

Compartmentalization and cyclic variation of immunoreactivity of renin and angiotensin converting enzyme in human endometrium throughout the menstrual cycle

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Renin and angiotensin converting enzymes (ACE) are responsible for the generation of angiotensin II which regulates blood pressure and fluid/electrolyte homeostasis. The cellular localization and cyclic distribution of renin and ACE in human endometrium are demonstrated in this study. Immunohistochemical studies revealed that both renin and ACE were consistently localized in the endometrial glandular epithelia throughout the menstrual cycle; however, the immunostainings respectively for ACE and renin were weak and moderate in stromal cells of proliferative endometrium and negligible in secretory endometrium. No renin immunostaining was detected around endometrial blood vessels. Although endothelial cells consistently stained for ACE, no renin immunoreactivity was detected in these cells during the menstrual cycle. Western blot analysis using ACE antibody directed against human kidney identified a single protein band with a relative molecular mass of ~153 kDa. The intensity of this band showed cyclic variation during the menstrual cycle with the highest ACE expression during the late secretory phase and at menses suggesting that ACE plays a role in the initiation of menstruation. The differences in the cellular distribution patterns of these two enzymes further supports our previous proposition that angiotensin II has different functions at the different stages of the menstrual cycle.

Key words: ACE/angiotensin/endometrium/menstruation/renin

Introduction

Angiotensin II (ANG II) is a potent vasoconstrictor and a regulator of blood pressure and, fluid and electrolyte homeostasis. Its physiological actions are mediated via two known receptor subtypes (AT₁ and AT₂) and its formation involves two key enzymes, renin and ANG II converting enzyme (ACE). Renin is a glycoproteolytic enzyme that is a single chain aspartyl protease with molecular weight 37–40 kDa and catalyses the proteolytic conversion of angiotensinogen, a large globular protein that serves as the substrate for renin, to the decapeptide ANG I (Griendling *et al.*, 1993). ACE is a dipeptidyl carboxypeptidase that converts ANG I to the potent vasoconstrictor ANG II and inactivates the vasodilator bradykinin (Griendling *et al.*, 1993). There are two distinct iso-

enzymes of ACE. The larger form (140–180 kDa, depending on species) is known as somatic ACE and is present throughout the body on both endothelial and epithelial cell surfaces; the highest concentrations have been found in lung and kidney (Ehlers and Riordan, 1990; Williams *et al.*, 1992). A second smaller form (90–110 kDa), germinal ACE, which contains only one active site, is associated exclusively with spermatozoa (Velletri *et al.*, 1985).

Locally active tissue renin–angiotensin system with organ-specific effects has been reported in many organs, including human decidua (Shaw *et al.*, 1989), the ovary (Palumbo *et al.*, 1993) and in part of the human endometrium (Ahmed *et al.*, 1995; Svane *et al.*, 1995). Active and total renin and its substrate are found in endometrium in considerably higher concentrations (>200 times) than those found in the plasma (Johnson, 1980). Renin production was significantly increased by progesterone (Johnson, 1980), and the maximum potential to produce ANG II coincides with the triggering events of menstruation, indicating a potential function in the control of endometrial bleeding (for review, see Li and Ahmed, 1996a).

We have previously shown that ANG II-like immunoreactivity shows changes in the cellular distribution in human endometrium throughout the menstrual cycle and AT₁ and AT₂ receptor subtypes display cyclic quantitative changes in human endometrium during cyclic menstruation (Ahmed *et al.*, 1995). In this study, the distribution of renin and ACE has been localized in specific endometrial cell types at different stages of the menstrual cycle; using Western blot analysis, cyclic changes in endometrial ACE expression has been shown during the menstrual cycle, supporting our hypothesis that the components of the renin–angiotensin system play an important role in cyclic menstruation.

