IL1RA mRNA stability and synthesis in KLE cells**

To determine whether the IL1B-mediated up-regulation of IL1R1, IL1R2 and IL1RA mRNA steady state levels in KLE cells occurred at the transcriptional and/or the posttranscriptional level, we evaluated IL1R1, IL1R2 and IL1RA mRNA stability and nuclear transcription. Our results showed no difference in IL1R1 or IL1RA mRNA stability between IL1B-treated and untreated control cells following the arrest of de novo RNA transcription by actinomycin D (10 μg/ml) (Fig. 5A and C), although a significant increase in IL1R2 mRNA stability in response to IL1B (P ≤ 0.05) (Fig. 5B) was noted. In contrast, nuclear run-on analysis showed a noticeable increase in IL1R1 and IL1RA mRNA nuclear transcription in cell exposed to IL1B, whereas a faint effect on IL1R2 mRNA nuclear transcription was observed (Fig. 5D).

**Increased IL1R1, IL1R2 and IL1RA expression in endometrial epithelial cells following IL1B treatment**

Because of the difficulty maintaining and expanding primary human endometrial epithelial cell cultures, the results showing an up-regulatory effect of IL1B on its type I and II receptors in KLE cells were then confirmed on primary endometrial epithelial cells with a limited number of conditions. These cells express IL1R1, IL1R2 and IL1RA in the endometrium as previously reported (Fukuda et al., 1995; Bigonnesse et al., 2001). Our western blot data showed a dose-dependent up-regulation of mbIL1R1, mbIL1R2 and IL1RA in response to IL1B (Fig. 6A). Densitometric analysis showed a significant increase in the intensity of the mbIL1R1 (90 kDa), mbIL1R2 (68 kDa) and IL1RA in cells treated for 12 h with 1 ng/ml IL1B (P ≤ 0.05, P ≤ 0.01 and P ≤ 0.05, respectively), as compared with cells incubated with the culture medium alone for an equivalent time (Fig. 6B). Measurement of the soluble forms of IL1Rs and IL1RA by ELISA showed a dose-dependent release of sIL1R2 and IL1RA following IL1B treatment and a statistically significant increase at 1 ng/ml (P ≤ 0.05) (Fig. 6C and D). However, no sIL1R1 was detectable in the culture medium either without or with IL1B (data not shown). Immunoprecipitation of sIL1R1, sIL1R2 and IL1RA was then performed on culture supernatants from endometrial epithelial cells which have been treated with different concentrations of IL1B (0–10 ng/ml) for 24 h in the presence of 35S-cysteine in

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**Figure 3** Immunocytofluorescence of IL1R1, IL1R2 and IL1RA in KLE cells.

Cells cultured in chamber slides were incubated for 24 h with the culture medium alone or containing 0.1 ng/ml IL1B. Detection of IL1R1 (A and C), IL1R2 (E and G) and IL1RA (I and K) was performed by immunocytochemical staining using specific monoclonal mouse antibodies. Note the increase in IL1R1 (C), IL1R2 (G) and IL1RA (K) immunofluorescent signal in KLE cells exposed to IL1B by comparison with non-stimulated cells (A, E and I, respectively). No immunofluorescence was observed in the absence of primary antibodies (B, F and J) or the presence of mouse IgGs (D, H and L) (controls). Data are representative of four different experiments.
cysteine-free culture medium. Our results showed that the newly synthesized sIL1R2 and IL1RA were markedly released from endometrial epithelial cells following treatment with IL1, whereas sIL1R1 was weakly released from these cells (Fig. 6E). A dose-dependent effect of IL1B on IL1R1, IL1R2 and IL1RA mRNA levels was also observed by real-time PCR as shown in Fig. 7, with statistically significant difference observed at 1 ng/ml (P < 0.01, P < 0.01 and P < 0.05, respectively).

