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Article

Differential regulation of Akt phosphorylation in endometriosis

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Abstract

Protein kinase B (PKB/Akt), a serine/threonine kinase, regulates the function of many cellular proteins involved in apoptosis and proliferation. It was postulated that there is a higher Akt activity in endometriosis compared with normal endometrium, and that oestrogen may be one of the factors responsible for the high Akt activation in endometriotic cells. Phospho-Akt (pAkt) concentrations in normal, eutopic and ectopic endometrial tissues were compared by immunohistochemistry, and a higher pAkt immunoreactivity was revealed in eutopic and ectopic endometrium compared with normal endometrium, *in vivo*. Higher Akt phosphorylation in stromal cells from eutopic endometrium was observed, when compared with normal, *in vitro* ($P < 0.05$). Akt phosphorylation was rapidly (2–10 min) stimulated when endometrial stromal cells from normal and endometriosis patients were treated with 17β-oestradiol. In endometrial stromal cells from the endometriosis group, ICI 182,780 (ICI, a specific oestrogen receptor antagonist) failed to antagonize the effect of oestradiol when combined with oestradiol, and revealed a stimulatory effect on Akt phosphorylation when given alone ($P < 0.05$). In conclusion, since Akt affects cell survival, it is suggested that increased Akt phosphorylation may be related to the altered apoptosis/proliferation harmony in endometriosis, and therefore Akt may play a critical role in the pathogenesis of endometriosis.

Keywords: *Akt phosphorylation, endometriosis, oestradiol*

Introduction

The presence of endometrial tissue outside the uterine cavity is referred to as endometriosis, and is an oestrogen-dependent phenomenon (Seli and Arici, 2003). Endometriosis is a chronic painful inflammatory disease representing one of the most common benign gynaecological disorders. Estimates of its frequency vary from 2% to 10% in women of reproductive age and up to 30% in women with subfertility problems (Mahmood and Templeton, 1991; Moen and Schei, 1997; Witz and Burns, 2002; Vigano *et al.*, 2004).

Oestrogen and other sex steroids influence many physiological functions, including reproductive (Can and Semiz, 2000; Can *et al.*, 2005) and non-reproductive systems, such as the central nervous system, skeletal system, and cardiovascular system (Enmark and Gustafsson, 1999; DeMayo *et al.*, 2002). Oestrogen has various effects, via genomic and non-genomic pathways (Pedram *et al.*, 2002), through several different mechanisms, on cell viability, survival and proliferation, and up- or down-regulates the expression of genes depending on cell type and environmental milieu. This phenomenon, beginning from the transfer of oestrogen

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into the cell, binding to its receptor in the cell nucleus, and activating the oestrogen-responsive genes, is called the classical, or 'genomic', mechanism of oestrogen action, and occurs over the course of hours. In contrast, oestrogen can act more quickly (within seconds or minutes) via 'non-genomic' mechanisms (Mena *et al.*, 1985), either through the oestrogen receptor located in or adjacent to the plasma membrane, or through other non-oestrogen receptor, plasma membrane-associated oestrogen-binding proteins, resulting in cellular responses such as increased concentrations of Ca^{2+} or nitric oxide (NO), and activation of kinases, including protein kinase A (PKA), mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3-kinase (PI3K), the last of which activates the Akt signalling pathway (Honda *et al.*, 2000; Levin, 2001). It has been shown that oestrogen rapidly induces phosphorylation and activation of NO synthase through the PI3K/Akt pathway in human endothelial cells (Haynes *et al.*, 2000).

Protein kinase B (PKB/Akt) is a serine/threonine kinase, which phosphorylates and thus regulates the function of many cellular proteins involved in cell survival, growth, malignant transformation, vesicle trafficking and cytoskeletal regulation (Zingg *et al.*, 2004). The main pathway for Akt phosphorylation is the PI3K secondary messenger system. After the activation of certain growth factor receptors, protein tyrosine kinases result in autophosphorylation of tyrosine residues, triggering phosphatidylinositol-4,5-biphosphate to generate phosphatidylinositol-3,4,5-triphosphate, which is a secondary messenger. This in turn induces Akt phosphorylation on serine 473 and/or threonine 308, transforming it to its active form, pAkt (Testa and Bellacosa, 2001).

The expression of oestrogen receptors in the endometrium of women without endometriosis is similar to that of eutopic endometrium from women with endometriosis (Jones *et al.*, 1995). On the other hand, oestrogen receptor expression in both epithelium and stroma of ectopic endometrium is significantly higher than in eutopic endometrium and normal endometrium throughout the menstrual cycle. Oestrogen receptor expression in the ectopic endometrium increases from the proliferative to the late secretory phase (Jones *et al.*, 1995).

The expression pattern of Akt in normal endometrium and the effect of oestrogen on Akt phosphorylation was reported (Guzeloglu Kayisli *et al.*, 2004). The present study tested the hypothesis that there is higher Akt activity in endometriosis compared with normal endometrium, and that oestrogen may be one of the factors responsible for the high Akt activation in endometriotic cells. Therefore, levels of Akt phosphorylation were compared in normal, eutopic and ectopic endometrium using in-vivo and in-vitro techniques.