Materials and methods

Tissue preparation

Normal endometrial tissues were obtained from fertile subjects ($n = 17$) undergoing sterilization or from infertile couples with solely tubal damage and/or male infertility ($n = 8$). They all had regular menstrual bleeding with a menstrual cycle of 30 ± 5 days for the previous 3 months. Women who had received any form of exogenous hormones or had used an intrauterine device in the previous 3 months, or exhibited demonstrable uterine pathology were excluded from the study. Endometrial biopsies were collected by routine dilatation and curettage. The tissue was rinsed in sterile saline and immediately immersed in 4% formaldehyde and routinely processed for paraffin wax embedding for histological evaluation and immunocytochemical study. Tissue was also rapidly frozen over dry ice, wrapped in Parafilm to prevent dehydration and stored at -70°C until used for Western blotting studies. All subjects were recruited on the basis of

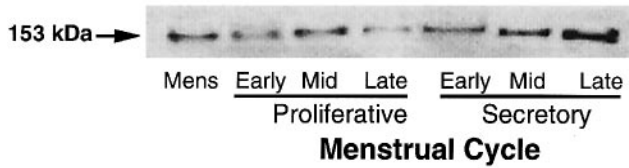


Figure 1. Western blot analysis of endometrial lysate throughout the menstrual cycle using antibody directed against human angiotensin II converting enzyme (ACE). A single protein band with the molecular mass of ~153 kDa is seen for all samples. Lane 1 is menstrual (Mens) endometrial sample, lanes 2–4 represent early, mid and late proliferative endometria respectively and lanes 5–7 represent early, mid and late secretory endometrial samples respectively. Position of the molecular weight marker is indicated on the left margin. At each menstrual stage, endometrial protein samples from three women were pooled.

Table I. Intensity score of endometrial renin immunostaining during the menstrual cycle

| Menstrual cycle | Glands | Stroma | Vessel |
|----------------------|--------|--------|--------|
| <i>Proliferative</i> | | | |
| Early | +++ | ++ | ++ |
| | +++ | + | + |
| | +++ | + | – |
| Mid | ++++ | + | + |
| | ++++ | ++ | – |
| | ++++ | + | + |
| | +++ | ++ | – |
| | ++++ | ++ | – |
| | ++++ | +++ | – |
| Late | +++ | + | + |
| | ++++ | +++ | – |
| | ++++ | ++ | – |
| | +++ | ++ | – |
| | +++ | ++ | – |
| <i>Secretory</i> | | | |
| Early | +++ | + | – |
| | +++ | + | – |
| | +++ | + | – |
| | ++++ | ++ | – |
| Mid | ++++ | ++ | – |
| | ++++ | + | – |
| | ++ | – | – |
| | ++++ | + | – |
| | ++++ | + | + |
| Late | ++++ | + | – |
| | ++ | + | – |
| | ++++ | – | – |
| | ++++ | + | – |

full consent and ethical committee approval was obtained from the South Birmingham Ethical Committee.

Western blot analysis

Protein was extracted from frozen endometrial tissue using high salt buffer (KCl 0.4 M, HEPES pH 7.4, 20 mM, dithiothreitol 1 mM, glycerol 20%, bacitracin 0.5 mg/ml, phenylmethylsulphonyl fluoride 40 µg/ml, pepstatin 5 µg/ml, leupeptin 5 µg/ml). Tissue was washed in ice-cold phosphate-buffered saline (PBS) before homogenization on ice. The homogenate was centrifuged at 3000 g at 4°C for 10 min. The supernatant was stored at –70°C until further use. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories Inc., Herts, UK) using bovine serum albumin (BSA) as standard.

The non-radioactive ECL Western Blotting System (Amersham International plc, Bucks, UK) was used to detect ACE in endometrial

Table II. Intensity score of endometrial ACE immunostaining during the menstrual cycle

| Menstrual cycle | Glands | Stroma | Vessel |
|----------------------|--------|--------|--------|
| <i>Proliferative</i> | | | |
| Early | ++++ | ++ | ++++ |
| | +++ | ++ | +++ |
| | ++++ | +++ | ++ |
| Mid | ++++ | ++ | +++ |
| | ++++ | ++ | ++ |
| | +++ | ++ | ++ |
| | ++++ | ++ | +++ |
| | ++++ | ++ | +++ |
| Late | +++ | + | ++ |
| | ++++ | ++ | +++ |
| | ++++ | ++ | +++ |
| | ++++ | ++ | +++ |
| | ++++ | ++ | ++ |
| <i>Secretory</i> | | | |
| Early | +++ | + | ++ |
| | +++ | + | ++ |
| | +++ | + | ++ |
| | +++ | + | + |
| Mid | +++ | ++ | +++ |
| | +++ | + | +++ |
| | +++ | + | ++ |
| | +++ | + | + |
| Late | +++ | + | +++ |
| | +++ | + | ++ |
| | +++ | ++ | +++ |
| | ++++ | + | ++ |

