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Triphasic pattern in the ex vivo response of human proliferative phase endometrium to oestrogens

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Abstract

The aim of this study was to evaluate the ex vivo oestrogen responsiveness of human proliferative phase endometrium using short-term explant cultures. The effects of oestrogen (17β-E2) on proliferation and the expression of oestrogen-responsive genes known to be involved in regulating endometrial function were evaluated.

Three distinct response patterns could be distinguished: (1) the menstrual (M) phase pattern (cycle days 2–5), which is characterised by a complete lack in the proliferative response to 17β-E2, while an increased expression of AR (2.6-fold, $P < 0.01$), PR (2.7-fold, $P < 0.01$) and COX-2 (3.5-fold, $P < 0.01$) at the mRNA level was observed and a similar upregulation was also found for AR, PR and COX-2 at the protein level; (2) the early proliferative (EP) phase pattern (cycle days 6–10) with 17β-E2 enhanced proliferation in the stroma (1.7-fold, $P < 0.05$), whereas the expression of AR, PR and COX-2 were not affected at the mRNA and protein levels and ER-α mRNA and protein levels were significantly reduced by 17β-E2; (3) the late proliferative (LP) phase pattern (cycle days 11–14), which is characterised by a moderate stimulation of proliferation (1.4-fold, $P < 0.05$) and PR mRNA expression (1.7-fold, $P < 0.01$) by 17β-E2.

In conclusion, three distinct response patterns to 17β-E2 could be identified with respect to proliferation and the expression of known oestrogen-responsive genes in human proliferative phase endometrium explant cultures.
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Keywords: Steroid receptors; Steroid hormones; Maturation; Explant cultures

1. Introduction

Various model systems have been employed to accurately assess steroid responsiveness in human endometrium ex vivo. Initially, studies of steroid effects were carried out using primary cultures of either epithelium or stroma cells [1]. Even though these cultures were shown to respond to oestrogen, proliferation could not be stimulated in these cultured cells. As an alternative, cancer cell lines were used to study steroid effects [2,3]. The use of cancer cell lines, however, cannot be reliably compared to the normal in vivo situation, since these cells demonstrate aberrant biological behaviour and genomic instability, which affect cellular physiology and function in time.

It was shown that the regulation of proliferation and differentiation in the endometrium by oestrogen is largely mediated by paracrine factors produced by stroma [4,5]. Co-cultures of both stroma and epithelium [5–7] were therefore used to study steroid effects on human endometrium [6,8–11]. Even though responsiveness to oestrogens was reported, application of these co-culture systems is cumbersome and time-consuming. In contrast to these model systems, the immediate use after tissue sampling and the

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better preservation of paracrine, juxtacrine and matricrine interactions makes cultured explants behave closer to the in vivo status of the tissue compared to the other model systems.

Oestrogen responsiveness in an explant culture system has previously been reported. Oestrogen enhanced the production of PGF-2α in secretory endometrium but not in proliferative endometrium [10], whereas the MMP-suppressing effect of oestradiol plus progesterone was much more dramatic in proliferative endometria [12]. These differences can be attributed to multiple causes, i.e. endogenous steroid hormone concentrations, differences in the receptor levels, and/or the presence of steroid receptor co-activators or repressors [13].

Using cultures of human endometrium explants, we investigated the effects of oestrogen on the expression of various biologically relevant oestrogen responsive markers, i.e. Ki-67, oestrogen receptor-α (ER-α), oestrogen receptor-β (ER-β), progesterone receptor (PR), androgen receptor (AR) and cyclooxygenase-2 (COX-2) [10]. We demonstrate three distinct oestrogen response patterns in the human proliferative endometrium ex vivo.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade and were purchased from commercial suppliers. Radiolabelled [2,4,6,7,16,17-^{3}H] 17β-oestradiol (140 Ci/mmol) was purchased from Perkin-Elmer (UK). Oestradiol (17β-E2), and an oestrogen receptor antagonist (ICI 164384), were gifts from Organon Pharmaceuticals (Organon N.V., Oss, The Netherlands). Millicell-CM culture inserts (pore size of 0.4 μm, 30 mm diameter) were purchased from Millipore (France). Phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) or DMEM/Ham's F12 medium (Gibco, Life Technologies, Grand Island, NY, USA), devoid of serum, but supplemented with L-glutamine (1%), penicillin and streptomycin (1%, P/S) was used in all stages of explant preparations.

2.2. Human endometrium tissue

The study was approved by the Medical Ethical Committee of the University Hospital, Maastricht. All women gave their written consent. Human endometrium tissue was obtained from female volunteers either by aspiration biopsy using a Pipelle (Unimar Inc., Prodimed, Neuilly-Enthelle, France) or from hysterectomy specimens. All surgeries were performed for benign indications. Women were 20–45 years of age, did not receive any hormonal treatment and had normal, regular menstrual cycles of 25–35 days. In no case did women contribute more than one tissue sample. Dating of the tissue was performed first according to the established histological criteria [14] and was finely adjusted according to clinical information of the start of the last menstrual period. An experienced pathologist observed no abnormalities in the collected tissues. Endometrium tissues were collected on each day between cycle day 2 and cycle day 14 of the menstrual cycle. After tissue collection, part of the endometrium tissue was rapidly frozen in lysis buffer (Promega, USA). The rest of the tissue was transported on ice to the laboratory in DMEM/Ham's F-12 medium for culture.

2.3. Comparison of explant culture systems

Proliferative endometrial tissue ($n = 3$) was placed in a petridish on ice. Human endometrium was cut into tissue pieces of about 2 mm^3 with a sterile surgical blade and placed on either lens paper or in tissue culture inserts (24 explants/30 mm insert).