Materials and methods

Tissue collection

Endometrial tissues ($n = 14$, mean age, 39.5; range, 26–52) were obtained from human uteri after surgery for benign diseases excluding endometrial disease to be used as control. Histological dating was performed by grouping the samples as early proliferative (EP, days 1–6; $n = 3$), late proliferative (LP, days 7–14; $n = 3$), early secretory (ES, days 15–20; $n = 5$) and late secretory (LS, days 21–28; $n = 3$). Paired eutopic and ectopic endometriotic tissues were obtained from 15 (mean age, 40.4; range, 29–53) (EP, $n = 3$; LP, $n = 5$; ES, $n = 4$ and LS, $n = 3$) women with mild endometriosis during laparoscopy. Distribution of endometriosis tissues was as follows: ovarian endometriomas ($n = 8$), peritoneal implants ($n = 5$) and Fallopian tube implants ($n = 2$). Prior to surgery, informed consent approved by the Human Investigation Committee of Yale University was obtained from each patient. The collected tissue samples were carefully divided into two pieces, which were treated as follows; one for histological dating and immunohistochemistry (IHC), and the other for isolation and culture of endometrial stromal cells.

Isolation and culture of human endometrial stromal cells

Endometrial tissues were obtained from the control group ($n = 3$, ES phase) and endometriosis group ($n = 3$, ES phase), and then rinsed and minced in Hanks' Balanced Salt Solution (HBSS; Gibco BRL, Rockville, MD, USA) to remove blood cells and cellular debris. Then tissues were incubated in a digestion mixture of HBSS that contained HEPES (25 mmol/l; Sigma Chemical Co., St Louis, MO, USA), penicillin (200 IU/ml; Sigma), streptomycin (200 mg/ml; Sigma), collagenase I (1 mg/ml; Sigma) and deoxyribonuclease (150 IU/ml; Sigma) for 45 min at 37°C in a shaking bath. The dispersed endometrial cells were filtered through a wire sieve (73 μm diameter pore; Sigma). The cells that passed through the filter were considered as endometrial stromal cells and were plated in medium consisting of Dulbecco's modified Eagle's medium Ham's F12 containing 10% fetal bovine serum (FBS) and antibiotic–antimycotic (10,000 IU/ml penicillin G sodium, 10,000 μg/ml streptomycin sulphate and 25 μg/ml amphotericin B; Gibco BRL) in T-75 polystyrene flasks (75 cm^2; Falcon, Franklin Lakes, NJ, USA), maintained at 37°C in a 5% CO_2 in an air humidified incubator. Following 80% cellular confluence, trypsin–EDTA (0.5 g/l trypsin and 0.2 g/l EDTA.4Na; Gibco BRL) mixture was used for cell detachment, and cells were subcultured to six-well plates (2.5 cm^2/well; Falcon). Cells were allowed to replicate up to 80% confluence. Endometrial stromal cells were identified by immunocytochemistry among other cell types using cell-surface specific markers (Arici *et al.*, 1993). The confluent cells were incubated in serum-free, phenol red-free media (Sigma) for 24 h prior to treatment with oestradiol (Sigma) and/or a specific oestrogen receptor antagonist, ICI 182,780 (ICI). Ethanol (Sigma) was used as the vehicle in oestradiol dilutions at 10^{-8} mol/l or in ICI 10^{-6} mol/l. Cells were treated with oestradiol for 2, 5, 10, 20, or 30 min. Wortmannin (Sigma) at 10^{-6} mol/l was used to test the PI3K pathway-dependent Akt phosphorylation.

Immunohistochemistry and immunofluorescent staining

Paraffin embedded tissues were cut into 8 μm slices. Slides were deparaffinized and incubated in 10 mmol/l pH = 6.0

citrate buffer (citrate in phosphate-buffered saline; PBS) in a microwave oven at 400 W for 20 min for antigen retrieval, followed by washing with PBS for 15 min. Endogenous peroxidase activity was quenched by incubation of sections in 3% H_2O_2 for 10 min. Sections were incubated with Ultra V Block (Lab vision, Fremont, CA, USA) for 7 min at room temperature to decrease non-specific antibody binding. Thereafter, sections were incubated for 2 h at room temperature with pAkt (Ser473) mouse monoclonal antibody (Cell Signaling, Beverly, MA, USA) diluted 1:100 in PBS. Normal mouse immunoglobulin G2b (IgG2b) (Vector, Laboratories, Burlingame, CA, USA) was used instead of primary antibody as negative control. Sections were washed in PBS, incubated with biotinylated goat anti-mouse IgG (Vector Laboratories) for 1 h at room temperature and washed in PBS. Thereafter, they were incubated with streptavidin–peroxidase complex (LabVision) for 20 min at room temperature. 3,3′-Diaminobenzidine (DAB) reagent (LabVision) was used for the chromogenic reaction, which was terminated with tap water when the staining appeared. Slides were counterstained with Mayers haematoxylin (Sigma). After mounting slides were observed under a bright-field microscope (Nikon Eclipse E600, Japan). Immunostaining was evaluated using the HSCORE histological scoring system. HSCORE was calculated using the following equation: $\text{HSCORE} = \sum \text{P}i\ (i+1)$; where i is the intensity of staining with a value of 1, 2 or 3 (weak, moderate or strong respectively), Pi is the percentage of stained epithelial or stromal cells for each intensity varying from 0% to 100%, and 1 is a correction for optical density. This yielded a range of results from 0 for no staining to 4 for maximal staining.