Discussion

The human endometrium is a special site of cytokine production and action. Cytokines play a critical role in the dynamic processes of tissue remodeling, growth, and differentiation occurring during a normal menstrual cycle and throughout embryonic implantation and development (Kelly et al., 2001; Salamonsen et al., 2007). IL1 is one of the major cytokines involved in these complex processes (Salamonsen and Woolley, 1996; Kelly et al., 2001; Krussel et al., 2003; Fazleabas et al., 2004; Rossi et al., 2005; Herrmann-Lavoie et al., 2007). The molecule is a potent proinflammatory factor and an important player in the inflammatory-like process of immune cell invasion and tissue disintegration taking place in the endometrium during the menstrual period (Salamonsen and Woolley, 1996; Kelly et al., 2001; Rossi et al., 2005). However, owing to the wide spectrum of its biological properties, including promotion of angiogenesis, cell growth and tissue remodeling (Singer et al., 1997; Bischof et al., 2000; Krussel et al., 2003; Turzanski et al., 2004; Voronov et al., 2007; Wang et al., 2007), IL1 is recognized as a major and early embryonic signal (Krussel et al., 2003; Fazleabas et al., 2004; Strakova et al., 2005). In connection with key embryonic signals such as hCG, IL1 triggers a cascade of intricate events at the feto-maternal interface, which facilitate embryonic implantation and growth within the endometrial host tissue (Strakova et al., 2005; Herrmann-Lavoie et al., 2007). Accordingly, it is not surprising to see elaborate mechanisms operating locally at the cytokine’s target level, which tightly regulate and counter-regulate its biological effects in order to ensure normal reproductive functions. Our present study suggests that IL1B may modulate endometrial cell receptivity to its own action. Actually, endometrial epithelial cells of the KLE cell line and primary cultures appeared to react to IL1B by up-regulating the cytokine’s type 1 functional and signaling receptor, but they also appeared to up-regulate in parallel the expression of the cytokine’s decoy inhibitory receptor type 2 and receptor antagonist. It is worth mentioning that the present study’s limitation is that the majority of the work has been carried out using the KLE epithelial cell line, although some of the experiments have been repeated using primary epithelial cells. Nevertheless, our results showed a significant increase in IL1R1, IL1R2 and IL1RA protein and mRNA levels, although KLE and primary endometrial epithelial cells did not similarly respond to all IL1B doses. They further indicated that ILIRs and IL1RA were differently regulated. IL1R1 and IL1RA expression were clearly regulated at the transcriptional level as observed by run on analysis of de novo nuclear mRNA transcription.
However, IL1R2 regulation by IL1B was mainly due to an increase in IL1R2 mRNA stability, as transcriptional activation of IL1R2 gene was relatively less pronounced as detected by run-on. It is important to note that both IL1Rs, once they have been internalized after binding to IL1, follow different processing pathways (Mizel et al., 1987; Curtis et al., 1990; Bourke et al., 2003; Brissoni et al., 2006). IL1R2 is rapidly recycled back to the membrane (Bourke et al., 2003). The internalization of IL1 by IL1R2 and the surface re-expression of the receptors is thought to represent a mechanism by which IL1 is efficiently removed from inflammatory sites, suggesting that the type II IL1R not only acts as a decoy but also as a scavenger of IL1 (Bourke et al., 2003). The transcriptional up-regulation of IL1RA by IL1B and the absence of any detectable mRNA stabilization. Interestingly, the earliest detection, by comparison to IL1RA, of significant IL1B-induced elevation IL1R2 mRNA steady-state levels and sIL1R2 in the culture medium would suggest the deployment of at least two consecutive mechanisms of counter-regulation of IL1 by endometrial cells. IL1R1 and IL1R2 have opposite effects in IL1 signaling; IL1R2 is considered a decoy and scavenger receptor that inhibits the cell response to IL1, although IL1R1 is known as the effective signaling receptor that mediates cell activation by IL1 (Dinarello, 1998). The localization and the expression patterns of IL1 receptors in the endometrium throughout the menstrual cycle have been investigated (Simon et al., 1993; Bigonnesse et al., 2001; Boucher et al., 2001), but little is known about how these receptors are regulated in endometrial cells. IL1R1 expression has been shown to be up-regulated by IL1B and leptin in human endometrial cells (Simon et al., 1994; Gonzalez et al., 2003), but the mechanisms/factors involved in the regulation of IL1R2 and/or the concomitant regulation of both IL1R1 and IL1R2 in these cells remained unknown. Therefore, together with the above cited literature, the present findings would indicate that although endometrial cell receptivity to IL1 is increased in response to the cytokine, endometrial cells deploy a counter-regulatory mechanism which may help moderate and buffer the cytokine’s excessive effects. Naturally occurring sIL1Rs are produced by