Lens paper method: Strips of lens paper were placed on stainless steel grids in 6-well-plates with the paper edges in serum-free phenol-red free DMEM (2 ml) supplemented with glutamine (Gibco), sodium pyruvate (1 mM) (Merck, UK) P/S (1%) (Gibco), fungizone (1%) (Gibco).

Tissue culture inserts: Millicell culture inserts were placed in 6-well-plates, and DMEM/Ham's F12 medium (1.2 ml) devoid of serum and phenol red was added to the lower chamber. Cultures were performed in duplicate for 24 h.

In this study the culture/incubation period was limited to 24 h. Previous experiments have shown that the tissue architecture remained intact during the first 24 h of culture [15]. The advantage of culturing in serum-free conditions is that the traces of steroid hormones present in charcoal-stripped sera cannot interfere with the experiment. Studies using CHO cells transfected with plasmids expressing ER-α and plasmids containing oestrogen responsive elements showed that double-stripped FCS still contains oestrogenic activity.

2.4. Oestradiol uptake by explants cultured on lens paper and in Millicell inserts

The efficacy of oestradiol uptake was monitored by comparing the uptake of radiolabeled oestrogen. Treatments included: [1] control (0.1% ethanol + 0.01% toluene) and [2] [2,4,6,7,16,17-^{3}H]-17β-E2 (1 nM) (Amersham Pharmacia Biotech, UK). After 24 h, the culture medium and explants were collected and stored at −20 °C for scintillation counting. Prior to scintillation counting the explants were homogenized in 200 μl Tris buffer.

2.5. Protein release by explant cultured on lens paper and in Millicell inserts

The efficacy of protein release into the culture medium was determined by analysing the release of matrix metalloproteinases (MMP) using gelatin-substrate zymography [16]. In addition, the enzyme activity released into the culture media was evaluated by measuring the total collagenase activity [17]. Briefly, the culture medium was treated with 4-aminophenylmercuric acetate (APMA, 2mM) during 2 h at 37 °C to artificially activate the latent collagenases and the

total collagenase activity was assayed using [^{3}H]-acetylated collagen as a substrate. One unit of collagenase is defined as the amount of enzyme that degrades 1 μg of soluble collagen per minute. The total collagenase activity released into the culture medium was normalised to the total amount of proteins present in the medium. Protein assay was performed as described [18].

2.6. In vitro tissue response to 17β-E2

Multiple oestrogen responsive parameters were analysed in the cultured explants. Explants were cultured for 24 h with the following treatments: control (0.1% ethanol), 17β-E2 (1 nM) or 17β-E2 (1 nM) + ICI 164384 (1 μM). The oestrogen receptor antagonist ICI 164384 was included in the experimental design to confirm that the observed oestrogen response is mediated via the oestrogen receptor.

After incubation, part of the explants was harvested for total RNA isolation, and the rest was fixed in 10% phosphate-buffered formalin for 2 h and embedded in paraffin for morphological evaluation and immunohistochemistry (IHC). The presence of ER-α, ER-β, PR, AR and COX-2 mRNA was measured by real-time PCR. Protein expression was evaluated by IHC (except for ER-β). Oestradiol effects on proliferation were evaluated by IHC for Ki-67.

2.7. RNA isolation

Total RNA from explants was isolated using the SV total RNA isolation kit (Promega, USA) according to the manufacturer's protocol, except that the concentration of DNase-1 was doubled and the incubation time extended for 15 min during the DNase treatment of the RNA in order to completely remove genomic DNA. Total RNA was eluted from the column in 50 μl RNase-free water and stored at −70 °C until further analysis. The quality of the RNA samples was determined by evaluating the 18S and 28S RNA bands under UV light, after agarose gel electrophoresis and ethidium bromide staining. A GAPDH PCR was performed to test for genomic DNA contamination of the isolated RNA.

2.8. Complementary DNA synthesis (cDNA)

Total RNA (1 μg) was incubated with random hexamers (1 μg/μl; Promega, USA) at 70 °C for 10 min. The samples were chilled on ice for 5 min. A reverse transcriptase (RT)-mix consisting of 5× RT-buffer (4 μl), 10 mM dNTP mix (1 μl) (Pharmacia, Uppsala, Sweden), 0.1 M DTT (2 μl) (Invitrogen, California, USA) and superscript II reverse transcriptase (200 U/μl) (Invitrogen, California, USA) was added and the samples were incubated at 42 °C for 1 h. Reverse transcriptase was then inactivated by heating the samples at 95 °C for 5 min. The cDNA was stored at −20 °C until further use. About 50 ng of cDNA template was used in each real-time PCR reaction.

Table 1
Sequences of the primers and probes used in the QRT-PCR analyses

Gene	Primer/probe	Sequence
ER-α	Forward	agggaagctactgtttgctcctaa
	Reverse	aagatctccaccatgccctctac
	Probe	ttgctcttggacaggaaccagggaaaat (FAM-TAMRA)
ER-β	Forward	cgacttcggaagtgttacgaagt
	Reverse	caaggcggtacccacatctc
	Probe	tggtgaagtgtggctcccggagag (FAM-TAMRA)
PR	Forward	cggacaccttgcctgaagtt
	Reverse	cagggccgagggaagagtag
	Probe	cggccatacctatctccctggacgg (FAM-TAMRA)

2.9. Real-time PCR

Human cyclophylin A (**Hs 99999904-m1**), AR (**Hs 00171172-m1**) and COX-2 (**Hs 00153133-m1**) primers and probes were purchased from Perkin-Elmer Applied Biosystems as pre-developed assays. Human cyclophylin A (**Hs 99999904-m1**) was used as an endogenous control. Primers and TaqmanTM probes for human ER-α, ER-β, and PR were designed using the Primer ExpresssTM software (Perkin-Elmer Applied Biosystems, Foster City, CA). We conducted BLASTN searches against dbEST, htgs and nr (the non-redundant set of GenBank, EMBL, and DDBJ data sequences) to confirm the gene specificity of the nucleotide sequences chosen as the primers. The nucleotide sequences of the primers and probes used in this study are shown in Table 1. Human cyclophylin A was selected as an endogenous RNA control to normalize for the differences in the amount of total RNA added to each reaction. Uncultured human endometrium tissue was used as a positive control, with the exception of the ER-α and ER-β real-time PCRs for which CHO cells transiently transfected with either ER-α or ER-β expression vectors were used as positive controls.