Isolated endometrial stromal cells were labelled with a monoclonal anti-pAkt antibody (Cell Signaling, 1:100 in PBS) for 90 min at 37°C, then washed three times in PBS and incubated in a Texas red-conjugated goat anti-rabbit IgG (Sigma, 1:100 in PBS) for 60 min in a dark humidified chamber at 37°C. Slides were mounted in a 1:1 PBS/glycerol medium containing 1 μg/ml of Hoechst 33258 (Polyscience Inc., PA, USA) for nuclear staining and 25 mg/ml sodium azide as an anti-fading reagent. Slides were analysed in a fluorescent equipped light microscope (Nikon).

Western blot analysis

Total protein extraction from cultured endometrial stromal cells obtained from normal and endometriosis patients was performed using a cell extraction buffer (Biosource International Inc., Camarillo, CA, USA) with proteinase inhibitor cocktail (Sigma) and 1 mmol/l phenylmethylsulphonyl fluoride (Sigma). The protein concentration was determined using a detergent compatible protein assay kit (BioRad Laboratories, Hercules, CA, USA). Each lane of a 10% Tris–HCl sodium dodecyl sulphate polyacrylamide gel electrophoresis gel (BioRad) was loaded with 20 μg protein for electrophoresis. The proteins were electro blotted onto a nitrocellulose membrane (BioRad) (Towbin *et al.*, 1979). The membrane was then incubated in 5% non-fat dry milk in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBS-T) for 1 h to reduce non-specific antibody binding. The membranes were incubated with anti-pAkt (Ser473) mouse monoclonal antibody (Cell Signaling) for 2 h at room temperature and then were washed with TBS-T for 1 h. Peroxidase labelled anti-mouse IgG (Vector) in TBS-T incubation for 1 h at room temperature was followed by washing with TBS-T for 1 h. Chemiluminescent detecting reagent (Amersham-Biosciences, NJ, USA) and 25 × 25 cm film (Kodak, Rochester, NY, USA) were used to show antibody-bound bands. Membranes were stripped and marked with rabbit polyclonal anti-human Akt antibody (Cell Signaling). Similar steps were applied to stripped membranes as described above. Each experiment was repeated three times using the proteins that were isolated from six independent endometrial cell cultures, three for the normal group and three for the endometriosis group.

Statistical analysis

HSCORE assessments and signal intensities from Akt and pAkt bands were compared using the computer-based SigmaStat software package (version 3.0; Jandel Scientific Corporation, San Rafael, CA, USA). The differences among each group were tested using one-way analysis of variance (ANOVA) and post hoc Ranks tests. Significance level was set at $P < 0.05$.

Results

Regulation of pAkt in endometriosis *in vivo*

To compare in-vivo Akt activity among normal, eutopic and ectopic endometrial cells, immunohistochemistry was performed using pAkt (Ser473) antibody on paraffin sections obtained from women with or without endometriosis (**Figure 1**). Localization of pAkt was restricted to nuclear and perinuclear regions (**Figure 1H**).

In normal endometrial tissues, late proliferative stromal and glandular cells revealed significantly higher pAkt immunoreactivity compared with those from early proliferative phase ($P < 0.05$; **Figures 1** and **2A**). On the other hand, in eutopic endometrial tissues early secretory phase stromal and glandular cells revealed a significantly higher immunoreactivity when compared with those from early proliferative phase ($P < 0.05$; **Figures 1C**, **D** and **2A**). Moreover, eutopic endometrial stromal cells and endometrial glandular cells showed significantly higher pAkt immunoreactivity compared with normal endometrial stromal cells and endometrial glandular cells during early secretory phase ($P < 0.05$; **Figures 1B**, **D** and **2A**).

Ectopic and eutopic endometrial stromal cells revealed significantly stronger pAkt immunoreactivity compared with normal endometrial stromal cells when all phases of the menstrual cycle were considered together ($P < 0.05$; **Figure 2B**). However, no difference was noted between ectopic and eutopic endometrial stromal cells (**Figures 1E**, **F** and **2B**). Furthermore, ectopic endometrial glandular cells showed significantly stronger pAkt immunoreactivity when compared the both eutopic and normal

