Characterization of endothelin-1 and nitric oxide generating systems in corpus luteum-derived endothelial cells

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Abstract

Endothelium-derived endothelin-1 (ET-1) and nitric oxide (NO) are pivotal regulators of corpus luteum (CL) function. To have a better insight into their synthesis and action, members of the ET system (ET-1, ET converting enzyme (ECE-1) isoforms a–d, ET_A and ET_B receptors) along with NO synthase (NOS) isoforms – endothelial (e)NOS and inducible (i)NOS – were quantified in CL-derived endothelial cells (CLEC). The expression of these genes in microvascular CLEC, obtained by lectin-coated magnetic beads, was compared with cells removed from the luteal microenvironment and maintained in culture for different durations, and with endothelial cells (EC) derived from a large blood vessel (i.e. bovine aortic endothelial cells, BAEC). The profile of gene expression in the different EC types was determined by quantitative real-time PCR. Freshly isolated EC from mid-cycle CL exhibited high ET-1 receptor expression (both ET_A and ET_B), low ET-1 synthesizing ability (both prepro (pp) ET-1 and ECE-1), but elevated iNOS – the high throughput NOS isoform. The distinct phenotype of CLEC was lost soon after an overnight culture. ET_A and ET_B receptor levels declined, ppET-1 levels increased while iNOS was reduced. These changes were extenuated during long-term culture of CLEC. The general pattern of gene expression in BAEC and long-term cultured CLEC was similar yet some differences, reminiscent of freshly isolated CLEC, remained: ECE-1c, ET_B receptor and NOS isoforms were expressed differently in BAEC as compared with lines of CLEC.

This study suggests that the luteal microenvironment is necessary to sustain the selective phenotype of its resident endothelial cells. The inverse relationship between ppET-1 and iNOS observed in freshly isolated CLEC and in cultured cells is physiologically significant and suggests that ET-1 and NO may modulate the production of each other.

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Introduction

The corpus luteum is a highly vascular endocrine gland endowed with one of the highest blood flows in the adult organism. The dense network of blood capillaries irrigating the corpus luteum allows an intricate cross talk between luteal steroidogenic and endothelial cells (Grazul Bilska *et al.* 1997, Reynolds *et al.* 2000). Endothelium-derived compounds, endothelin-1 (ET-1) and nitric oxide (NO), have been demonstrated to play pivotal roles in corpus luteum function. These vasoactive compounds inhibit progesterone production and it has been proposed that they act as mediators during the process of luteolysis (Miyamoto *et al.* 1997, Meidan *et al.* 1999, Jaroszewski & Hansel 2000, Skarzynski & Okuda 2000, Hinckley & Milvae 2001). The microvascular system is locally specialized to accommodate the needs of individual tissues, and

endothelial cells lining these blood vessels exhibit distinct gene expression profiles (Chi *et al.* 2003). Therefore, understanding the characteristics of microvascular luteal endothelial cells may lead to a better insight into corpus luteum physiology.

ET-1 and NO are produced within the corpus luteum; however the characteristic profiles of ET-1 and NO generating systems in corpus luteum-derived microvascular cells are unknown. The formation of NO from L-arginine is catalyzed by several NO synthase (NOS) isoenzymes (Alderton *et al.* 2001, Albrecht *et al.* 2003). Endothelial cells were shown to contain a constitutive isoform, designated endothelial (e)NOS, and an inducible (i) isoform, iNOS. The constitutive isoform, eNOS, contributes a low NO output, while iNOS, induced by a variety of inflammatory cues, produces much higher levels of NO. The ET system encodes a family of signaling peptides, G-protein-coupled

