Cyclic and characteristic expression of phosphorylated Akt in human endometrium and decidual cells in vivo and in vitro

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BACKGROUND: Akt is activated by phosphorylation and plays an important role in cell survival and maintenance of structure. METHODS: We investigated whether phosphorylated Akt was characteristically expressed in human endometrium in vivo and whether insulin-like growth factor-I (IGF-I) can activate Akt using cultured decidualized human stromal cells in vitro, using immunohistochemistry and Western blotting analysis. RESULTS: The levels of phosphorylated Akt protein increased markedly in the decidual tissues from ectopic pregnancy. The expression of phosphorylated Akt protein in stromal cells increased with the decidualization. The decidual cells showed strong cytoplasmic staining for phosphorylated Akt. However, cultured decidualized human stromal cells diminished phosphorylated Akt expression compared to control cells. IGF-I administration to decidualized human stromal cells significantly recovered pAkt expression. The effect of IGF-I on decidualized human stromal cells was blocked by an inhibitor of phosphatidylinositol-3 kinase (PI3K) (LY294,002). These results suggest that IGF-I may activate Akt via PI3K in human endometrium and decidua. The expression of phosphorylated Akt in stromal cells was only detected in the functional layer, where tissue remodelling occurs during menstruation or implantation. CONCLUSIONS: Akt activation may be involved in cell survival and extracellular matrix remodelling in human endometrium and decidua.

Key words: Akt/decidualization/endometrium/IGF-I

Introduction

Human endometrium exhibits cyclic change and tissue reconstruction throughout the menstrual cycle accompanied by cell renewal and extracellular matrix (ECM) remodelling (Noyes et al., 1950; Nakano et al., 2001; Noguchi et al., 2003). The cyclic change and tissue reconstruction are also essential for decidualization of endometrium and embryo implantation. Sex steroid hormones as well as many kinds of growth factors such as insulin-like growth factor-I (IGF-I) and epidermal growth factor affect decidualization and implantation (Irwin et al., 1991; Henemeyer and Markoff, 1998; Nayak and Giudice, 2003). Several studies have suggested that apoptosis of cells and reconstruction of ECM in decidua can contribute to early placental formation when extravilous trophoblasts secrete matrix metalloproteinases (MMPs) (Alexander et al., 1996; Das et al., 1997) and invade maternal endometrial tissues (Cross et al., 1994; Smith et al., 1997). Furthermore, decidua may provide a firm scaffold on which to form well-developed placenta in order to prevent trophoblasts from invasion that is too deep.

Akt is an approximately 60 kDa serine/threonine kinase (Bellacosa et al., 1991) and is activated through the activation of phosphatidylinositol-3 kinase (PI3K) stimulated by several growth factors, cytokines and sex steroids, such as insulin, tumour necrosis factor, platelet-derived growth factor (PDGF), estrogen, prolactin, leukaemia inhibitory factor (LIF) and IGF-I (Burgering and Coffer, 1995; Franke et al., 1995; Alessi and Cohen, 1998; Pastorino et al., 1999; Negoro et al., 2001; Tessier et al., 2001; Ivanova et al., 2002). The activation of Akt is accompanied by the phosphorylation of Thr308 and Ser473. The initially defined functions of Akt were glycogen synthesis, glycolysis and glucose uptake. However, there have been some reports suggesting that Akt is transferred into various regions including the cytoplasm and nucleus and then plays an important role in cell survival and maintenance of physiological function in neural cells, endothelial cells and others by phosphorylating various substances (Yao and Cooper, 1994; Deprez et al., 1997; Dudek et al., 1997; Kulik et al., 1997).

The role of Akt activation in human endometrium is not yet clear. Previously, we have identified Akt as a gene that shows increased expression in the uterus during embryo implantation...
in mice, by means of a cDNA array system. Although the expression of Akt protein was localized in luminal and glandular epithelium in the mouse uterus before implantation of an embryo, the expression of Akt protein appeared in the decidualized stromal cells (unpublished data). In recent years, the expression of pAkt in human stromal cells and cultured human decidualized stromal cells has been demonstrated (Yoshino et al., 2003; Guzeloglu Kayisli et al., 2004), while there are few reports concerning relationship between the cyclic expression of pAkt and decidual reaction in endometrial stromal cells in vivo without a direct cell–cell interaction with trophoblasts. Some investigators reported that estradiol stimulated Akt activation in cultured endometrial stromal cells (Guzeloglu Kayisli et al., 2004). Not only sex steroid hormones but also various cytokines including growth hormones exist in endometrial tissues. IGF-I is also well known to play an important role in activating Akt in the mouse uterus (Klotz et al., 2000; 2002; Lathi et al., 2005). In the present study, we examined the expression of pAkt in human endometrial stromal cells and ectopic pregnancy deciduas in vivo. We also investigated whether IGF-I can modify the expression of pAkt in cultured human endometrial stromal cells.