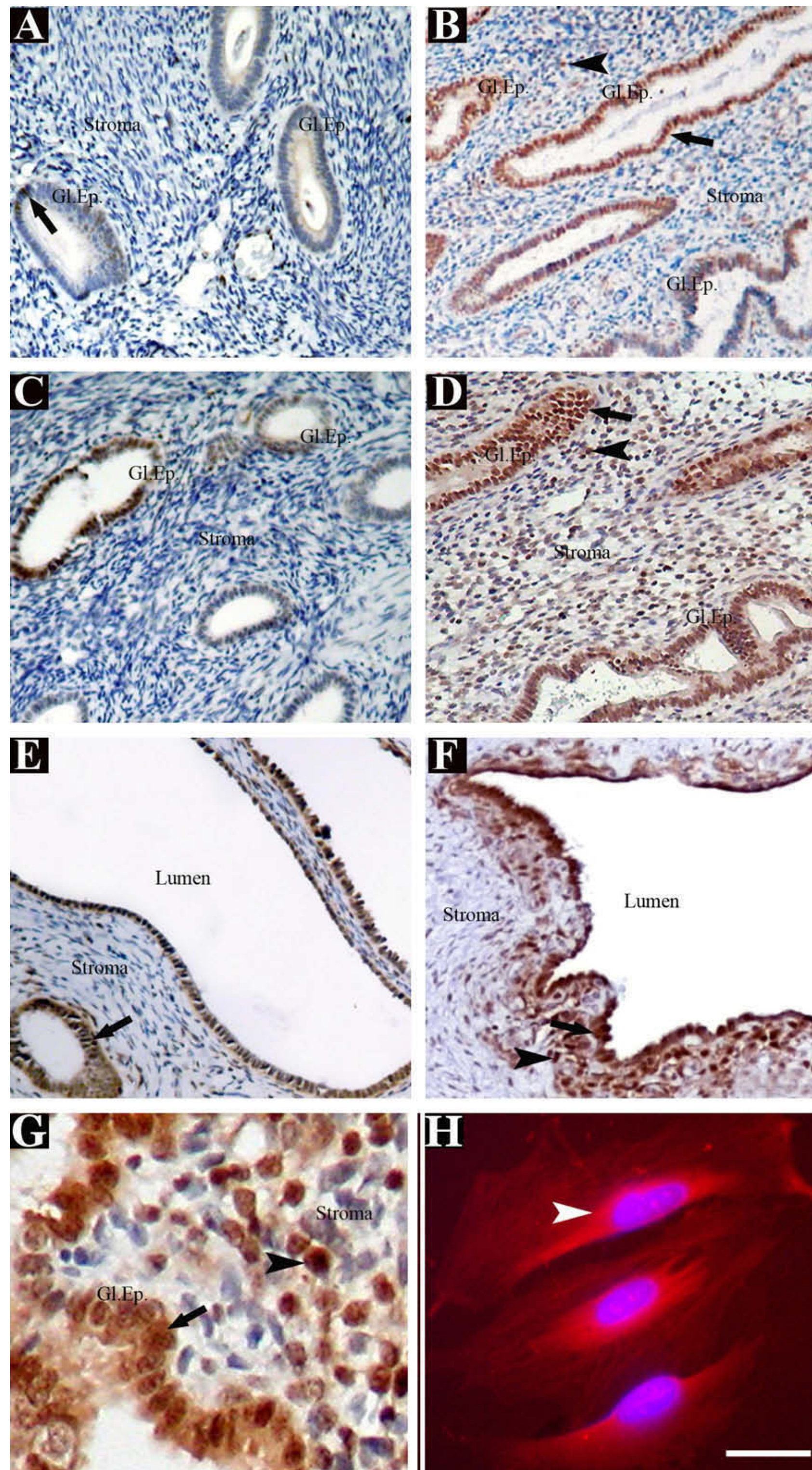

Figure 1. Immunolocalization of protein kinase B (pAkt) in human endometrium. Normal endometrium from early proliferative (**A**) and early secretory (**B**) phases. Eutopic endometrium from early proliferative (**C**) and early secretory (**D** and **G**) phases. Ectopic endometrium from early proliferative (**E**) and early secretory (**F**) phases. Immunofluorescent staining of pAkt in cultured endometriotic stromal cells after 2 min oestradiol (10^{-8} mol/l) exposure (**H**). Red signals indicate pAkt immunostaining and blue signals indicate nucleus with Hoechst 33258. Gl.Ep. = glandular epithelium, arrow = positive stained glandular epithelium, arrowhead (black) = positive stained stromal cell/s, arrowhead (white) = perinuclear pAkt immunopositivity. Bar = 200 μm for **A**–**F**, 40 μm for **G** and 7 μm for **H**.

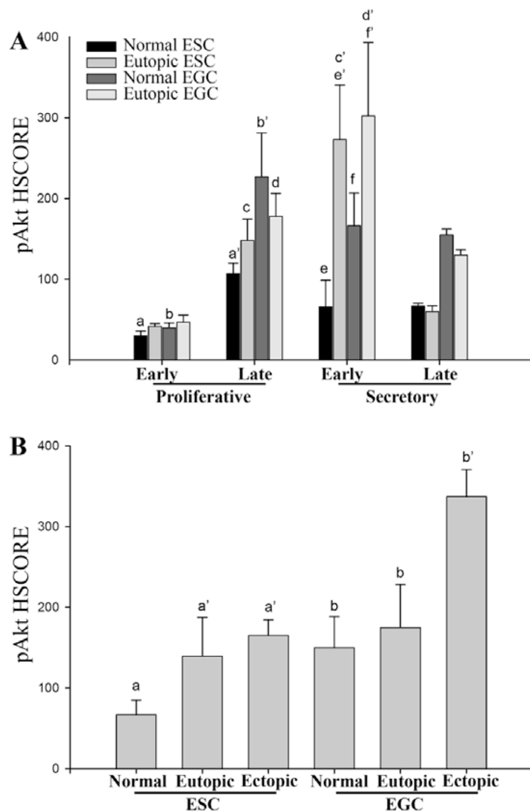

Figure 2. Histological scoring system (HSCORE) analysis of protein kinase B (pAkt) immunostaining in stromal and glandular cells. (**A**) Comparison of the HSCOREs in normal and eutopic endometrial tissues according to menstrual cycle phases. (**B**) Comparison of the HSCOREs in normal, eutopic and ectopic paired of eutopic endometrial stromal and glandular cells. Values are expressed as means ± SEM. $P < 0.05$ for (A) a, b, c, d, e, f versus a′, b′, c′, d′, e′, f′ and (**B**) a, b versus a′, b′ respectively. EGC = endometrial glandular cells; ESC = endometrial stromal cells.