receptors (ET_A and ET_B) and converting enzymes (ECE) which are expressed in the ovaries of several domestic animal species (Mamluk *et al.* 1999, Wright *et al.* 2001, Meidan & Levy 2002). ET-1 is produced as a large prepro (pp) endothelin polypeptide of ~200 amino acids. Following removal of the signal peptides, the inactive intermediate, big ET, consisting of 38–41 amino acids is formed (Schmidt *et al.* 1994, Xu *et al.* 1994). ECE-1 then cleaves big ET between Trp21 and Val22/Ile22 to generate the mature, active 21-amino acid peptide (Schmidt *et al.* 1994, Xu *et al.* 1994). The importance of this cleavage site is demonstrated by the fact that the vasoconstricting activity of either ET-1 (1–20) amino acids or ET-1 (1–22) amino acids is three orders of magnitude weaker than ET-1 (1–21) amino acids (Okada *et al.* 1990). Four isoforms of ECE-1 (1a, 1b, 1c, and 1d) have been identified (Schweizer *et al.* 1997, Turner *et al.* 1998, Valdenaire *et al.* 1999b). The four proteins are encoded by one gene and share a common carboxyl-terminal portion. Each is expressed from a distinct promoter that regulates expression of four unique amino termini (Turner *et al.* 1998, Valdenaire *et al.* 1999b). Although the ectodomain containing the active site is identical in each of the isoforms, the diverse amino-terminal sequences may be responsible for differences in subcellular localization, directing different intracellular sites for ET-1 production (Turner *et al.* 1998, Emoto *et al.* 1999, Valdenaire *et al.* 1999b). ET-1 acts via two G-protein-coupled receptors termed ET_A and ET_B (Arai *et al.* 1990, Aramori & Nakanishi 1992) mediating its diverse biological responses (Neylon 1999).

This study was designed to characterize the expression of the ET-1 system and NOS isoforms in corpus luteum-derived microvascular endothelial cells using freshly isolated cells obtained by lectin-coated magnetic beads. To better characterize the phenotype of luteal endothelial cells, we compared their expression profile with cells removed from the luteal microenvironment and maintained in culture for different durations and also with endothelial cells derived from a large blood vessel (i.e. bovine aortic endothelial cells, BAEC).

Materials and Methods

Materials

M-199 nutrient mixture, Dulbecco's Minimum Essential Medium (DMEM) with Ham's F12 1:1 (v/v) nutrient mixture, SuperScriptII RNase H⁻ reverse transcriptase, calf serum (CS) and Ultra pure electrophoresis agarose gel were from Gibco BRL Life Technologies (Gaithersburg, MD, USA). Vitrogen, type I collagen, was from Cohesion Technologies (Palo Alto, CA, USA). *Bandeiraea simplicifolia* lectin-1 (BS-1) was from Vector Laboratories (Burlingame, CA, USA). Collagenase type IV was from Worthington Biochemical Corp. (Freehold, NJ, USA). Uncoated magnetic beads (Dynabeads M-450) were from Dynal (Oslo, OM, Norway). Penicillin, streptomycin, and

fetal calf serum (FCS) were from Biological Industries (Beit Haemek, Israel). Deoxynucleotide triphosphates, random hexamer oligodeoxynucleotides, and Taq DNA polymerase were from Fermentas (Vilnius, Lithuania). Oligo-dT and oligonucleotide primers were synthesized by MWG Biotech AG (Ebersberg, Germany). Real-time PCR SYBR Green master-mix kit was from Eurogentec (Seraing, Belgium). Rabbit anti-human von Willebrand factor antibody was from Dako (Glostrup, Denmark). Cy3-conjugated donkey anti-rabbit IgG fluorescent antibody was from Jackson (West Grove, PA, USA). Protease inhibitor cocktail for mammalian cell extracts and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Sigma (St Louis, MO, USA). Rabbit anti-ET_A and anti-ET_B were from Alomone Labs (Jerusalem, Israel). Protein quantification kit was from Bio-Rad Laboratories (Hercules, CA, USA).

Corpus luteum collection, luteal cell dispersion and endothelial cells enrichment

Corpora lutea were collected at a local slaughterhouse and the luteal stage was determined by macroscopic examination, as described by Fields and Fields (1996). Corpora lutea at the mid-luteal phase (day 9–14) were dispersed using collagenase as previously described (Levy *et al.* 2001, 2003). Briefly, corpora lutea were washed, sliced with a tissue slicer, and preincubated in a shaking bath at 37°C for 10 min in M-199. Slices were then incubated in M-199 containing 0.5% BSA and collagenase (420 U/ml). Every 10 min, dissociated cells were removed, and fresh media containing collagenase were added. This procedure was repeated 8–10 times. Dissociated cells were kept in media containing 5% FCS until the end of the procedure. Magnetic tosylactivated beads were coated with BS-1 lectin (0.15 mg/ml) which specifically binds glycoproteins expressed by bovine endothelial cells. Dispersed luteal cells were suspended in 1% BSA in M-199, mixed with beads and incubated for 25 min at 4°C on a rocking platform. The adherent cells (BS-1 positive) were washed with M-199 containing 1% BSA and concentrated using a magnet until the supernatant was free of BS-1 negative cells. The adherent cells were subsequently eluted by 0.2 M lactose solution in PBS. Enriched endothelial cells were seeded on plates pre-coated with Vitrogen (2%) and cultured overnight in DMEM Ham's F12 containing 10% FCS. Freshly isolated (dispersed) and cultured endothelial cells were collected for RNA extraction.