samples as previously described (Khaliq *et al.*, 1996). Briefly, 50 µg total protein was separated by electrophoresis on a polyacrylamide gel consisting of a stacking gel overlaying the separating gel. The samples were loaded in a final volume of 25 µl with sample buffer containing 0.002% bromophenol blue and electrophoresed at 50 V for ~2 h. A kaleidoscope protein marker (Bio-rad Laboratories, Hercules, CA, USA) was run alongside the samples. This protein marker was a protein standard calibrated molecular weight and prestained which allowed accurate molecular weight determination of unknown protein. After electrophoresis the protein was transferred onto a nitrocellulose membrane in a cooling system (10°C) overnight at 36 V. The filter was blocked to reduce non-specific binding of the antibody using Tris-buffered saline–Tween (TBS-T) containing 10% Marvel and 2.5% BSA for 4 h. After washing in TBS-T the membrane was incubated overnight at 4°C with the ACE antibody. This rabbit polyclonal antiserum was raised against the purified human kidney ACE (Littlewood *et al.*, 1989) used at a dilution of 1:1000. The filter was washed and incubated with the secondary anti-rabbit antibody for 1.5 h at room temperature. After a final wash in TBS-T the filter was incubated for 1 min at room temperature in detection reagent, immediately wrapped in Saranwrap and exposed for periods of 30 s, 1 min, 5 min and 10 min to an X-ray film.

Immunocytochemistry

Paraffin sections (4 µm) were cut, mounted on glass slides and placed in a 60°C oven for 30 min. Haematoxylin and eosin sections were dated, based on the criteria of Fox and Buckley (1983). Sections from each endometrial specimen were dated from the last menstrual period and were used in the study only if there was corroboration by independent histological dating. The phase of the endometrial cycle was histologically classified as early, mid or late proliferative and early, mid or late secretory endometrium in terms of its morphological changes (Fox and Buckley, 1983).