All PCR reactions were performed using an ABI Prism 7700 sequence detection system (Perkin-Elmer). The thermal cycling conditions comprised an initial decontamination step at 50 °C for 2 min, a denaturation step at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Experiments were performed in duplicate for each sample. Quantitative values were obtained from the threshold cycle number (C_t), at which the increase in the signal associated with exponential growth of PCR products is first detected with the ABI Prism 7700 sequence detector software (Perkin-Elmer). As the targets and cyclophylin A have similar amplification efficiencies, we used the comparative C_t method ($\Delta\Delta C_t$) to perform relative quantification of our target genes (for details, see user bulletin #2 for the ABI PRISM 7700 Sequence Detection System, available at http://www.uk1.unifreiburg.de/core/facility/tagman/user_bulletin_2.pdf.

Briefly, the difference in the number of cycles, ΔC_t, was determined as the difference between the target gene and cyclophylin A within each experiment. Next, the $\Delta\Delta C_t$ was

Table 2
Antibodies used in the immunohistochemical staining procedures

Antigen	Clone	Company	Dilution	Incubation time
ER-α	ID5	DAKO	1:50	2 h RT
PR	PgR 636	DAKO	1:500	2 h RT
AR	AR441	DAKO	1:50	2 h RT
COX-2	33	BD Biosciences	1:400	2 h RT
Ki-67	S5	Roche	1:100	O/N 4 °C

calculated between the treated and control samples within each experiment.

The fold change was calculated as $FC = 2^{-\Delta\Delta C_t}$.

2.10. Immunohistochemistry

Paraffin-embedded endometrial explants were sectioned at 5 μm. Sections were deparaffinised and rehydrated in a graded series of alcohols. For the evaluation of tissue morphology, sections were stained with haematoxylin and eosin. For immunohistochemical staining, a tissue antigen retrieval step was carried out by boiling the slides in Tris-EDTA buffer (pH 9) for 20 min. After cooling down the slides to room temperature for 15 min, the sections were washed three times with PBS, and treated with 0.3% H_2O_2 in methanol for 20 min to block endogenous peroxidase activity. After washing in PBS, the primary antibodies were applied (Table 2). Antibody binding was visualized with the ChemMate™ DAKO EnVision™ Detection Kit (DAKO, Carpinteria, CA, USA). This reagent is a peroxidase-conjugated polymer, which also carries antibodies to rabbit and mouse immunoglobulins. The reaction is visualised by the ChemMate™ DAB+ chromogen are also included in this kit. Negative controls included sections incubated with normal mouse IgGs (DAKO, USA) of the same isotype and concentration as the primary antibody. Tissue sections of uncultured proliferative endometrium were used as positive staining controls.

2.11. Evaluation of ER-α, PR and AR immunostaining

Staining was semi-quantitatively assessed using the H-score [19]. In a pilot study we determined the inter- and intra-observer variations and were <5%. Assessment of the staining was random and blind. To account for the heterogeneity within the human endometrium explants, at least two explants were evaluated per treatment. Staining intensity was assessed in three separate cohorts of 100 cells in three different high power fields (400×).

The receptor H-score was calculated as described [19]:

$$\text{Receptor score} = \sum_{i=0}^{i=3} P(i)Xi,$$

where i is the intensity of staining from 0 (no staining) to 3 (strong) and $P(i)$ the percentage of stained cells in each category. The final receptor scores were obtained by calculating the sum of the specific staining of the three cohorts. For ER-α and PR, H-scores were calculated separately for glands and stroma. For AR, the H-score was calculated only in stroma, since hardly any staining was detected in the glands.

2.12. Evaluation of COX-2 immunostaining

The COX-2 protein was localised predominantly in the cytoplasm of glandular epithelium cells. Using the Qwin image analysis software from Leica (Bensheim, Germany), the stained surface area was measured and expressed as a percentage of the total selected glandular epithelium area. This is referred to as the staining index (SI).

2.13. Evaluation of Ki-67 immunostaining

The proliferative fraction in the cultured endometrial explants was assessed by counting the number of Ki-67 positive cells. In a pilot series, we determined the inter- and intra-observer variation as <3%. A total of 900 cells were counted in three separate intact glands and 300 cells in three fields of stroma, as described [20]. Any nuclear staining was regarded as positive, regardless of the staining intensity.

2.14. Statistical analysis

Statistical tests were carried out using the SPSS 10 (SPSS Inc., Chicago, IL) statistical analysis package. For the real-time PCR data, the fold change of the 17β-E2-treated samples over the controls within each experiment was calculated. The effects of treatments on fold change in mRNA expression, proliferation and protein expression (H-scores) were analysed using the non-parametric paired Wilcoxon signed rank test for paired samples at a confidence level of 95%.

3. Results

3.1. Explant cultures using inserts or grids

Suitable model systems require efficient uptake of the compounds to be tested, and a rapid secretion of the response parameters that are to be measured into the culture medium. Two culture systems were evaluated to see whether they met these criteria: the lens paper/grid method, in which the edges of lens paper were submerged in culture medium [21], and Millicell culture inserts [15]. Steroid uptake ($n = 3$) was evaluated by determining the accumulation of ^{3}H-17β-E2 in the tissue. Total tissue uptake of ^{3}H-17β-E2 after 24 h of incubation was significantly higher (5-fold, $P < 0.05$) in explants cultured on Millicell culture inserts as compared to the explants cultured on the lens paper/grids.