Long-term endothelial cell cultures

BAEC were kindly provided by I Vlodavsky of the Hadasah-Hebrew University Hospital, (Jerusalem, Israel). These cells were grown in complete DMEM low-glucose medium containing 10% calf serum and 2 mM glutamine. Cytokeratin-positive (CK+) and cytokeratin-negative (CK-) endothelial cells (EC) derived from the bovine corpus luteum (Spanel Borowski & van der Bosch 1990, Spanel Borowski & Bein 1993, Spanel Borowski & Fenyses

1994) were used. These cell types maintain a stable phenotype during culture, and differ in morphological appearance, intercellular adhesion molecules, angiogenic genes and in the ability to form capillary-like structures. These cells were grown on plates pre-coated with 2% Vitrogen in complete DMEM Ham's F12 containing 10% FCS and 2 mM glutamine. Experiments were carried out on cells from passages 5 to 12, with 70–80% confluence.

RNA extraction and RT-PCR

Total RNA was extracted from cells (dispersed and cultured) by tri-reagent. One microgram total RNA was reverse transcribed in a total volume of 20 µl using 200 U reverse transcriptase, 50 pmol random hexamer and 0.075 nmol oligo-dT. Semi-quantitative PCR was performed as described before with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) used as an internal standard (Mamluk *et al.* 1999). The sequences of the primers used in the PCR reactions are shown in Table 1.

Real-time PCR

PCR reactions were performed using the PE Biosystems (Foster City, CA, USA) GeneAmp 5700 sequence detection system using the SYBR Green I PCR kit. Standard curves of sample cDNA were generated using serial dilutions (1:2 to 1:100). In initial experiments, PCR product identities were verified by agarose gel electrophoresis and DNA sequencing. Real-time PCR master mix reaction contained 12.5 µl of the 2 × SYBR Green buffer containing ROX passive reference, 200 µM dNTPs including dUTP, 5 mM MgCl₂, uracil N-glycosylase and Amplitaq HotGoldStar DNA polymerase, 0.55 pmol forward and reverse primers (list of real time PCR primers is shown in Table 2), 0.75 µl of a 1:10 000 dilution of SYBR Green stock solution and 1 µl (1 µg/µl) cDNA in a total volume of 25 µl. The reactions were performed in 0.2 ml PCR tubes capped with Micro-Amp optical caps. The reactions were incubated at 95 °C for 10 min to activate the Amplitaq Gold polymerase, followed by 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for both annealing and elongation. The PCR reactions were subjected to a heat dissociation protocol present in the PE Biosystems 5700 software to monitor PCR product purity. Relative expression of the RT-PCR products was determined using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001). This method calculates relative

Table 2 Primers used in real-time PCR.

Gene	Primer	Sequence	Product length (bp)
G3PDH	Forward	5'-GGCGTGACACGAGAAGTAT-3'	141
	Reverse	5'-CGTGGACAGTGGTCATAAGT-3'	
ECE-total	Forward	5'-TGTGGCGGCTGGATCAAAGC-3'	103
	Reverse	5'-AGGTGCTTGATGATGGCTTG-3'	
ECE-1a	Forward	5'-GTTCTCTCTGGATTAG-3'	178
	Reverse	5'-CTTGTCTGGTATTGGATGC-3'	
ECE-1b	Forward	5'-TGTCGGTGGGGATGTC-3'	113
ECE-1c	Forward	5'-CGGAGCGCGAGCGAT-3'	109
ECE-1d	Forward	5'-CCATGGAGGCGCTAAGAGAGT-3'	142
ECE 1b; c; d	Reverse	5'-GAAGTTCACCTGCAGGTGGT-3'	
ET-1	Forward	5'-TGTCTTCATCAGCAGCTC-3'	98
	Reverse	5'-TCAGCTTGGCAACACGAA-3'	
ET _a	Forward	5'-TACCAGAACAAAGTGATGAG-3'	194
	Reverse	5'-CACTGAGGACTTCTGCAA-3'	
ET _b	Forward	5'-AAGTACATCAACCGGTGGT-3'	194
	Reverse	5'-GGCAAGCAGCTTGTAGGTAT-3'	
iNOS	Forward	5'-GGTGAAGCAGTAACAAAGGA-3'	235
	Reverse	5'-GACCTGATGTTGCCGTTGTTG-3'	
eNOS	Forward	5'-CCTCACCCTACAATATCCT-3'	199
	Reverse	5'-TGCTCGTTGTCCAGGTGCTTC-3'	