Figure 5 Effect of IL1B on IL1R1, IL1R2 and IL1RA mRNA stability and gene expression in KLE cells. Confluent KLE cell cultures were stimulated with IL1B (1 ng/ml) for 12 h. Actinomycin D (10 μg/ml) was added to stop the de novo RNA synthesis, and cells were harvested after 0, 2, 4 and 8 h of incubation with actinomycin D. Total RNA was extracted and reverse transcribed. cDNA was analyzed by real-time PCR with specific primers for IL1R1, IL1R2, IL1RA and GAPDH. Levels of IL1R1, IL1R2 and IL1RA mRNA were normalized to those of GAPDH to assess the kinetics of IL1R1 (A), IL1R2 (B) and IL1RA (C) mRNA degradation. Data were expressed as % of control (ratio of IL1R1, IL1R2 or IL1RA mRNA levels found at different periods of time following the arrest of de novo mRNA transcription to that found at the time of arrest); data are from three different experiments. To evaluate IL1R1, IL1R2 and IL1RA transcriptional activation (D), confluent KLE cell cultures were stimulated with IL1B (1 ng/ml) for 6 h, cell nuclei were isolated and nuclear mRNA transcription was analyzed by nuclear run-on as described in Materials and Methods. DNA samples immobilized onto nylon membranes were as follows: lane 1, IL1R1 cDNA; lane 2, IL1R2 cDNA; lane 3, IL1RA; lane 4, 28S cDNA; and lane 5, pBluescript plasmid DNA. Radioactive transcripts were from KLE cells stimulated in MM or with 1 ng/ml IL1B. Data are representative of four different experiments.
proteolysis and shedding from the extracellular domain of mbIL1Rs (Arend et al., 1994; Fernandez-Botran et al., 1996; Orlando et al., 1997; Cui et al., 2003; Bellehumeur et al., 2005; Mantovani et al., 2007). Soluble IL1R2 has been found in body fluids and culture media (Arend et al., 1994). Both soluble and mb IL1R2 have the capability to bind to IL1, thereby preventing its interaction with IL1R1 and consequently the IL1-mediated cell activation (Boraschi and Tagliaabue, 2006). However, there are also data showing the presence of sIL1R1 in serum, plasma, synovial fluids and urine of healthy humans (Arend et al., 1994; Sporri et al., 2001; Gustot et al., 2005). Although some studies suggest an anti-inflammatory and immunosuppressive effect of sIL1R1 in vivo, other studies unexpectedly demonstrated agonistic effects, as soluble IL1R1 has been reported to enhance IL1B activity (Sporri et al., 2001). Our study showed that endometrial cells up-regulate mbIL1R1, mbIL1R2 and sIL1R2. Actually, although sIL1R2 was detectable in the culture supernatants and possibly naturally released by KLE and primary endometrial epithelial cells, sIL1R1 was not detectable in the same culture supernatants, which makes its involvement in the regulatory or the counter-regulation of IL1 activity by these cells unlikely. These data were corroborated by the analysis of the release of the de novo synthesized receptors in the culture medium following metabolic labeling. Actually, the newly synthesized sIL1R1 was faintly detected, whereas the newly synthesized sIL1R2 and IL1RA were markedly increased and in a dose-dependent manner following IL1B treatment. This suggests that sIL1R2 and IL1RA may act in an autocrine/paracrine fashion and operate within the immediate vicinity of cells to minimize the inflammatory response in the endometrium following exposure to IL1.

Figure 6 Effect of IL1B on mbIL1R1, mbIL1R2 and IL1RA expression in endometrial epithelial cells. Confluent endometrial epithelial cell cultures were treated with different concentrations of IL1B (0, 0.1, 1 and 10 ng/ml) for 12 h. Cells were recovered to evaluate IL1R1, IL1R2 and IL1RA protein expression in total cell protein extracts by Western blot (A). α-Tubulin was probed on the same membranes to ensure equal protein loading. Culture supernatants were recovered to evaluate sIL1R2 release (C) and IL1RA secretion (D) by ELISA; data were from four different endometrial samples and expressed as % of control (ratio of sIL1R2 or IL1RA concentrations detected in the presence of IL1 to those detected in the control basal culture medium for an equivalent period of 12 h). *P < 0.05, as compared with control. The intensity of mbIL1Rs, IL1RA and corresponding α-tubulin bands was evaluated by densitometric analysis (B). Values were normalized to α-tubulin band intensity and expressed as % of control (ratio of normalized mbIL1R1, mbIL1R2 or IL1RA band intensity detected following treatment with IL1B to that detected following incubation with the control culture medium alone for an equivalent period of time). *P < 0.05 and **P < 0.01 as compared with control. Data were from three different endometrial samples. sIL1R1 and sIL1R2 release and IL1RA secretion from endometrial epithelial cell cultures was also analyzed by metabolic labeling with 35S-cysteine and immunoprecipitation using a goat anti-IL1R1, goat anti-IL1R2 or rabbit anti-IL1RA antibody (E). Immunoprecipitated proteins were then analyzed by SDS-PAGE as described in Materials and Methods (representative data from three different endometrial samples); mb, membrane-bound; s, soluble.
In summary, the findings of the present study point toward possible regulatory mechanisms by which endometrial cells may react to IL1 by increasing their responsiveness to the cytokine and deploying counter-regulatory mechanisms. This may help buffer IL1 excessive effects and minimize the inflammatory response in the endometrium following exposure to this cytokine. This is of interest taking into account IL1’s major immuno-inflammatory, angiogenic, remodeling and growth promoting effects. They further indicate that the endometrial tissue possesses the appropriate regulatory mechanisms that can operate locally and maintain tight control on the local level of IL1. Disruption of such an imbalance, by embryonic signals such as hCG as shown in our previous studies may further favor IL1 effects (Herrmann-Lavoie et al., 2007), or contribute to the pathophysiology of disorders like endometriosis (Akoum et al., 2001, 2007).

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References


Figure 7 Effect of IL1B on IL1R1, IL1R2 and IL1RA mRNA expression in endometrial epithelial cells. Confluent cultures were treated with 0.1 ng/ml IL1B with different concentrations of IL1B (0, 0.1, 1 and 10 ng/ml) for 12 h. Total RNA was extracted and reverse transcribed, IL1R1, IL1R2, IL1RA and GAPDH cDNAs were amplified by Real-Time PCR as described in Materials and Methods and IL1R1 (A), IL1R2 (B) and IL1RA (C) mRNA levels were normalized to GAPDH mRNA levels. Data were from three different endometrial samples and expressed as % of control (ratio of IL1R1, IL1R2 or IL1RA mRNA levels found in cells incubated with IL1 to those found in cells incubated with the control basal culture medium for an equivalent period of 12 h). *P < 0.05 and **P < 0.01 as compared with control.
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