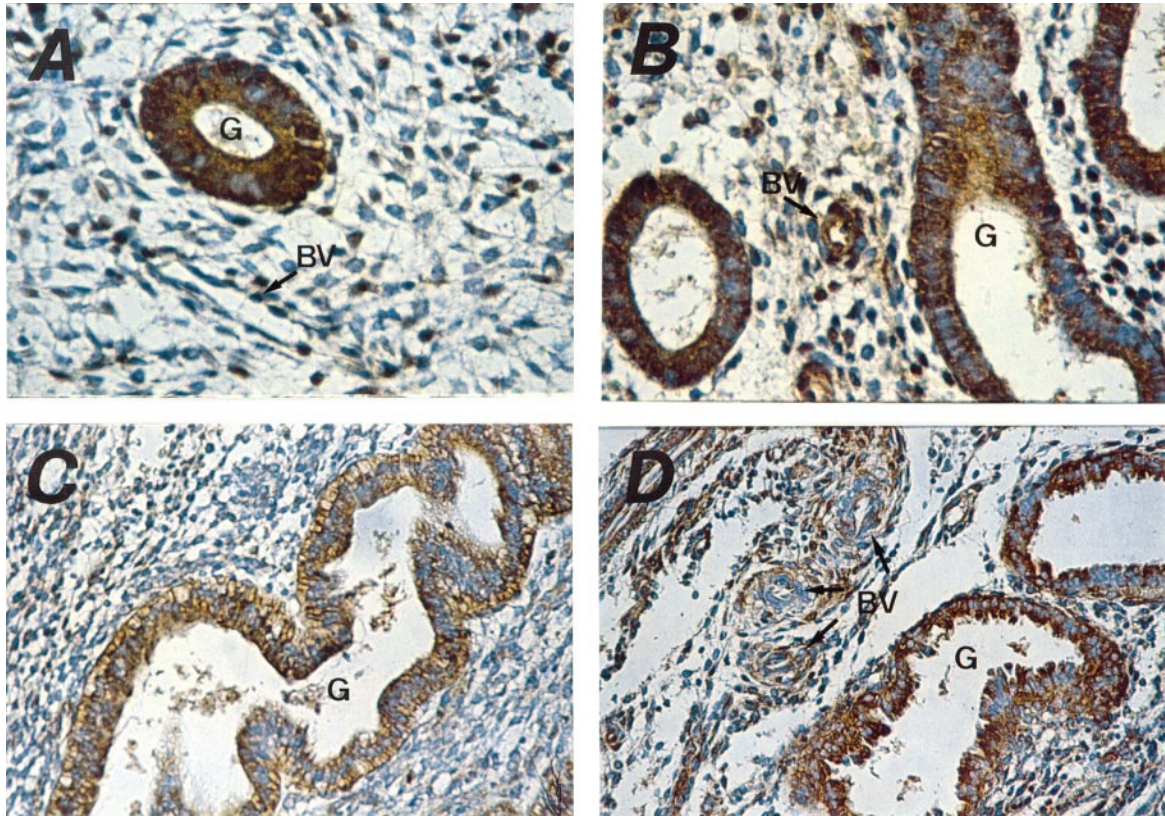


Figure 2.

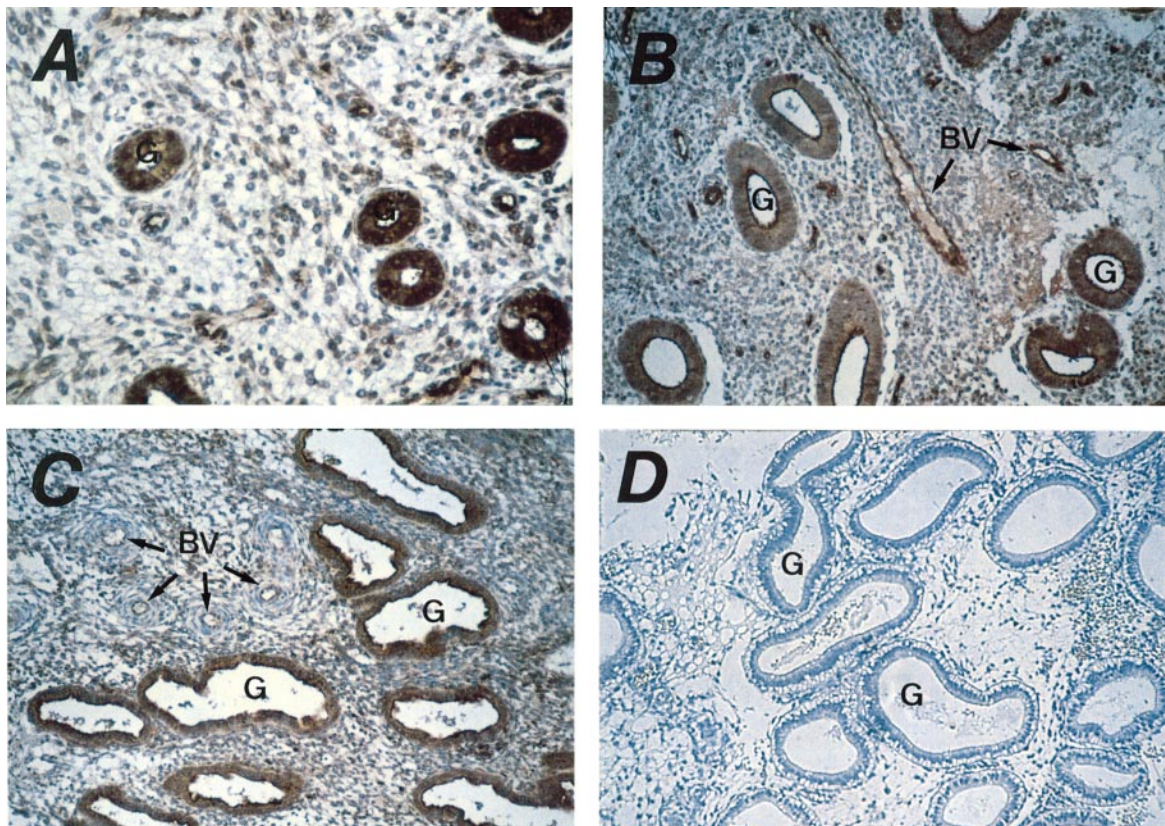


Figure 3.