expression using the equation: fold induction = $2^{-[\Delta\Delta C_t]}$, where C_t = the threshold cycle, i.e. the cycle number at which the sample's relative fluorescence increases above the background fluorescence and $\Delta\Delta C_t = [C_t \text{ gene of interest (unknown sample)} - C_t \text{ G3PDH (unknown sample)}] - [C_t \text{ gene of interest (calibrator sample)} - C_t \text{ G3PDH (calibrator sample)}]$. Each sample was run in duplicate and the mean C_t was used in the $\Delta\Delta C_t$ equation. For each data set the calibrator chosen was the one with the lowest expression.

Factor-VIII staining

Long-term cultured endothelial cells (CK-EC), BAEC and isolated luteal endothelial cells after overnight culture were fixed in methanol (4 °C, 5 min) and subsequently washed 3 times with PBS. After 45 min blocking with 2% gelatin at room temperature, cells were incubated with rabbit anti-human von Willebrand factor (factor VIII – endothelial cell marker) for 2 h. The cells were washed, and then incubated with Cy3-conjugated donkey anti-rabbit IgG fluorescent antibody for 1 h and washed again. Cells were photographed using a fluorescent microscope (magnification × 640, 543 nm).

Western blot analyses

The procedure was carried out as previously described (Levy *et al.* 2001). Endothelial cells (dispersed and cultured) were homogenized in lysis buffer (25 mM Tris HCl, 100 mM NaCl, 0.5% deoxycholate, 0.5% NP40, 5 mM EDTA at pH 7.5 and protease inhibitor cocktail) and were immediately sonicated on ice for 2 min. Homogenates were centrifuged for 1 min at 2000 g and the protein concentration of the supernatant was determined using Bio-Rad DC reagents. Samples containing 30 µg protein

Table 1 Primers used in semi-quantitative PCR.

Gene	Primer	Sequence	Product length (bp)
G3PDH	Forward	5'-TGTTCCAGTATGATTCCACCC-3'	850
	Reverse	5'-TCCACCACCCTGTTGCTGTA-3'	
CD-31	Forward	5'-TGAGGAGCAAGACCGTACCATA-3'	987
	Reverse	5'-ACGGAGCAGTAAACACAATGG-3'	
Tie-2	Forward	5'-CCAGTGTGAGAAAGAAGGTGT-3'	523
	Reverse	5'-CCGTCCGAGGCTCCAAATAG-3'	

were separated by 7.5% SDS/PAGE under reducing conditions and were electrically transferred to nitrocellulose membranes. After 2-h blocking in Tris-buffered saline-Tween-20 + 5% casein, membranes were incubated with the respective antibodies – anti ECE-1, ET_A and ET_B – for 2 h. Anti-ECE-1 antiserum, produced in our laboratory, was raised against a synthetic C-terminal peptide of ECE-1 and affinity purified using the immunizing peptide immobilized on a Sepharose 4B column. The membranes were washed, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature, and binding was detected with an enhanced chemiluminescence detection system (Amersham Biosciences).

Statistical analysis

The data are presented as means $\pm$ S.E.M. Data on dispersed and overnight cultured luteal endothelial cells were from four different corpora lutea and data on long-term cultured cells were from three to four different batches of each cell type used (BAEC, CK+ and CK– luteal endothelial cells). The differences between cell types were analyzed by one-way ANOVA, employing the *post hoc* multiple comparisons Dunnett's test. Analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA, v 10.05 for Windows). Differences were considered significant if $P \leq 0.05$.

Results

Endothelial cell markers: CD-31, Tie-2 and factor VIII

Endothelial cell markers, CD-31 and Tie-2 – the tyrosine kinase receptor for angiopoietins – were used to identify cells utilized in this study as endothelial cells (Fig. 1). All cell types expressed high levels of CD-31 and Tie-2 mRNA except CK+ EC which, as reported before (Lehmann *et al.* 2000), exhibited lower expression. The identification of freshly isolated luteal endothelial cells was further confirmed by the immunofluorescence staining for von Willerband factor (factor VIII; Fig. 1); these cells showed typical factor VIII staining as did luteal CK–EC (Fig. 1) and BAEC (data not shown).