Total collagenase activity in culture medium was higher when explants were placed on Millicell culture inserts compared to culturing explants on grids (2.98 versus 0.01 U/ml, $n = 1$). Gelatin zymography showed that explants cultured in Millicell culture inserts release more latent and active MMP-

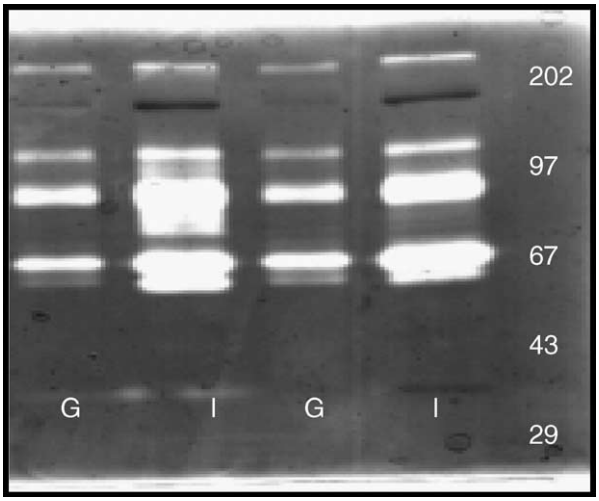

Fig. 1. Gelatin zymogram showing the presence of latent and active MMP-2 and MMP-9 in the culture medium after culturing explants using Millicell culture inserts (I) or the grid method (G). 10 μg of protein was loaded in each lane.

2 and MMP-9 ($n = 6$) as compared to explants cultured on grids (Fig. 1). Millicell culture inserts were thus used in this study.

3.2. *In vitro tissue response to 17β-E2: Cellular proliferation*

In M phase explants, 17β-E2 decreased the number of Ki-67 positive cells in stromal cells by 12% ($P < 0.05$). The decrease was more pronounced when combining ICI 164384 to 17β-E2. In EP phase explants, 17β-E2 enhanced the Ki-67 positive fraction by 75% ($P < 0.05$) in the stroma cells only. There was a tendency to induce Ki-67 expression in the glandular epithelium ($P = 0.06$). Co-incubating 17β-E2 with the pure anti-oestrogen ICI 164384 antagonised the inductive effect of 17β-E2 and even decreased the number of Ki-67 positive cells compared to controls. In LP phase explants, addition of 17β-E2 to the culture medium increased the fraction of Ki-67 positive cells in the glandular epithelial cells by 42% ($P < 0.05$, Fig. 2). Co-incubation with ICI 164384 nullified this effect.

3.3. *In vitro tissue response to 17β-E2: expression of oestrogen-regulated genes*

3.3.1. *Oestrogen receptor-α and -β*

Expression of ER-α mRNA was not affected in M phase and LP phase explants by incubating explants with 17β-E2 for 24 h, but ER-α mRNA expression was significantly reduced by 38% in EP phase explants ($P < 0.05$, Fig. 3). Co-incubating 17β-E2 with ICI 164384 reduced the expression of ER-α mRNA in M phase and slightly increased ER-α mRNA in LP phase explants compared to the 17β-E2 treated explants. In the CHO-ER-β cells the average ΔC_t was 31, when using 10 ng of cDNA in the real-time PCR analysis. However, no ER-β mRNA transcripts were detected, either in cultured or in uncultured human endometrium explants.

Nuclear staining of ER-α was observed in both the glandular and stroma cell compartments (Figs. 4 and 5). Treating EP phase explants with 17β-E2 reduced the ER-α protein expression both in the glands (38%) and stroma (31%). Co-incubating M phase explants with 17β-E2 and ICI 164384 further reduced ER-α expression in both the glandular epithelium and stroma to levels below the controls. This additional suppressing effect of ICI 164384 on ER-α was not observed in the EP and LP phase explants.

3.3.2. *Progesterone receptor*

Treating explants with 17β-E2 increased PR mRNA levels in M (2.7-fold, $P < 0.01$) and LP phase explants (1.7-fold, $P < 0.01$) (Fig. 3). These effects were counteracted when 17β-E2 was co-incubated with ICI 164384. Progesterone receptor mRNA levels were increased by 17β-E2 treatments in 70% of M phase biopsies and 45% of LP phase biopsies. Only 13% of EP phase biopsies responded to 17β-E2 treatment.

Nuclear staining of PR was observed in both the glandular and stroma cell compartments (Figs. 4 and 5). Treating explants with 17β-E2 significantly increased the PR content (1.5-fold, $P < 0.05$) both in the stroma and the glandular ep-

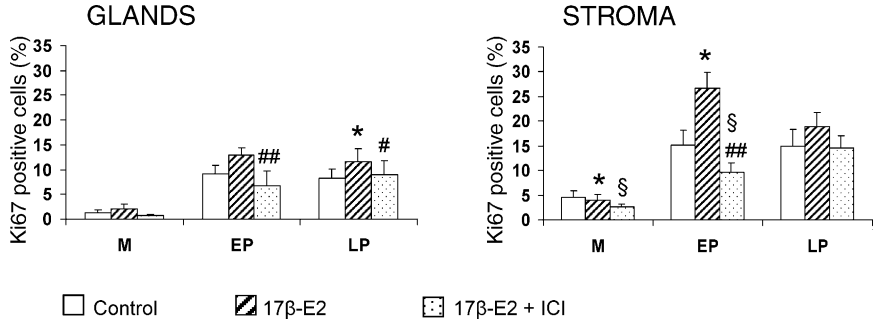

Fig. 2. Effect of 17β-E2 and the oestrogen receptor antagonist ICI 164384 on proliferation in M phase ($n = 6$), EP phase ($n = 12$), and LP phase ($n = 10$) explants as determined by the number of Ki-67 positive cells in glands and in stroma. The results are presented as the percentage of Ki-67 positive cells. Error bars represent S.E. of the mean. $^{*}P < 0.05$: control vs. 17β-E2; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$: 17β-E2 vs. 17β-E2 + ICI; $^{\S}P < 0.05$: 17β-E2 + ICI vs. control; □, Controls; ▨, 17β-E2; ⁘, 17β-E2 + ICI.