Paraffin sections were used and immunostaining was carried out by the streptavidin–biotin complex method described previously (Li *et al.*, 1994). Briefly, sections were deparaffinized by incubation for 5 min with xylene. The tissues were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity and incubated for 30 min with 10% non-immune goat serum to reduce non-specific background staining. The sections were then incubated with primary antibodies for 45 min at room temperature. The polyclonal rabbit antiserum was raised against human renin, which was kindly provided by Dr M.Horiuchi and was used at 1:200 dilution. The ACE antibody was used at 1:100 (Littlewood *et al.*, 1989). Amplification of the primary antibody reaction was achieved using a goat anti-rabbit secondary antibody (diluted 1:200 in PBS, pH 7.4) for 30 min followed by a complex of streptavidin (Dako Ltd, Bucks, UK) and biotinylated peroxidase (Dako) for another 30 min. Finally, the binding was visualized by the addition of 0.5 mg/ml diaminobenzidine (Sigma Chemical Co. Ltd, Poole, Dorset, UK) and 0.01% hydrogen peroxide in PBS to form the insoluble antigen–antibody complex. Between each step, the sections were washed in PBS over a period of 15 min. All incubations of antisera were carried out at room temperature in a wet chamber mounted on a rocking tray which ensures a movement of antiserum over the whole section. Thereafter, the sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. To test the specificity of the immunohistochemical staining, the primary antibody was replaced with rabbit non-immune serum in control experiments.

An arbitrary 4-point scale was used to grade the intensity and the proportion of positively stained cells. A score of 4 indicated very intense staining of >95% for stromal cells, glandular epithelium and blood vessels compared with a score of zero which indicated an absence of staining. Scores of 1, 2 and 3 indicated occasional, very weak, and moderate intensity of staining respectively. Both the immunohistochemistry and the scoring of positively stained cells were performed by a single individual without prior knowledge of the phase of the menstrual cycle.

Results

A total of 25 endometrial samples were collected from women with normal menstrual cycles for this study, including the proliferative and secretory phases (13 and 12 respectively). Sections from each endometrial specimen were staged using

the date of the last menstrual period and histological criteria (Fox and Buckley, 1983)

Western blot analysis

The antibody to ACE incubated with protein was extracted from endometrium, confirming that the ACE immunoactivity consistently existed in the endometrial samples from different stages of menstrual cycles. Determination of the size of the protein species from the position of the band in relation to the molecular weight marker showed a protein of 140–160 kDa size in all endometrial samples studied (Figure 1). Western blot comparing different stages of endometrial samples consistently showed higher amounts of ACE protein in menstruation and mid to late secretory endometrium. The lowest amount of ACE protein was detected in late proliferative and early secretory endometrium, which was confirmed in a separate experiment on endometrial protein obtained from another set of endometrial samples from different stages of menstrual cycles. Chemiluminescence signals were obtained following immunoblotting of 50 µg total endometrial protein from three women in each case throughout all phases of the normal menstrual cycle. As samples in each subgroup were pooled, individual variation was not relevant and therefore not examined.

Immunocytochemistry

Renin and ACE immunoreactivities were detected in the endometrial glandular epithelia of all sections studied and the intense immunostaining was consistently displayed throughout the menstrual cycle (Figures 2 and 3; Tables I and II). Cytoplasmic staining was observed in stromal cells and glandular epithelial cells. The intensity of immunostaining for renin in stroma varied from very weak to moderate and the renin immunoreactivity was restricted to some stromal cells (Figure 2). The proliferative endometrium showed moderate immunostaining compared with the weak immunostaining in most secretory endometrial sections (Figure 2A–C). There was no immunostaining to renin antibody detected around endometrial blood vessels in most endometrial sections (Figure 2A) except