Expression of ET_A and ET_B in corpus luteum-derived endothelial cells and BAEC

We first determined the patterns of ET_A and ET_B mRNA levels in different corpus luteum-derived endothelial cells and compared them with BAEC using real-time PCR (Fig. 2A, B). Interestingly, both receptor types were highly expressed in freshly isolated luteal endothelial cells. An overnight culture significantly reduced ET receptor levels, with the decline in ET_B being more pronounced than that in ET_A (four- vs twofold reduction respectively, $P < 0.05$). Lower levels of ET_A and ET_B expression were present in long-term cultured corpus luteum-derived endothelial cells (CK+ and CK– respectively; Fig. 2). In contrast,

neither ET_A nor ET_B mRNA could be detected in BAEC. Taken together, these data demonstrate that ET receptors are not maintained in culture. A similar pattern of ET_A and ET_B receptors was also apparent at the protein level (Fig. 2C). Freshly isolated luteal endothelial cells exhibited the highest levels of both ET_A and ET_B receptor protein as compared with CK– and CK+ cells, while ET_A could not be detected in either CK– or CK+ cells or BAEC (data not shown). In contrast, although lower than the amount present in dispersed endothelial cells, ET_B protein could be detected in the typical luteal endothelial cells (CK– cells). These differences are probably caused by different affinities of anti-ET receptor antibodies.

Relative mRNA expression of ppET-1 and total ECE-1 in different endothelial cell types

BAEC, CK– cells and overnight cultured luteal endothelial cells uniformly exhibited high ppET-1 mRNA expression (Fig. 3), whereas levels in freshly isolated endothelial cells were considerably lower (27.46 ± 2.94 and 6.68 ± 1.38 for cultured endothelial cells and dispersed endothelial cells respectively; Fig. 3), indicating that ppET-1 is upregulated during culture regardless of its length.

Using primers spanning the common region of all ECE-1 forms, we found that long-term cultured cells, in spite of their origin – namely CK–EC and BAEC – both demonstrated the highest ECE-1 mRNA levels (Fig. 4) as also observed for ppET-1 (Fig. 3). Also, similar to ppET-1, freshly dispersed endothelial cells had lower levels of ECE-1 than either CK–EC or BAEC (Figs 3, 4A). However, in contrast to ppET-1, ECE-1 mRNA decreased after an overnight culture (16.94 ± 3.10 and 5.96 ± 1.61 in freshly dispersed endothelial cells before and after overnight culture respectively; Fig. 4). ECE-1 protein in endothelial cells is depicted in Fig. 4B. The two bands represent different glycosylation patterns of the enzyme (Xu *et al.* 1994). In agreement with its gene expression, the highest protein levels of ECE-1 were found in CK– cells and BAEC, while dispersed endothelial cells (cultured and non-cultured) and CK+ cells exhibited low ECE-1 protein levels (Fig. 4B).

Relative mRNA levels of ECE-1 isoforms in corpus luteum-derived endothelial cells and BAEC

To have a better insight of the ET-1 generating system, we examined the relative levels of each ECE-1 isoform in the various endothelial cell types (Fig. 5). All ECE-1 isoforms were present in the various endothelial cell types but their abundance differed. Although the pattern of their expression was similar, some differences could be observed: ECE-1d and -1b were highest in CK–EC and BAEC, however, a clear disparity between these two cell types was observed in terms of isoform ECE-1c expression and to a lesser extent in ECE-1a. Of the different cell types examined, BAEC expressed the highest levels of ECE-1c. CK+ cells had relatively high ECE-1c levels, while all

Figure 1 Endothelial cell (EC) identification by CD31 and Tie-2 mRNA expression (upper panel) and immunofluorescent staining for von Willebrand factor (factor VIII; lower panel). (Upper panel) Inverse images of ethidium bromide-stained agarose gel show amplification of Tie-2 and CD31 mRNA by RT-PCR with G3PDH as an internal standard. (Lower panel) Immunofluorescent staining for factor VIII in overnight cultured luteal endothelial cells and CK- endothelial cells showing a distinctive granular appearance (magnification $\times 640$; 543 nm).

other isoforms in this cell type were low compared with the other cell types. Levels of ECE-1a, -1b and -1c in luteal endothelial cells were not different before and after overnight culture. ECE-1d, on the other hand, the isoform which appeared to be highly expressed, fell significantly in cultured cells as compared with the dispersed cells, suggesting that reduction of this isoform may be responsible for the decline in total ECE-1 observed in these cells. In fact the pattern of ECE-1d (Fig. 5) in the various cell types was similar to that of total ECE-1 (Fig. 4).