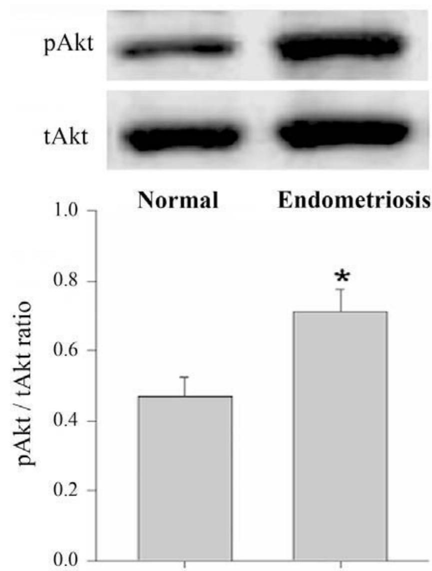

Figure 3. Western blot analysis of phosphorylated protein kinase B (pAkt) and total Akt (tAkt) in untreated normal and eutopic endometrial stromal cells in culture. Experiments were repeated three times with similar results and values are expressed as means ± SEM. $^{*}P < 0.05$.

endometrial glandular cells ($P < 0.05$; **Figures 1E**, **F** and **2B**).

Regulation of pAkt by oestrogen in endometriotic cells *in vitro*

To compare total and phosphorylation level of Akt in endometrial stromal cells from normal and endometriosis groups, cell culture was used and Western blot analysis was performed. First, total (t) and pAkt concentrations were semi-quantified in endometrial stromal cells obtained from normal endometrium and eutopic endometrium with-

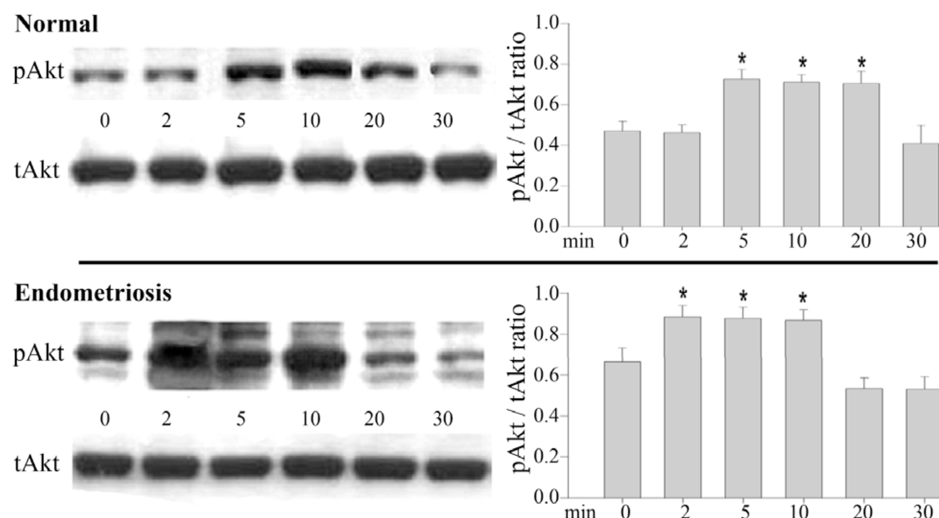

Figure 4. Western blot analysis of oestradiol (10^{-8} mol/l)-induced protein kinase B (Akt) phosphorylation in cultured normal and endometriotic stromal cells in a time-dependent (0–30 min) manner. Experiments were repeated three times with similar results and values are expressed as means ± SEM. $^{*}P < 0.05$.

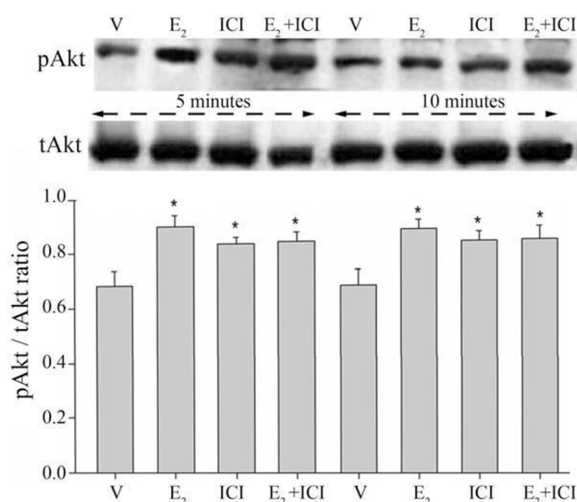

Figure 5. Analysis of the temporal effects (5–10 min) of oestradiol (E_2; 10^{-8} mol/l), oestrogen receptor inhibitor ICI (10^{-6} mol/l) on protein kinase B (Akt) phosphorylation in eutopic endometriosis cells (V = vehicle). $^{*}P < 0.05$, oestradiol and/or ICI treated cells versus control cells.

out any treatment. Endometrial stromal cells from the endometriosis group revealed a significantly higher pAkt/tAkt ratio, when compared with normal ($P < 0.05$; **Figure 3**).

The effect of oestradiol on Akt phosphorylation was tested by treating the cultured endometriotic and normal endometrial stromal cells with oestradiol (10^{-8} mol/l) in a time-dependent manner (0–30 min). Akt phosphorylation was significantly higher at 5–20 min in the normal group and 2–10 min in the endometriosis group compared with the other time points ($P < 0.05$; **Figure 4**). Moreover, in both normal and endometriotic endometrial stromal cells treated with oestradiol, pAkt concentrations returned to their baseline level after 30 min (**Figure 4**).