Materials and methods

Antibodies and reagents

Phenylmethylsulphonyl fluoride (PMSF), sodium vanadate, pepstatin-A and IGF-I were purchased from Sigma (St. Louis, MO, USA). Complete protease inhibitor cocktail was obtained from Boehringer Mannheim (Mannheim, Germany). Horse-radish peroxidase (HRP)-conjugated secondary antibody and enhanced chemiluminescence (ECL) kit were from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). The EnVision+ kit for rabbit immunoglobulin G (IgG) was from DAKO (Glostrup, Denmark). Mouse anti-human GAPDH antibody for Western blotting was obtained from Santa Cruz (Santa Cruz, CA, USA). Rabbit polyclonal anti-Akt and anti-phospho Akt (Ser473) antibodies for Western blotting and rabbit polyclonal anti-phospho Akt (Ser473) (HIC-specific) and LY294,002 were obtained from Cell Signaling Technology (Beverly, MA, USA). Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F12 medium (F-12) and dextran-coated charcoal (DCC)-treated fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA, USA). HEPES, gentamicin, 17β-estradiol (E2) and progesterone were from Sigma.

Samples

Human endometrial samples were obtained at hysterectomy or endometrial biopsy from 42 premenopausal non-pregnant women with regular menstrual cycles (age range, 30–51 years) after obtaining written consent. The hysterectomies were performed to treat leiomyomas or adenomyosis. Specimens for immunohistochemistry (n = 28), which contained myometrium and endometrium, were obtained from the corpus of the uterus with a cold knife. Specimens for protein extraction (n = 20), which contained only the functional layer of the endometrium, were obtained from the corpus of the uterus by curettage. Only histologically normal tissues were used in this study. Endometrial samples were dated with respect to the menstrual cycle using Noyes’ histological criteria (Noyes et al., 1950). If there was any disagreement between the histological dating and the announced day of last menstrual bleeding, the specimens were excluded. Women who had received any exogenous hormones were also excluded. The endometria were dated to the early-proliferative (n = 6), late-proliferative (n = 5), early-secretory (days 17–20, n = 5), mid-secretory (days 21–23, n = 10) and late-secretory (days 24–28, n = 8) stages of the menstrual cycle. The uterine decidual tissues in early ectopic tubal pregnancy used for immunohistochemistry (n = 5) and protein extraction (n = 5) were obtained from eight cases (age range, 26–37 years) by curettage after obtaining written consent. Tissue fragments were flash-frozen for protein extraction in liquid nitrogen and stored at −80°C until the assay. The tissues for immunohistochemistry were fixed in 4% paraformaldehyde for 1 h then dehydrated and embedded in paraffin wax. A part of the tissues in the late-proliferative phase was prepared for cell culture (n = 5).

These procedures were approved by the Ethics Committee of the Graduate School of Biomedical Sciences, Hiroshima University.

Culture of endometrial stromal cells

Endometrial stromal cells were prepared from human endometrium at the late-proliferative phase by a previously described method (Noguchi et al., 2003; Kudo et al., 2004). Briefly, minced endometrial tissues were incubated for 1 h at 37°C in a 1 : 1 mixture of DMEM and F-12 containing 0.25% collagenase, 0.002% deoxyribonuclease I, 100 IU/ml penicillin, 100 IU/ml streptomycin and 10% DCC-treated FBS. The digested tissues were filtered using double layers of monofilament nylon mesh membranes (pore size: 37 and 105 μm). The filtrate was washed three times, and the number of viable cells was counted by Trypan Blue dye exclusion. The isolated endometrial stromal cells were cultured at 37°C in DMEM/F-12 (1 : 1) supplemented with 10% DCC-treated FBS, 20 mM HEPES and 100 ng/ml gentamicin in a humidified atmosphere of 5% CO2 and 95% air for 5 days to the stage of sub-confluence. Some of the cells were starved in serum-free medium for 24 h, and then incubated with IGF-I for 10, 20 and 40 min at 40 ng/ml or for 20 min at 1, 10 and 100 ng/ml (n = 3). The other cells were parallel cultured with 10−6 mol/l 17β-E2 and 10−7 mol/l progesterone (P4) or vehicle for 7 days, with the medium being changed every 48 h (n = 3). Cells were then cultured in serum-free medium for 24 h. The next day, cells were stimulated with IGF-I for 20 min at 40 ng/ml. Parallel cultures were treated with LY294,002 (LY) for 30 min at 10 μM before stimulation with IGF-I. Total RNA and protein were extracted as described below.