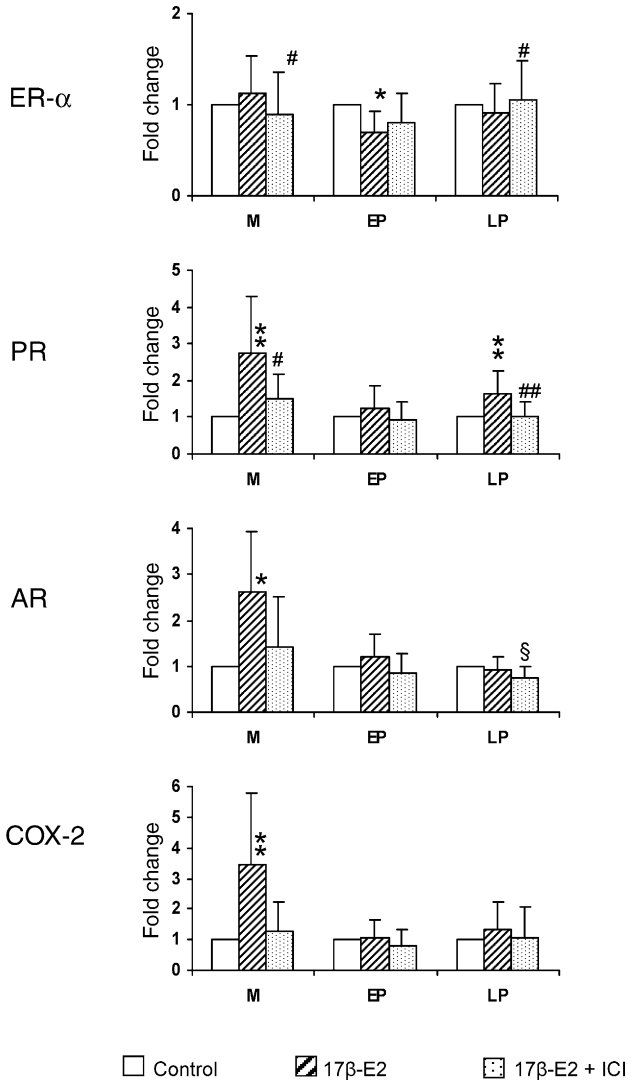

Fig. 3. Effects of 17β-E2 and the oestrogen receptor antagonist ICI 164384 on the expression of ER-α, PR, AR and COX-2 mRNA in M phase ($n = 6$), EP phase ($n = 12$), and LP ($n = 10$) phase explants as measured with real-time PCR $^{*}P < 0.05$, $^{**}P < 0.01$: control vs. 17β-17β-E2; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$: 17β-E2 vs. 17β-E2 + ICI; $^{\S}P < 0.05$: control vs. 17β-E2 + ICI; □, controls; ▨, 17β-E2; ⊡, 17β-E2 + ICI.

ithelium of M phase explants (Fig. 5). No stimulation was observed in the EP and LP phase explants. In M phase explants, co-incubation of 17β-E2 with ICI 164384 reduced PR levels to far below the control levels in both the glands and the stroma. This was not observed in the EP phase explants. In stromal cells of LP phase explants PR immunostaining was significantly lower in the presence of ICI 164384 and 17β-E2 compared to 17β-E2 alone.

3.3.3. Androgen receptor

When culturing explants in the presence of 17β-E2, the expression of AR mRNA was significantly increased (2.6-fold; $P < 0.05$) in M phase explants compared to controls (Fig. 3). In EP and LP phase explants 17β-E2 treatment did not affect AR mRNA.

AR staining was predominantly localised in the stroma with sporadic staining in the glands (Fig. 4), which agrees with other reports [20]. Immunostaining of AR in M phase biopsies was enhanced after incubation with 17β-E2, even though statistical significance was not achieved (Fig. 5). This stimulatory effect was antagonised by the addition of ICI 164384.

3.3.4. Cyclooxygenase-2

In M phase explants, 17β-E2 treatment increased COX-2 mRNA levels (3.4-fold $P < 0.01$) compared to the controls (Fig. 3). This effect was antagonised by ICI 164384. Incubating EP and LP phase explants with 17β-E2 had no effect on the expression of COX-2 mRNA levels.

Cyclooxygenase staining was predominantly located in the glandular epithelium (Fig. 4). Image analysis revealed that 17β-E2 treatment enhanced (2.1-fold, $P < 0.05$) the COX-2 staining index in the glandular epithelium of only M phase explants (Fig. 5). No changes were detected in the EP and LP phase explants. Including ICI 164384 antagonised the stimulatory effect of 17β-E2.

4. Discussion

Human proliferative phase endometrium in short-term explant culture responds to oestrogen treatment in a distinct triphasic pattern. In endometrium from the menstrual (M) phase (cycle days 2–5), 17β-oestradiol (17β-E2) had no effect on proliferation as measured by Ki-67, but the expression of AR mRNA, and PR and COX-2 mRNA and protein increased in response to 17β-E2. In contrast, endometrial tissue from the early proliferative (EP) phase (cycle days 6–10) responded to 17β-E2 with increased proliferation, the expression of the oestrogen-responsive genes was hardly affected, and the expression of ER-α was reduced both at the mRNA and protein level. In endometrium from the late proliferative (LP) phase (cycle days 11–14), 17β-E2 had little effect on proliferation and gene expression, since only PR mRNA expression was moderately enhanced. To our knowledge, the stimulation of gene expression in human menstrual endometrium cultured ex vivo as explants, the down regulation of ER-α mRNA and protein by its ligand in the EP phase, as well as the opposite effects of 17β-E2 in M versus EP phase endometrium on the regulation of proliferation and the expression of AR, PR and COX-2 has not been previously reported.