Figure 2. Immunocytochemical localization of renin in human endometrium with normal regular cyclic menstruation. Endometrial sections A–D were incubated with rabbit antihuman renin antibody. (A) The early–mid proliferative endometrial section shows very intense renin-like immunostaining in the glandular epithelial cells and moderate immunostaining in some stromal cells; no renin immunoactivity was detected around/in blood vessels (arrow). (B) The late proliferative endometrial section shows intense renin immunostaining in the glandular epithelia and endothelial cells in the inner layer of blood vessels (arrow), with weak or moderate staining in some stromal cells. (C) The early secretory endometrial section displays intense staining in the glandular epithelia with vacuoles but the stroma was relatively negative with respect to staining. (D) The late secretory endometrial section also showed intense renin immunostaining in the glands and there was no staining in the perivascular smooth cells around endometrial blood vessels (arrow). G = endometrial gland; BV = endometrial blood vessel. Original magnifications: ×560 for A and B; ×280 for C and D.

Figure 3. Immunocytochemical localization of angiotensin converting enzymes (ACE) in human endometrium with normal regular cyclic menstruation. Endometrial sections A–C were incubated with rabbit antihuman ACE antibody. (A) The early–mid proliferative endometrial section showed very intense ACE immunostaining in the glandular epithelial cells and intense immunostaining in stroma and endothelial cells of endometrial blood vessels. (B) The late proliferative endometrial section showed intense ACE immunostaining in the glandular cells of endometrial blood vessels. (C) The late secretory endometrial section displayed intense staining in the glandular epithelia and weak staining in the endothelial cells of blood vessels (arrows). (D) The negative control section which showed no immunostaining was incubated with non-immune serum. G = endometrial gland; BV = endometrial blood vessel. Original magnifications: ×280 for A; ×140 for B–D.

for three proliferative endometria which showed weak or moderate immunostaining in endothelial cells of blood vessels (Figure 2B). In contrast, very weak immunostaining to ACE antibody was visible in stroma at the early to mid proliferative endometrium (Figure 3A). In stroma, ACE immunoactivity was reduced substantially in late proliferative and was negative throughout secretory endometrium (Figure 3B and C). Endothelial cells of endometrial blood vessels stained for ACE consistently throughout the menstrual cycle while it was negative for renin. In the endothelial cells, moderate to intense immunostaining was detected in proliferative endometria (Figure 3A and B) and secretory endometria showed weak or moderate immunostaining to ACE antibody (Figure 3C).

The cyclic changes in the pattern of staining for renin and ACE immunoreactivity in the stroma, glands and blood vessels through the menstrual cycle were tabulated according to an arbitrary grading system (Tables I and II). No specific staining was seen when pre-immune serum was substituted (Figure 3D).

Discussion

This study provides evidence of renin and ACE immunoreactivity coexisting consistently in endometrial epithelial cells throughout the menstrual cycle and suggests that the glandular epithelium is likely to be the source of the pre-menstrual increases in endometrial renin concentration reported previously (Johnson, 1980). In contrast with an earlier report indicating that endometrial stromal cells are the source of active renin and that progesterone markedly increased renin production (Shah *et al.*, 1991), this study demonstrates the localization of moderate renin immunostaining in proliferative endometrial stroma, with weak or absent staining in the secretory stromal endometrium. This difference probably relates to the fact that Shah *et al.* (1991) measured renin production in endometrial stromal cells in culture, which excludes the in-vivo environment. The present study also shows that renin immunoreactivity in endometrial glandular epithelial cells persists throughout the menstrual cycle, thus conflicting with an earlier report indicating immunostaining of stromal and glandular epithelial cells in proliferative but not secretory endometrium (Raju and Lee, 1989). The negative reactivity in some sections may relate to loss of antigenicity due to tissue preparation, specificity of the antisera employed, or due to low sensitivity of the immunohistochemical technique employed. Although renin immunoreactivity does not guarantee that the particular cell is the source of the renin, renin mRNA has been detected in endometrial and decidual homogenates (Shaw *et al.*, 1989).