Protein extraction and Western analysis

To isolate protein from uterine tissue and harvested cells, specimen and cells were homogenized in an ice-cold lysis buffer (25 mM Tris-HCl pH 7.4, 2% Nonidet P-40, 50 mM NaCl, 0.2% SDS, 1 mM PMSF, 5 μM pepstatin-A, 1 mM sodium vanadate and complete protease inhibitor cocktail) and then centrifuged at 15 000 × g for 30 min at 4°C to remove insoluble material. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA). Protein extract samples were boiled for 3 min in sample buffer (0.1 M dithiothreitol, 2% SDS, 15% glycerol, 0.006% bromophenol blue and 0.08 M Tris–HCl pH 6.8). Equal amounts of total protein (30–50 μg/lane) were resolved by 8% polyacrylamide gels under reducing conditions in 25 mM Trisma base, 192 mM glycine and 0.1% SDS. Proteins then were electrotransferred to nitrocellulose membranes (110 V, 60 min). The membranes were incubated at room temperature for 1 h in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% non-fat dry milk to block non-specific binding, then washed and incubated with antibody against Akt (1 : 1000) or Phospho-Akt (Ser473) (1 : 1000) overnight at 4°C. The membranes were washed and incubated with a HRP-conjugated secondary antibody (1 : 2000) at room temperature for 50 min. Peroxidase activity was visualized with the ECL kit and exposed for 1–5 min to X-ray film (MEDICAL X-RAY FILM, FUJI). Equal loading of
Endogenous peroxidase activity was blocked by incubating the sections for 30 min at room temperature in 0.3% H₂O₂/methanol. The retrieved by heating the sections in a microwave in citrate buffer followed by cooling in the buffer for 60 min at room temperature. Endogenous peroxidase activity was blocked by incubating the sections for 30 min at room temperature in 0.3% H₂O₂/methanol. The sections were incubated with 5% normal goat serum for 30 min at room temperature to block non-specific staining. Immunohistochemistry was performed using polyclonal antibody for phospho-Akt (Ser473) at a dilution of 1:50 (90 min). Detection of antibody binding was done using goat anti-rabbit Ig conjugated to peroxidase-labelled dextran polymer (EnVision+). Peroxidase activity was revealed by incubation with 3,3′-diaminobenzidine tetrahydroxychloride (DAB), and all sections were counter-stained with haematoxylin. Negative control sections were processed by substituting non-immune serum for the primary antibody.

Two investigators observed each samples blinded to the stage of menstrual cycle and evaluated positively stained cells using the following intensity categories: – (no staining), +/- (weak but detectable staining), + (moderate staining) and ++ (intense staining). If there was any disagreement in the evaluation between investigators, the specimens were excluded.

RNA isolation and RT–PCR analysis
Total RNA from harvested cells was isolated using an RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. The RNA concentration was measured spectrophotometrically in all samples. Total RNA (0.1 μg) was reverse transcribed and amplified by PCR using a one-step RT–PCR kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. RT was carried out at 60°C for 30 min, and PCR amplification was performed at 94°C for 90 s, 63°C for 60 s and 60°C for 6 min for 25 cycles (glycerol-3-phosphatedehydrogenase: G3PDH, PRL), using primers for G3PDH supplied by the manufacturer and for PRL (GenBank accession no.V00566 J00299) (sense 5′-CGA AGA CAA GGA GCA AGC-3′, antisense 5′-AAG CAG AAA GGC GAG ACT-3′). Reaction products were electrophoresed on a 2% agarose gel, which was stained with ethidium bromide. The predicted sizes of the PCR-amplified products were 288 and 450 bp for PRL and G3PDH, respectively.

Statistical analysis
Western blot analysis and RT–PCR were repeated a minimum of three times using three to five different samples. Mann–Whitney’s U-test and t-test were used for statistical analysis employing STAT VIEW 5.0 software (SAS Institute, Cary, NC, USA). A significance level of 0.05 was used for all tests.