Reports on explant cultures to evaluate the oestrogen responsiveness of proliferative phase endometrium are rare, and thus far no studies have been published on the effects of oestrogen on proliferation in this model system. Short-term explant cultures have several features which are relevant for understanding the complex intra- and inter-cellular mechanisms of tissue responsiveness to oestrogen (reviewed in [13]). Essential to the short-term explant cultures is the preservation of the tissue architecture, which is a prerequisite

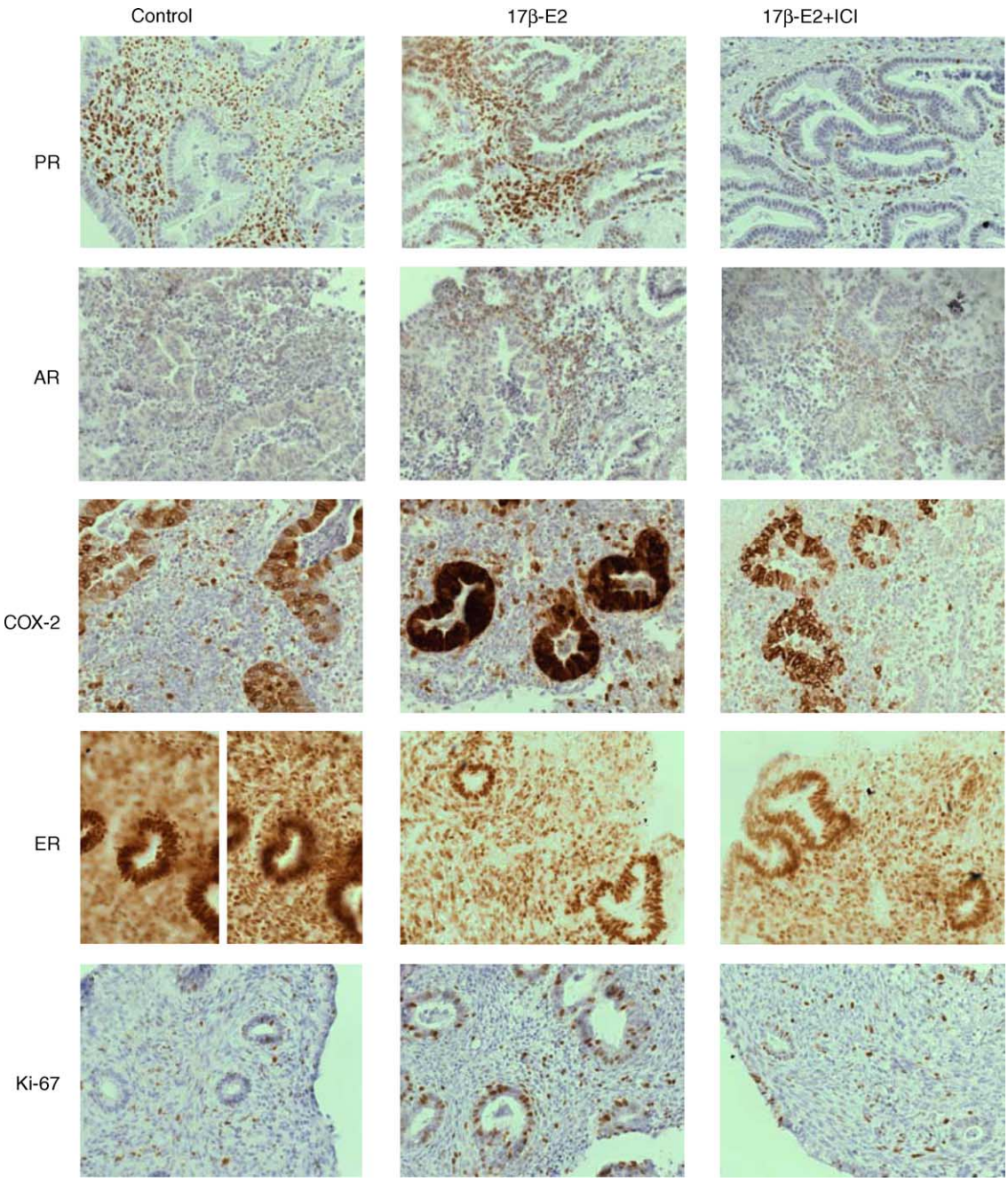

Fig. 4. Effect of 17β-E2 in the presence and absence of the oestrogen receptor antagonist ICI 164384 on the expression of ER-α, PR, AR, COX-2 and Ki-67. Representative immunohistochemical staining of explants cultured in the presence of vehicle (control), 17β-E2 and 17β-E2 + ICI. Scale bar represents 20 μm.

for the interactions between the epithelium, stroma, and extracellular matrix. Presumably the short-term in vitro response reflects the physiological in vivo state of the tissue at the time of tissue collection. After 24 h of culture under serum-free conditions the viability of the tissue is unchanged [15] and the proliferative activity remains within the physiological range [26]. From these studies it is clear that the absence of serum has no adverse effects on the viability and responsiveness of the endometrial tissue in short-term cultures.