Relative mRNA levels of NOS isoforms in corpus luteum-derived endothelial cells and BAEC

Endothelial cells freshly isolated from the corpus luteum had the highest levels of iNOS expression as compared with long-term cultured endothelial cells, luteal or aortic

endothelial cells (Fig. 6), but even an overnight culture reduced iNOS expression tenfold. An opposite pattern was observed for eNOS expression: highest levels were expressed by long-term cultured CK- cells and BAEC, while freshly dispersed cells had much lower levels of expression (approx. fivefold less than in long-term cultured cells; Fig. 6).

When eNOS expression was depicted relative to that of iNOS (where iNOS served as calibrator; Fig. 7), it became clear that eNOS was the predominant form, with levels ranging from 2 and 5 in freshly isolated cells and in cells cultured overnight respectively, to an excess in eNOS of a 3 to 4 times order of magnitude in the long-term cultured cells. This also demonstrated that eNOS and iNOS are inversely regulated during culture: the constitutive type, eNOS, is upregulated, while the inducible type, iNOS, disappears almost completely. A difference in NOS isoform expression in CK-EC and

Figure 2 Characterization of ET_A (ETA) and ET_B (ETB) receptor mRNA expression (A and B) in dispersed and overnight cultured endothelial cells (EC; left panel) and long-term cultured EC (right panel). Data of ET_A (A) and ET_B (B) are the means $\pm$ S.E.M. from three different separate experiments. Following real-time RT-PCR, relative mean mRNA levels for each cell type were calculated using the $\Delta\Delta C_t$ method (see Materials and Methods). Expression is normalized with G3PDH and presented as relative gene expression. Different letters indicate significant differences among cell types ($P < 0.05$). (C) ET_A and ET_B protein levels were determined in dispersed luteal EC, CK + EC and CK – EC by Western blot analysis using specific antibodies. A representative Western blot is shown.

Figure 3 ppET-1 mRNA expression in dispersed and overnight cultured endothelial cells (EC; left panel) and long-term cultured EC (right panel). Results are the means $\pm$ S.E.M. from three different experiments. For details see legend to Fig. 2. Different letters indicate significant differences among cell types ($P < 0.05$).

BAEC was noted: corpus luteum-derived cells had higher iNOS and lower eNOS than aortic-derived endothelial cells (Figs 6 and 7).

Relative mRNA levels of NOS isoforms and ppET-1 in luteal steroidogenic and endothelial cells

The corpus luteum is a heterogeneous gland comprised mainly of endothelial (>50% of total cells) and luteal steroidogenic (~30%) cells. To determine the cellular site of NOS isoforms and ppET-1 mRNA expression within the gland, these genes were quantified in enriched endothelial and steroidogenic cell types (Levy *et al.* 2001) using real-time PCR. Data presented in Table 3 demonstrate that iNOS, eNOS and ppET-1 were expressed in orders of magnitude one or two times higher in endothelial cells than in steroidogenic cells. The residual expression of these genes in steroidogenic cells is most probably due to contamination by endothelial cells, as it corresponded to CD31 expression (data not shown).

Figure 4 Total ECE-1 expression (mRNA in A and protein in B) in dispersed and overnight cultured luteal EC, CK+EC, CK-EC and BAEC. PCR results of ECE-1 are the means $\pm$ S.E.M. from four different experiments. For details see legend to Fig. 2. Different letters indicate significant differences among cell types ($P < 0.05$). (B) ECE-1 protein levels were determined by Western blot analysis using a specific C-terminal antibody. Data are the means $\pm$ S.E.M. from three different experiments.

Discussion

The present study characterized and quantified the expression of members of the ET-1 system and NOS isoforms in corpus luteum-derived microvascular endothelial cells. By comparing the expression of these genes in cells freshly isolated from the corpus luteum with luteal endothelial cells maintained in culture (overnight or for a long-term culture) and with endothelial cells of a large blood vessel (BAEC), a typical phenotype of freshly isolated luteal endothelial cells can be described. These cells exhibited high ET-1 receptor expression (both ET_A and ET_B), low ET-1 synthesizing ability (both ppET-1 and ECE-1), and elevated iNOS – the high throughput NOS isoform. This phenotype is most probably the combined result of the endocrine and cellular microenvironment present in the corpus luteum.