Results
The expression of Akt and pAkt (Ser473) protein in tissue during the menstrual cycle
To obtain detailed information about the difference in the level of Akt and phosphorylated Akt (Ser473) protein during the menstrual cycle, we classified samples as follows: early-proliferative phase, late-proliferative phase, early-secretory phase, mid-secretory phase, late-secretory phase and decidua. We then performed Western blot analysis. The level of Akt protein showed no statistically significant change during the menstrual cycle, whereas the level of pAkt protein significantly increased in decidual tissues compared to that in the early-proliferative phase and mid-secretory phase (P < 0.05) (Figure 1A and B).

The localization of pAkt (Ser473) protein in endometrium
The localization of pAkt (Ser473) protein was examined by immunohistochemistry. The specific patterns for pAkt (Ser473) protein are summarized in Table I. In the proliferative endometrium, immunostaining for pAkt was most intense in...
Characteristic expression of pAkt in endometrium

The intensity of pAkt protein was evaluated positively stained cells using the following intensity categories: – (no staining), +/- (weak but detectable staining), + (moderate staining) and ++ (intense staining). N, nucleus; C, cytoplasm.

Table 1. Cyclic expression of pAkt in the endometrium

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<tr>
<th>Phase</th>
<th>Functionalis</th>
<th>Basalis</th>
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<td></td>
<td>Epithelium</td>
<td>Stroma</td>
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<tr>
<td></td>
<td>N</td>
<td>C</td>
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<tr>
<td>Early-proliferative</td>
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<td>Late-proliferative</td>
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<tr>
<td>Early-secretory</td>
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<td>Mid-secretory</td>
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<td>Decidua</td>
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The expression of pAkt during decidual change of cultured endometrial stromal cells

To observe the expression of pAkt protein during decidual change in endometrial stromal cells, we examined protein level of pAkt in cultured cells treated with progesterone and E2 or vehicle. Decidualization of stromal cells was confirmed by their morphology and by increased expression of prolactin mRNA (Tabanelli et al., 1992) (Figure 3A). The amount of pAkt protein extracted from hormone-treated cells was apparently reduced compared with the relevant control (Figure 3B).

Regulation of pAkt expression by IGF-I in endometrial stromal cells and decidualized stromal cells

Treatment with IGF-I induced a significant increase in the phosphorylation of Akt compared to control cells. The level of pAkt was highest after 20-min exposure to IGF-I. IGF-I treatment...
resulted in a significant dose-dependent increase in pAkt (Figure 4). The diminished pAkt expression in decidualized stromal cells was recovered by administration of IGF-I. The ability of IGF-I to activate Akt was inhibited by LY294,002 (Figure 5).

Discussion

The total amount of Akt protein did not change in any stages of the menstrual cycle, while phosphorylated Akt tended to decrease in mid-secretory endometria and increase in decidual tissue, as determined using Western blot analysis. However, Western blotting detected total endometrial expression in the functional layer only and could not distinguish between epithelial and stromal expression, which may vary independently. Ideally, we should distinguish differences in the protein expressions between epithelial and stromal cells using samples obtained by a laser-captured microdissection (LCM) system. To date, however, we have not established a reliable LCM system for Western blotting. In immunohistochemistry, cytoplasmic pAkt expression of stromal cells in the functional layer increased in proportion to the pre-decidual reaction and was found to be prominent in decidual cells. Whereas the glandular epithelial cells showed strong nuclear staining, the immunoreactivity in pre-decidual and decidual cells was intense in the cytoplasm. Activated Akt near the cell membrane was reported to be transported and play an important physiological role in various regions including the nucleus in some tissues (Andjelkovic et al., 1997; Park et al., 2001; Lai et al., 2003). Guzeloglu Kayisli et al. (2004) reported that decidual cells expressed weak immunoreactivity for pAkt. However, our findings do not necessarily conflict with the results by Guzeloglu Kayisli et al. (2004). On immunohistochemistry, they reported semi-quantitatively that pAkt expression in decidual cells decreased using the N/C (nuclear/cytoplasmic) ratio. Similar to our study, they also reported the characteristic cytoplasmic expression of pAkt in decidual cells. As summarized in Table 1, the change in the N/C ratio during the menstrual cycle in our study similar to the data reported by Guzeloglu Kayisli et al. (2004).