Many studies have addressed the response of cultures of isolated stromal and epithelial cells from normal human endometrium either in separate or combined cultures [22,23]. From the studies of Pierro et al. [22], using LP phase endometrium, it became evident that 17β-E2 can stimulate proliferation in epithelial cells only in the presence of stromal cells, illustrating the importance of both cell types in controlling endometrial growth. This agrees with our finding that 17β-E2 enhanced proliferation in the glands of LP phase explants. Classen-Linke and co-workers also

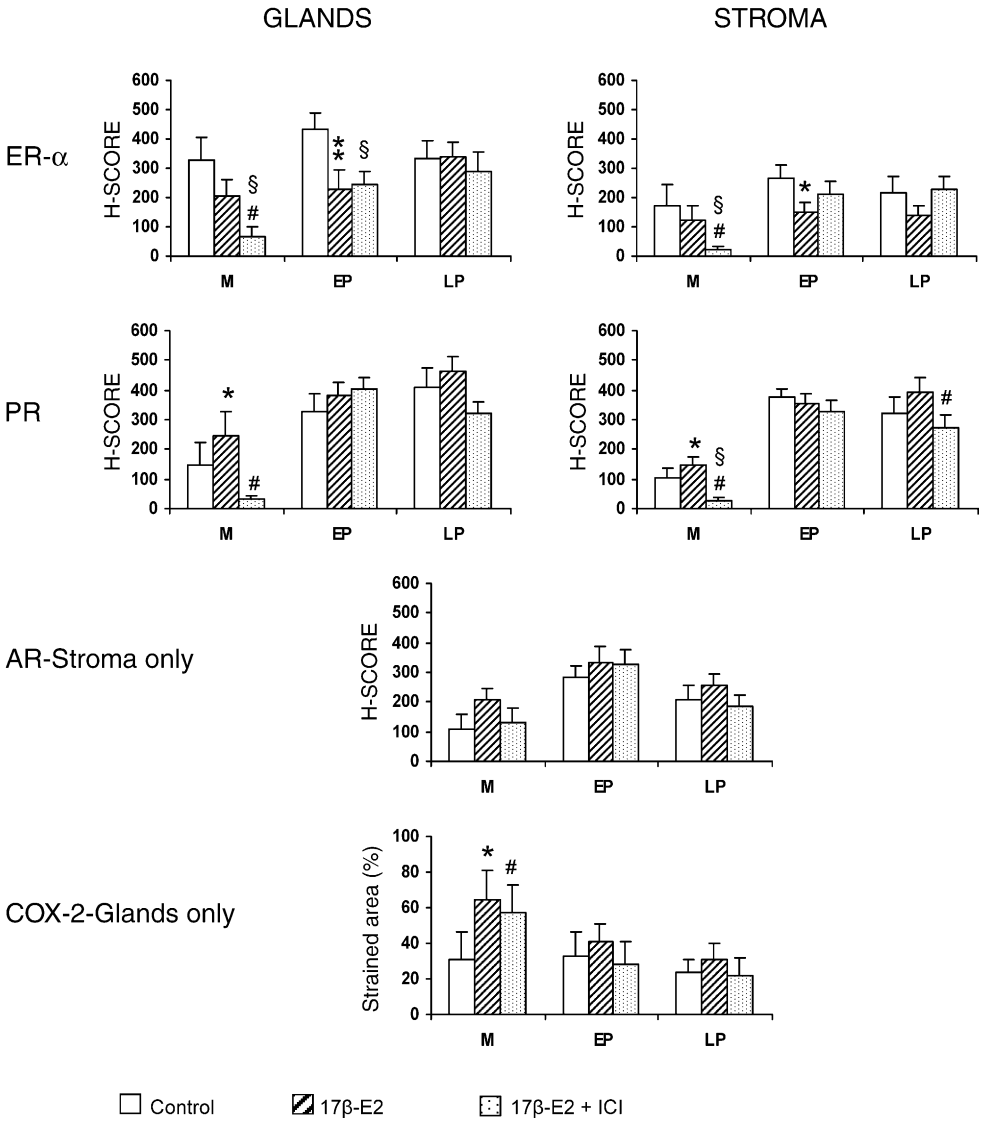

Fig. 5. Effects of 17β-E2 and the oestrogen receptor antagonist ICI 164384 on the expression of ER-α, PR, AR and COX-2 protein in M phase ($n = 6$), EP phase ($n = 12$), and LP ($n = 10$) phase explants. ER-α and PR protein expression was determined in both the glands and the stroma, AR protein was determined in the stroma, COX-2 protein expression was determined in the glandular epithelium. $^{*}P < 0.05$, $^{**}P < 0.01$: control vs. 17β-E2; $^{\#}P < 0.05$: 17β-E2 vs. 17β-E2 + ICI; $^{\S}P < 0.05$: control vs. 17β-E2 + ICI; □, controls; ▨, 17β-E2; ⊡, 17β-E2 + ICI.

showed that PR expression was enhanced in co cultures prepared from proliferative endometria treated with 17β-E2 [23]. In our study we observed little effects of 17β-E2 treatment in the EP and LP phase explants, indicating that the physical separation and extended culture periods of at least 6 days may disrupt the natural responsiveness of the tissue.

Indeed, the explants display a memory effect, i.e. the in vivo responsiveness to 17β-E2 is maintained in vitro. Oestrogens could not stimulate proliferation in endometria collected in the first 5 days of the menstrual cycle, when the surface epithelium that is damaged during menstruation is being repaired [24,25]. Only the basal layer, that contains the continuously dividing progenitor cells, is not (completely) shed during menstruation. These cells are apparently refractory to oestrogens, since the mitotic index of the basal layer remains constant during the proliferative and secretory phases [26]. Therefore, 17β-E2 will not increase proliferation in M phase tissue as found in this study. On the other hand, the daughter cells from the first cell divisions will constitute the lower functional layer and are presumably sensitive to oestrogen, since the mitotic index in this layer is increased in the mid-proliferative phase and is reduced in the luteal phase [26]. This responsiveness is apparently maintained during culture, as EP phase endometrial explants, predominantly consisting of functional layer tissue, were highly responsive to 17β-E2. The response of LP endometrium to 17β-E2 in vitro was slightly reduced compared to the response of EP phase endometrium. The mitotic index in vivo, was also shown to decrease after cycle day 10 [26]. This is presumably due to

prolonged exposure to oestrogen, which leads to inhibition of cell growth [27,28].