The relative levels of the NO and ET-1 generating systems in luteal endothelial cells are physiologically significant, as both compounds have been ascribed key roles in the control of steroidogenesis and in the process of corpus

luteum regression in several mammalian species (Apa *et al.* 1998, Friden *et al.* 2000, Skarzynski & Okuda 2000, Hinckley & Milvae 2001, Meidan & Levy 2002, Tognetti *et al.* 2003). Additionally, as demonstrated in this study, luteal endothelial cells are the predominant site of ppET-1 and NOS mRNA expression, therefore levels found in endothelial cells are indicative of tissue levels. Indeed, our findings in endothelial cells are corroborated by data available on iNOS and ET-1 in corpora lutea of mid cycle (Girsh *et al.* 1996, Milvae 2000, Skarzynski *et al.* 2003). Of particular interest are the inverse relationship between ppET-1 and iNOS observed here. Freshly isolated cells had high iNOS and low ppET-1, while during culture iNOS was progressively reduced with ppET-1 levels simultaneously increasing. These findings suggest that ET-1 and NO may interact at the level of luteal endothelial cells to modulate the production of each other; however, the mechanism for such mutual regulation awaits further research. An inverse relationship between ppET-1 and iNOS expression was also observed in a recent study which demonstrated an inhibition of iNOS mRNA in bovine corpus luteum during luteolysis at the time of the expected up-regulation in ppET-1 (Neuvians *et al.* 2004). The compound(s) emanating from the corpus luteum milieu which is responsible for the elevated iNOS expression in freshly isolated endothelial cells is at present unknown. Yet, the fact that iNOS is already elevated in luteal tissue at the mid-late luteal phase (Skarzynski *et al.* 2003) and in endothelial cells of mid cycle (this study) when ET-1 expression is still low (Girsh *et al.* 1996, Milvae 2000, Levy *et al.* 2003 and this study) may suggest that NO regulates steady state luteal function. ET-1, in contrast, is not elevated until luteolysis commences (Girsh *et al.* 1996, Hinckley & Milvae 2001) and therefore would be expected to act mainly during luteolysis.

ET-1 and NO function as mutual antagonists in the determination of arterial vascular tone, but are known to modulate various other physiological processes (Mawji & Marsden 2003). Imbalances in the endothelial NO and ET-1 are therefore of potential importance beyond the acute regulation of vascular tone in large blood vessels and definitely so in the microvascular circulation such as is present in the corpus luteum, where they can serve other functions as well.

We found that ppET-1 was low in dispersed endothelial cells and increased rapidly during an overnight culture suggesting that the surrounding luteal tissue may be involved in this phenomenon. Progesterone was shown to inhibit ET-1 gene expression in endothelial cells (Morey *et al.* 1998), therefore the increment in ppET-1 during culture could represent an alleviation from the high progesterone milieu prevailing in the corpus luteum. If ET-1 peptide is concomitantly elevated, it may cause the reduction of ECE-1 levels as observed previously for luteal regression – when high levels of ET-1 are present, ECE-1 levels decrease (Levy *et al.* 2003).

Figure 5 Pattern of mRNA expression of each ECE-1 isoform (ECE-1a, -1b, -1c and -1d) in different endothelial cell types. For details see legend to Fig. 2. Data are the means $\pm$ S.E.M. from four different experiments. Different letters indicate significant differences among cell types ($P < 0.05$).

One of the most notable characteristics of freshly dispersed luteal endothelial cells was their high levels of both ET_A and ET_B receptors. These receptors were detected at the mRNA and protein levels. The presence of ET_A in luteal endothelial cells was also demonstrated in one of our previous reports (Mamluk *et al.* 1999). Although the presence of pericytes or smooth muscle cells in the endothelial cell fraction was not ruled out, the fact that two different separation methods, i.e. lectin-coated magnetic beads (this study) and elutriation (Mamluk *et al.* 1999), were utilized for the enrichment of endothelial cells strengthen these findings. Conventionally, ET_A receptors are found on vascular smooth muscle cells and are responsible for the direct contractile effects of ET-1 (Luscher & Wenzel 1995, Greenberg 2002). Therefore, the presence of ET_A on luteal endothelial cells is rather unusual, although other microvascular cells (cardiac capillaries) were also reported to express ET_A receptors (Wendel Wellner *et al.* 2002). These receptors may be involved in microvascular permeability (Filep *et al.* 1993) or may modulate ET-1 production and action as suggested by the findings reported here. This

study also shows comparably high levels of ET_B expression in freshly isolated luteal endothelial cells. These receptors are expressed in the vascular smooth muscle cells and endothelium of various tissues (Tsukahara *et al.* 1994, Noiri *et al.* 1997). In addition, it was suggested that endothelial ET_B receptors are involved in the release of NO, thereby altering peripheral vasodilation (Liu *et al.* 2003).