In recent years, some investigators reported that Akt induced the synthesis of laminin-1 and collagen type IV isotypes and the remodelling of the basement membrane (Li et al., 2001). Furthermore, cells overexpressing Akt had elevated gelatinase
activity that was induced by MMP-2 and 9 (Thant et al., 2000; Park et al., 2001; Grille et al., 2003). It has also been reported that active Akt exhibited reduction of cell–cell adhesion and increased invasiveness in epithelial cells (Grille et al., 2003). Our study demonstrated that pAkt expression in stromal cells may result in dynamic changes in mainly functional layers where tissue remodelling occurs during menstrual cycle or implantation. In the basal layer of the endometrium, the stromal cells showed less immunoreactivity of pAkt at any stage of the menstrual cycle. These results show the possibility that Akt activation may be involved in remodelling of the ECM of the endometrium during menstruation and implantation as well as in inhibition of apoptosis of endometrial cells.

The factors regulating activation of Akt in endometrial stromal cells remain unclear. Sex steroid hormones, which regulate activation of Akt in many types of cells and induce decidualization of endometrial cells, may be involved. E2 was reported to activate some kinds of cells such as neural cells and endothelial cells via the PI3K/Akt pathway (Haynes et al., 2000; Ivanova et al., 2002). Guzeloglu Kayisli et al. (2004) also reported that E2 induced rapid activation of Akt in endometrial stromal cells. They reported that estrogen-mediated phosphorylation of Akt peaked in only 15 min, whereas they reported that long-term (3–24 h) estrogen treatment of endometrial stromal cells did not affect phosho-Akt levels. In preliminary study, we also found that long-term estrogen treatment (7 days) did not affect phosho-Akt levels. However, in this study we focused on the reaction of cultured decidualized stromal cells that were treated by both E2 and progesterone. Our current study demonstrated that the level of pAkt was decreased in decidualized stromal cells that were cultured for 7 days with E2 and progesterone, compared to untreated cells that were also cultured for the same number of days, Yoshino et al. (2003) and Pohnke et al. (2004) reported the same findings as our data that progesterone decreased pAkt expression in cultured decidualized cells, and the level of pAkt was decreased in cells undergoing in vitro decidualization. As for the expression of pAkt in decidual reaction, endometrium in vivo and cultured decidualized cells in vitro showed opposite results. There are several differences in in vivo and in vitro cellular environment such as an epithelial and a stromal cell interaction and immunological milieu constituted of bone marrow-derived cells, cytokines and growth factors in the endometrium. These factors may be able to explain the discrepancy of the expression of pAkt in vivo and in vitro.

Among many growth factors and cytokines, IGF-I is a candidate molecule that affects Akt activation in endometrial stromal cells. IGF-I may play a role in mediating the mitogenic effects of estrogen in uterine cells (Girvijan et al., 1994). It was also reported that IGF-I can be involved in decidualization of the endometrium (Hemenyre and Markoff, 1998) and stimulate the production of PRL in cultured decidualized stromal cells (Ben-Jonathan et al., 1996) as well as activate phosphorylation of Akt (Dudek et al., 1997; Kulik et al., 1997). Experimental data have already demonstrated that IGF-I can be locally produced by immune cells (Baxter et al., 1991). Our results clearly showed that IGF-I activates Akt via PI3K in cultured decidualized cells. Although detail examinations are required to determine the mechanism of activating Akt by IGFs in autocrine and paracrine fashion, IGF-I can explain at least partially the in vivo and in vitro discrepancy in expression of pAkt in decidual cells. Many investigators have determined the importance of IGFs in controlling the invasiveness of trophoblasts as well (Irwin et al., 2001; Crosse et al., 2002). The maintenance of expression of pAkt in decidual tissues may play a pivotal role in remodelling intercellular and extracellular structure for protecting the endometrial decidual cells against trophoblast invasion and provide a scaffold for migrating trophoblasts.

In summary, the level of pAkt (Ser473) protein was highest in the deciduals from ectopic pregnancy, though the level of Akt protein was unchanged during the menstrual cycle. The cytoplasm of pre-decidual and decidual cells showed intense immunostaining as well as nuclear staining. Endometrial stromal cells in the basal layer had little immunoreactivity of pAkt. The level of pAkt in cultured decidualized cells decreased after decidual change. The discrepancy of the results between in vivo and in vitro studies may be due to growth factors such as IGF-I that can activate Akt via PI3K. Further investigation may be required to examine the detailed mechanism of activation of Akt related to paracrine or autocrine regulation, epithelial–stromal interaction and the invasion of trophoblast in endometrium and deciduals.

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References