The most striking finding in this study is the distinct patterns in the M, EP and LP phases and the opposite effect of 17β-E2 on proliferation and gene expression. In order to clarify these differences in oestrogen responsiveness, the possible mediators that can affect oestrogen responsiveness in a cell, such as differences in tissue levels of steroid hormones and/or steroid hormone receptors, as well as the presence of intra-cellular regulatory (co)factors, will be discussed.

In this study endogenous tissue oestradiol concentrations were not measured, however, there are clear indications that the influence of local tissue concentrations on the responsiveness of the tissue in vitro can be neglected. Tissue concentrations throughout the proliferative phase and in postmenopausal endometria were shown to remain in the nM concentration range [29,30]. This suggests that tissue levels do not vary as dramatically as the plasma levels. In the uptake study we demonstrated that after incubation, the tissue accumulated a concentration of about 2.4 nM radiolabeled 17β-E2, which is similar to the physiological tissue concentrations [29]. Compared to the lens paper method, the Millicell culture system allows a much more efficient exchange of steroid hormones and secretory proteins; approximately 10% of the specific radioactivity was taken up by the explants compared to explants cultured on lens paper. Since the concentration in the medium was 1 nM, and the volume in the well 1.2 ml (1.2 pmol), approximately 0.12 pmol must have accumulated in the tissue. Assuming the 24 explants of approximately 2 mm^3 (μl) present in each insert have an approximate volume of 50 μl, the tissue concentration will be about 2.4 nM. Based on these observations it is not likely that variations in the concentration of endogenous 17β-E2 accounts for the opposite responses observed in M and EP phase endometria.

The responsiveness to 17β-E2 is largely determined by the intra-cellular oestrogen receptor activities. In vivo, the proliferation index increases parallel to the increases in ER levels [26,31]. In vitro, ER protein levels and basal proliferation activity were also increased in EP phase explant controls compared to controls of M phase endometria (30% and 500%, respectively). Surprisingly, 17β-E2 reduced ER-α levels in the EP phase explants, but enhanced proliferation. This underlines that the enhanced proliferative response cannot be attributed to the ER levels alone.

Oestrogen regulation of gene expression in primary cell cultures in different cell types has been extensively studied, but in explant cultures of proliferative endometrium, only one study reports induction of PR by 17β-E2 [22]. The production of PGF-2α could not be stimulated in explant cultures of human proliferative endometrium [1]. Similarly, in our study COX-2 expression in EP and LP phase explants was not stimulated by 17β-E2 treatment, whereas in M phase endometrium 17β-E2 significantly enhanced COX-2 expression. The mutual exclusive stimulation of proliferation and the expression of AR, PR and COX-2 by 17β-E2, illustrates that in human endometrium the regulation of cell growth and the acquisition of differentiated functions are inter-related. This requires a tight control of ER-α regulated gene expression, which depends on the presence and action of co-activators and co-repressors [32]. In human endometrium the expression of the steroid receptor co-activator (SRC-1, [32]), cAMP response element binding protein (CREB, [33]), the nuclear receptor co-repressor (N-CoR, [34]), and the silencing mediator of retinoid and thyroid receptors (SMRT, [34]) has previously been described. The expression of some of these cofactors shows cyclical variation. It is particularly interesting that the expression of SRC-1 and N-CoR mRNA is elevated in the M phase endometrium and is decreased in the proliferative and secretory endometrium [34]. In contrast, Shiozawa and co-workers demonstrated that the changes of SRC-1 during the menstrual cycle resemble the changes in the expression levels of PR and Ki-67. The exact mechanisms by which these co-regulators modulate the responsiveness of the endometrial cells remain obscure, however.

The effects of 17β-E2 were antagonized by the ER antagonist ICI 164384, which indicates that the effects are mediated by ER binding. In contrast to other studies, ER-β mRNA transcripts were not detected with real-time PCR in both cultured and uncultured explants [35,36]. Therefore, it is not likely that ER-β is involved in the regulation of proliferation and the expression of the markers studied in this research.

The antagonizing actions of ICI 164384 appear to involve more than just competition with 17β-E2 for binding to ER. The antagonizing effect of ICI 164384 on ER and PR expression was frequently of such magnitude that expression levels were significantly lower than those for the controls. Why this was limited to the M phase explants only remains unclear. It is tempting to suggest that the inhibitory effect of ICI 164384 causes rapid degradation of ER and PR protein. This is indicated by recent studies, showing that treatment with ICI 164384 resulted in a rapid loss of ER from 17β-E2-sensitive cells [37] and uterine tissue of rats [38], due to a decrease in the half-life of the ER protein (from 5 h to 1 h) [37]. Degradation of mRNA was not observed. Our findings also support absence of ER-α mRNA degradation.

In conclusion, human endometrium in short-term culture is responsive to oestrogens with respect to proliferation and the expression of known oestrogen-responsive genes. Three phases with distinct response patterns could be identified: (1) a menstrual phase in which 17β-E2 induces the expression of various 17β-E2 responsive genes, but does not induce proliferation; (2) an early proliferative phase in which the endometrium is highly responsive with respect to proliferation, but not the expression of 17β-E2 -regulated genes; (3) a late proliferative phase, or pre-ovulatory phase in which proliferation and the expression of 17β-E2-responsive genes is moderately enhanced. The in vitro proliferative response to 17β-E2 mimics the in vivo responses of the endometrium, which indicates that the in vivo tissue architecture and microenvironment are preserved in the cultured explants

allowing adequate interaction between the various cell types and the extracellular matrix.

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