To investigate if the effect of oestradiol on Akt phosphorylation is oestrogen receptor dependent, endometrial stromal cells were treated with oestradiol (10^{-8} mol/l), ICI (10^{-6} mol/l), a pure oestradiol receptor antagonist, or oestradiol + ICI. ICI alone, interestingly, revealed a stimulatory effect on Akt phosphorylation in endometriotic stromal cells (**Figure 5**). When ICI was combined with oestradiol, ICI did not inhibit oestradiol-induced Akt phosphorylation in endometriotic stromal cells (**Figure 5**). Moreover, wortmannin (10^{-6} mol/l), a specific PI3K inhibitor, completely blocked the phosphorylation of Akt in the presence or absence of oestradiol (data not shown).

Discussion

Endometriosis is a chronic inflammatory disease, characterized by implantation and growth of endometrial tissue outside the uterine cavity. Since decreased apoptosis exists in both eutopic and ectopic endometrium of women with endometriosis when compared with normal endometrium (Garcia-Velasco and Arici, 2003; Beliard *et al.*, 2004; Goumenou *et al.*, 2004), one could suggest that proteins affecting the apoptotic mechanism and cell proliferation may play a central role in the aetiopathogenesis of endometriosis. One such protein is protein kinase B (Akt). Following its activation, Akt binds to its substrates, which are known as survival/anti-apoptotic or cell growth/proliferation proteins (Ahmed *et al.*, 1997; Zingg *et al.*, 2004). For instance, an apoptotic protein BAD, a B-cell CLL/lymphoma 2 (Bcl-2) family member, has an apoptotic effect (Khor *et al.*, 2004) and its apoptotic activity is suppressed by Akt. Akt also inhibits proapoptotic proteins such as procaspase-9 and forkhead family transcription factors (Skurk *et al.*, 2004). Moreover, Akt phosphorylation has an anti-apoptotic role by inhibiting TRAIL (tumour necrosis factor alpha related apoptosis-inducing ligand)-induced apoptosis (Whang *et al.*, 2004).

In the present study, the aim was to evaluate the Akt phosphorylation pattern and its relation to the menstrual cycle in endometriosis, and to compare endometrial samples from women with and without endometriosis. For this purpose, pAkt immunoreactivity in normal, eutopic and ectopic endometrium throughout the menstrual cycle was analysed. It was shown that Akt phosphorylation changes temporally and spatially throughout the menstrual cycle in normal, eutopic and ectopic endometrium, and that oestrogen plays a role in its regulation changes.

Temporal–spatial changes of Akt phosphorylation during the menstrual cycle and early pregnancy were previously demonstrated in human endometrium (Guzeloglu Kayisli *et al.*, 2004). In the present study, significant differences were observed in the level of Akt phosphorylation between normal, eutopic and ectopic endometriotic samples *in vivo*. Akt phosphorylation was found to be higher in eutopic endometrium than in normal stroma and glands. Moreover, high levels of pAkt immunostaining were observed in both stromal and glandular cells in early secretory phase eutopic endometrium compared with normal endometrium. Dmowski *et al.* showed that the amount of apoptotic cells in early proliferative and late secretory phase was higher than late proliferative and early secretory phase eutopic endometrium, as observed in cycle-matched normal endometrium (Dmowski *et al.*, 2001). Dmowski *et al.* (2001) have also shown that eutopic endometrium has significantly fewer apoptotic cells compared with normal endometrium during early proliferative and late secretory phases. It was detected that the distribution and intensity of pAkt were higher in late proliferative and early secretory compared with early proliferative and late secretory phases, suggesting that the increase in pAkt may lead to a decrease in apoptotic cell frequency, which supports the anti-apoptotic mechanisms of Akt phosphorylation (Zhou *et al.*, 1998). Gebel *et al.* demonstrated that apoptosis decreased in ectopic endometrium compared with eutopic endometrium in paired samples (Gebel *et al.*, 1998). Here, it is illustrated that pAkt immunoreactivity increases in ectopic endometrium compared with normal endometrium. Goumenou *et al.* noted that the percentage of apoptotic cells was higher in stromal cells compared with glandular cells; however, Bcl-2/Bax ratio was higher in the glandular epithelium than endometriotic stroma (Goumenou *et al.*, 2004). No significant dif-

ference was observed in pAkt immunoreactivity between eutopic and ectopic endometrial stromal cells, but both were significantly higher than normal endometrial stromal cells. Moreover, ectopic glandular cells have significantly higher Akt activity than normal and eutopic endometrial glandular cells.

Higher pAkt concentration, but not tAkt concentration, in cultured endometrial stromal cells obtained from eutopic endometrial cells as detected by Western blot analysis supports IHC findings. Previously, an increase in Akt phosphorylation by oestrogen in neuronal cells was demonstrated (Honda *et al.*, 2000; Levin, 2001; Mannella and Brinton, 2006)). It was reported that the biological effects of oestradiol are exerted by two mechanisms, genomic and non-genomic (Mena *et al.*, 1985). The results suggest rather a rapid phosphorylation of Akt following oestradiol treatment, indicative of non-genomic activation by oestradiol. This finding is comparable with the Yoshino *et al.* report, in which the concentration of pAkt was increased by oestradiol in endometrial stromal cells (Yoshino *et al.*, 2003). Stoica *et al.* reported that oestradiol can rapidly activate PI3K/Akt, and this effect is mediated by the ErbB2 signalling pathway in the MCF-7 breast cancer cell line (Stoica *et al.*, 2003). They also noted that treatment of cells with oestradiol resulted in phosphorylation of Akt, a ninefold increase in Akt activity in 10 min; and Akt activation was blocked by wortmannin; by AG825, a selective ErbB2 inhibitor; and by the anti-oestrogens ICI and 4-hydroxy-tamoxifen. Here, it has been shown that wortmannin blocks phosphorylation of Akt, indicating the reaction was achieved via the PI3K pathway.