The accepted function of renin is the formation of the decapeptide ANG I by hydrolysing the bond between amino acid residues 10 and 11 at the N-terminal end of the angiotensinogen molecule (Ganong, 1995). However, ACE is a rather non-specific dipeptidylcarboxypeptidase that removes COOH-terminal dipeptides from a variety of substrates (Igc *et al.*, 1972). ACE is a widely distributed cell-surface peptidase which plays a key role in the control of biological function of the renin-angiotensin system. ACE does not always localize with other components of the renin-angiotensin system and

the enzyme displays a broad substrate specificity. In the present study, the endothelium of the blood vessels consistently stained for ACE but not renin. The ACE immunoactivity detected in the endometrium, however, localized with renin in the endometrial glandular epithelium, and in endometrial stromal cells displayed cyclic changes consistent with the obvious cyclic changes reported for ANG II immunostaining and AT receptor subtypes (Ahmed *et al.*, 1995; Saridogan *et al.*, 1996a). Although the distribution of renin and ACE detected in the present study was different in pattern of staining from that of ANG II (Ahmed *et al.*, 1995), in the stromal compartment of the endometrium they both appear to be under steroid regulation. This is further supported by Western blot analysis which showed that the highest ACE expression was during the late secretory phase and at menses suggesting that ACE plays a role in the initiation of menstruation. Corticosteroids can also induce ACE from cultured endothelial cells, alveolar macrophages and monocytes, and lung (Ehlers and Riordan, 1990), and the testicular form of ACE activity was attenuated by follicle stimulating hormone/luteinizing hormone, human chorionic gonadotrophin, and testosterone in hypophysectomized rats (Velletri *et al.*, 1985).

Rapid capillary growth associated with endometrial regeneration follows menstruation and thus it was proposed that ANG II may modulate cell growth and neovascularization (Ahmed *et al.*, 1995). The very intense ANG II immunostaining together with the expression of AT₁ receptor mRNA reported around the endometrial blood vessels in the late secretory phase was suggested to indicate its role in the initiation of menstruation by the vasoconstriction of the spiral arteriole prior to menstruation (Ahmed *et al.*, 1995). A recent study further confirmed the presence of AT₁ receptor by the localization of AT₁ receptor protein in human endometrium (Saridogan *et al.*, 1996a). Angiotensin II is not only a potent contractile agonist for vascular smooth muscle, but can also act as a growth factor (Powell *et al.*, 1990). Angiotensin II stimulates the expression of basic fibroblast growth factor in bovine luteal cells (Stirling *et al.*, 1990) which stimulates endothelial cell proliferation and angiogenesis (Ahmed *et al.*, 1994). Moreover, fibroblast growth factor receptor-1 is a critical component for endometrial remodelling (Sangha *et al.*, 1997). ACE activity in the uterus has been reported to be of the same order of magnitude as in the lung which contains the highest levels of ACE activity in humans (Lieberman and Sastre, 1983) and ACE inhibitors block neointima formation after vascular injury (Farhy *et al.*, 1993) due to inhibition of ANG II formation and kinin degradation. Kinins are known to stimulate synthesis of endothelium-derived relaxing factor such as nitric oxide and prostacyclin, both of which have anti-growth properties (Farhy *et al.*, 1993). An imbalance in the ACE activity may not only affect menstruation but could predispose women to hyperplasia (Li and Ahmed, 1996b) and endometriosis.

In addition to renin and ACE, however, a variety of acid proteases can form ANG II and ACE can act on a variety of different substrates in addition to ANG I. In human heart, a minor component (10–15%) of ANG II formation is through ACE and a major component (~75–80%) is through a serine proteinase called chymase (Urata *et al.*, 1994). High levels of

chymase enzymatic activity have been found in the skin, oesophagus, stomach and the uterus, but the highest level is in the uterus (Urata *et al.*, 1994). Its physiological function in uterine tissue is not known.

Current findings, taken together with our previous data on the expression of ANG II and its receptor subtypes in normal (Ahmed *et al.*, 1995) and hyperplastic endometria (Li and Ahmed, 1996), suggest that the renin-angiotensin system must play a key role in the initiation of menstruation. Moreover, the stimulatory actions of ANG II on Fallopian tube ciliary activity (Vinson *et al.*, 1995) suggest that ANG II may regulate secretory epithelial function during implantation (Saridogan *et al.*, 1996b). Plasma and follicular fluid renin concentrations are significantly elevated during the mid-luteal phase in women undergoing ovarian stimulation for in-vitro fertilization (Beerendonk *et al.*, 1996). Our previous proposition for the 'dual role hypothesis' for angiotensin II in human endometrium is supported by the current data and we suggest that ANG II has different functions at the different stages of the menstrual cycle.

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