Neither ET_A nor ET_B receptors were maintained in long-term cultured cells and an overnight incubation of isolated endothelial cells was sufficient to significantly reduce their levels. A further decline was observed in luteal endothelial cells kept in long-term cultures; however, these cells retained some low ET receptor expression. BAEC, on the other hand, expressed none of these receptors, either at the mRNA or protein level.

Several subpopulations of mechanically released endothelial cells derived from bovine corpus luteum were characterized by Spanel-Borowski and co-workers (Spanel Borowski & van der Bosch 1990, Spanel Borowski & Fenyves 1994). While CK- cells derive from the typical, predominant endothelial cell population in the bovine

Figure 6 The relative distribution of NOS isoforms mRNA expression within each endothelial cell type. Data are the means $\pm$ S.E.M. from four different experiments. For details see legend to Fig. 2. Different letters indicate significant differences among cell types ($P < 0.05$). Error bars were too small to be depicted.

corpus luteum, CK + cells comprise a rare luteal endothelial cell type. Data obtained in this study further confirm that CK + cells are atypical endothelial cells, as their expression of most genes examined was considerably lower than in the CK- cells.

Although both ppET-1 and ECE-1 act in concert to produce the bioactive peptide, these genes are controlled independently. Previously, this has been shown to occur in a tissue (Tripiciano *et al.* 1999, Levy *et al.* 2001) (where more than one cell type can express the genes); this study now shows that ET-1 and ECE-1 can also be differently regulated within the same cell. All four ECE-1 isoforms were expressed by the different endothelial cells utilized in this study, although there was a distinct distribution of

each isoform in the different cell types. The diverse amino-terminal sequences in the different isoforms directs tissue-specific expression, as well as different subcellular localization (Emoto *et al.* 1999, Tripiciano *et al.* 1999, Valdenaire *et al.* 1999a, Levy *et al.* 2003). Therefore, the identification of ECE-1 isoform distribution in luteal endothelial cells could elucidate the site of big ET-1 cleavage. The high levels of ECE-1d in luteal endothelial cells reported here favor an intracellular site of ET-1 activation (Muller *et al.* 2003).

Endothelial cells from different tissues display diverse characteristics imposed by their tissue microenvironment (LeCouter *et al.* 2002, Chi *et al.* 2003). One of the well known examples is the effect of astrocytes on the ability of brain endothelial cells to exhibit barrier properties (Descamps *et al.* 1996). We observed here that freshly isolated luteal endothelial cells have a characteristic gene expression profile for almost all members of the ET-1 system as well as for the two NOS isoforms. When removed from the corpus luteum microenvironment, endothelial cells gradually lose some of their selective characteristics. Nevertheless, some differences in gene expression remain

Figure 7 The expression of eNOS relative to iNOS mRNA expression in the different endothelial cell types. Data are the means $\pm$ S.E.M. from four different experiments. Expression is presented as relative gene expression using iNOS of each cell type as calibrator. Different letters indicate significant differences among cell types ($P < 0.05$).

Table 3 Relative levels of mRNA expression of NOS isoforms and ppET-1 in freshly isolated luteal steroidogenic and endothelial cells. Data are the means $\pm$ S.D. ($n = 5$ separate CL preparations).

	iNOS	eNOS	ppET-1
Steroidogenic cells	0.14 ± 0.07	0.3 ± 0.26	0.47 ± 0.11
Endothelial cells	8.56 ± 4.17	26.79 ± 8.50	14.67 ± 3.11

The relative expression for each gene was calculated using the ΔC_t method (see Materials and Methods).

even in the long-term cultured cells: ECE-1c, ET_B receptors and NOS isoforms were expressed differently in BAEC as compared with luteal endothelial cell lines. Recently, we have also noted that type II receptors of a novel pro-angiogenic factor, endocrine gland-vascular endothelial growth factor/prokineticin 1 (Kisliouk *et al.* 2003), are present in fresh and cultured luteal endothelial cells but absent in BAEC. These data highlight the intrinsic properties of endothelial cells originating from the corpus luteum.

The profile of gene expression in corpus luteum-derived endothelial cells described here will contribute to a better appreciation of the distinct characteristics in these cells. Such knowledge is mandatory for the design of novel approaches to control the function of the corpus luteum and perhaps other steroidogenic tissues as well.

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