It has previously been shown that ICI alone did not alter Akt phosphorylation, and when ICI combined with oestradiol, ICI did not inhibit oestradiol-induced Akt phosphorylation in normal endometrial stromal cells in culture (Guzeloglu Kayisli *et al.*, 2004). In contrast, here it was noted that ICI alone stimulated Akt phosphorylation in endometrial stromal cells obtained from the eutopic endometrium of endometriosis patients, suggesting that ICI behaves differently in normal versus endometriotic cells in terms of oestrogen-mediated Akt activation. One of the explanations for this aberrant behaviour in endometrial stromal cells of endometriosis could be related to the relative concentration of oestrogen α and β receptors, and suggests a non-genomic interaction between Akt, ICI and oestrogen receptors, since previous studies have also shown changes in the ratio of oestrogen α to β receptors in endometriotic cells compared with normal endometrial cells (Jackson *et al.*, 2007; Xue *et al.*, 2007; Bukulmez *et al.*, 2008). Anti-oestrogenic effects of ICI are known to occur by producing conformational changes in oestrogen receptors (Wakeling *et al.*, 1991). Moreover, in a recent study, Zhao *et al.* demonstrated that ICI induced significant activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt with efficacy comparable with neurons treated with oestradiol, and thus they concluded that the *in vitro* analyses of ICI provides direct evidence of an oestrogenic agonist profile of ICI action in rat hippocampal neurons (Zhao *et al.*, 2006). Similarly, investigation on the influence of oestradiol on developing neonatal rat cerebellar neurons in primary culture revealed that low concentrations of oestradiol, 17α-oestradiol and ICI stimulate phosphorylation of the ERK1/2 mitogen-activated protein kinase (Wong *et al.*, 2003). Mercier *et al.* noted that ICI treatment increased ERK1/2 phosphorylation in cardiac myocytes and cardiac fibroblasts via an oestrogen-independent pathway (Mercier *et al.*, 2003). When taken together, the present results suggest that ICI may not antagonize, and may in fact augment some of the non-genomic actions of oestrogen, including the oestradiol-mediated phosphorylation of Akt in endometrial stromal cells of endometriosis.

It is concluded that there is higher Akt activity (phosphorylation) in both eutopic and ectopic endometrial cells compared with normal endometrium, supporting the hypothesis that increased cell survival in endometriotic cells may be related to Akt activation. Furthermore, one of the factors responsible for higher Akt phosphorylation in endometriotic cells may be the continuous local stimulation of endometriotic cells by oestrogen due to its local production. Therefore, Akt may play a central role in endometriosis by increasing cell survival and up-regulating anti-apoptotic cascades.

References

Ahmed NN, Grimes HL, Bellacosa A *et al.* 1997 Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. *Proceedings of the National Academy of Sciences of the USA* **94**, 3627–3632.

Arici A, Head JR, MacDonald PC, Casey ML 1993 Regulation of interleukin-8 gene expression in human endometrial cells in culture. *Molecular and Cellular Endocrinology* **94**, 195–204.

Beliard A, Noel A, Foidart JM 2004 Reduction of apoptosis and proliferation in endometriosis. *Fertility and Sterility* **82**, 80–85.

Bukulmez O, Hardy DB, Carr BR *et al.* 2008 Inflammatory status influences aromatase and steroid receptor expression in endometriosis. *Endocrinology* **149**, 1190–1204.

Can A, Semiz O 2000 Diethylstilbestrol (DES)-induced cell cycle delay and meiotic spindle disruption in mouse oocytes during in-vitro maturation. *Molecular Human Reproduction* **6**, 154–162.

Can A, Semiz O, Cinar O 2005 Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis. *Molecular Human Reproduction* **11**, 389–396.

DeMayo FJ, Zhao B, Takamoto N, Tsai SY 2002 Mechanisms of action of estrogen and progesterone. *Annals of the New York Academy of Sciences* **955**, 48–59, discussion 86–8, 396–406.

Dmowski WP, Ding J, Shen J *et al.* 2001 Apoptosis in endometrial glandular and stromal cells in women with and without endometriosis. *Human Reproduction* **16**, 1802–1808.

Enmark E, Gustafsson JA 1999 Oestrogen receptors – an overview. *Journal of Internal Medicine* **246**, 133–138.

Garcia-Velasco JA, Arici A 2003 Apoptosis and the pathogenesis of endometriosis. *Seminars in Reproductive Medicine* **21**, 165–172.

Gebel HM, Braun DP, Tambur A *et al.* 1998 Spontaneous apoptosis of endometrial tissue is impaired in women with endometriosis. *Fertility and Sterility* **69**, 1042–1047.

Goumenou AG, Matalliotakis IM, Tzardi M *et al.* 2004 Apoptosis and differential expression of apoptosis-related proteins in endometriotic glandular and stromal cells. *Journal of the Society of Gynecologic Investigation* **11**, 318–322.

Guzeloglu Kayisli O, Kayisli UA, Luleci G, Arici A 2004 In vivo and in vitro regulation of Akt activation in human endometrial cells is estrogen dependent. *Biology of Reproduction* **71**, 714–721.

Haynes MP, Sinha D, Russell KS *et al.* 2000 Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. *Circulation Research* **87**, 677–682.

Honda K, Sawada H, Kihara T *et al.* 2000 Phosphatidylinositol 3-kinase mediates neuroprotection by estrogen in cultured cortical neurons. *Journal of Neuroscience Research* **60**, 321–327.

Jackson KS, Brudney A, Hastings JM *et al.* 2007 The altered distribution of the steroid hormone receptors and the chaperone immunophilin FKBP52 in a baboon model of endometriosis is associated with progesterone resistance during the window of uterine receptivity. *Reproductive Sciences* **14**, 137–150.

Jones RK, Bulmer JN, Searle RF 1995 Immunohistochemical characterization of proliferation, oestrogen receptor and progesterone receptor expression in endometriosis: comparison of eutopic and ectopic endometrium with normal cycling endometrium. *Human Reproduction* **10**, 3272–3279.

Khor TO, Gul YA, Ithnin H, Seow HF 2004 Positive correlation between overexpression of phospho-BAD with phosphorylated Akt at serine 473 but not threonine 308 in colorectal carcinoma. *Cancer Letters* **210**, 139–150.

Levin ER 2001 Cell localization, physiology, and nongenomic actions of estrogen receptors. *Journal of Applied Physiology* **91**, 1860–1867.

Mahmood TA, Templeton A 1991 Prevalence and genesis of endometriosis. *Human Reproduction* **6**, 544–549.

Mannella P, Brinton RD 2006 Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. *Journal of Neuroscience* **26**, 9439–9447.

Mena MA, Grunert G, Mansilla MS *et al.* 1985 Inhibition of non-genomic responses to oestrogen in the rat uterus by testosterone propionate. *Journal of Reproduction and Fertility* **74**, 1–7.

Mercier I, Mader S, Calderone A 2003 Tamoxifen and ICI 182,780 negatively influenced cardiac cell growth via an estrogen receptor-independent mechanism. *Cardiovascular Research* **59**, 883–892.

Moen MH, Schei B 1997 Epidemiology of endometriosis in a Norwegian county. *Acta Obstetricia et Gynecologica Scandinavica* **76**, 559–562.

Pedram A, Razandi M, Aitkenhead M *et al.* 2002 Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. *Journal of Biological Chemistry* **277**, 50768–50775.

Seli E, Arici A 2003 Endometriosis: interaction of immune and endocrine systems. *Seminars in Reproductive Medicine* **21**, 135–144.

Skurk C, Maatz H, Kim HS *et al.* 2004 The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. *Journal of Biological Chemistry* **279**, 1513–1525.

Stoica GE, Franke TF, Wellstein A *et al.* 2003 Estradiol rapidly activates Akt via the ErbB2 signaling pathway. *Molecular Endocrinology* **17**, 818–830.

Testa JR, Bellacosa A 2001 AKT plays a central role in tumorigenesis. *Proceedings of the National Academy of Sciences of the USA* **98**, 10983–10985.

Towbin H, Staehelin T, Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the USA* **76**, 4350–4354.

Vigano P, Parazzini F, Somigliana E, Vercellini P 2004 Endometriosis: epidemiology and aetiological factors. *Best Practice and Research in Clinical Obstetrics and Gynaecology* **18**, 177–200.

Wakeling AE, Dukes M, Bowler J 1991 A potent specific pure antiestrogen with clinical potential. *Cancer Research* **51**, 3867–3873.

Whang YE, Yuan XJ, Liu Y *et al.* 2004 Regulation of sensitivity to TRAIL by the PTEN tumor suppressor. *Vitamins and Hormones* **67**, 409–426.

Witz CA, Burns WN 2002 Endometriosis and infertility: is there a cause and effect relationship? *Gynecologic and Obstetric Investigation* **53**(Suppl. 1), 2–11.

Wong JK, Le HH, Zsarnovszky A, Belcher SM 2003 Estrogens and ICI182,780 (Faslodex) modulate mitosis and cell death in immature cerebellar neurons via rapid activation of p44/p42 mitogen-activated protein kinase. *Journal of Neuroscience* **23**, 4984–4995.

Xue Q, Lin Z, Cheng YH *et al.* 2007 Promoter methylation regulates estrogen receptor 2 in human endometrium and endometriosis. *Biology of Reproduction* **77**, 681–687.

Yoshino O, Osuga Y, Hirota Y *et al.* 2003 Akt as a possible intracellular mediator for decidualization in human endometrial stromal cells. *Molecular Human Reproduction* **9**, 265–269.

Zhao L, O'Neill K, Brinton RD 2006 Estrogenic agonist activity of ICI 182,780 (Faslodex) in hippocampal neurons: implications for basic science understanding of estrogen signaling and development of estrogen modulators with a dual therapeutic profile. *Journal of Pharmacology and Experimental Therapeutics* **319**, 1124–1132.

Zhou H, Summers SA, Birnbaum MJ, Pittman RN 1998 Inhibition of Akt kinase by cell-permeable ceramide and its implications for ceramide-induced apoptosis. *Journal of Biological Chemistry* **273**, 16568–16575.

Zingg D, Riesterer O, Fabbro D *et al.* 2004 Differential activation of the phosphatidylinositol 3′-kinase/Akt survival pathway by ionizing radiation in tumor and primary endothelial cells. *Cancer Research* **64**, 5398–5406.

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