Ph.D. Thesis

MECHANISMS OF CELL PROLIFERATION IN THE UTERUS

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Introduction

Estradiol (E2) exerts its effects through multiple mechanisms. In the „classic" mode of action E2 binds to estrogen receptor, which in turn facilitates transcription of estrogen reponsive genes by binding to the DNA and/or other transcription factors. Other („nongenomic") effects of E2 can not be prevented by transcriptional inhibitors or are too rapid to be explained by transcriptional changes. These effects include facilitation of Ca^{2+} uptake and activation of MAP kinase or phosphatidylinositol-3 kinase (PI3K)-Akt pathways. The PI3K-Akt pathway has a central role in regulation of cell cycle, apoptosis and cellular metabolism. This pathway begins with the activation of PI3K leading to phosphorylation of Akt which activates antiapoptotic and inhibit proapoptotic processes and facilitates cell proliferation through phosphorylation if several proteins. The mechanism by which E2 activates PI3K is not clear yet. Though it has been demonstrated that the estrogen receptor can bind to the catalytic subunit of PI3K resulting in its activation, the significance of this mechanism in uterine tissue has not been proven. One of the substrates of Akt are the FOXO proteins which are members of the forkhead-box family of transcription factors. As transcription factors, they regulate cellular physiology by altering the transcriptional activity of the cell. Among others, they induce expression of proteins which arrest the cell cycle or have proapoptotic effects. FOXO proteins are regulated by posttranslational modifications. In cell culture systems phosphorylation of FOXO proteins by Akt inhibits their transcriptional activity, and it may contribute to the antiapoptotic effect of Akt.

Recently, PI3K-Akt pathway has been extensively studied in cell culture systems, and by now the major issues if its regulation and effect have been cleared. However, regulation of physiological an pathological processes in vivo is more complex because of the multicellular nature of tissues and the presence of a wide array of endocrine and paracrine mediators. Animal experiments are demanded to study cellular physiology in the context of the whole animal and to develop molecules that could be used to targeted manipulation signal transduction pathways in vivo.

Stimulation of cell proliferation is one of the most important effects of E2. Intensity of cell proliferation is determined by the ratio of survival (proliferative and antiapoptotic) effects and proapoptotic effects. The scientific literature and the prior work done in our laboratory suggests that the PI3K-Akt pathway plays a central role in the mechanism of E2 action, regulation of uterine cell proliferation, and development of different pathological conditions of the reproductive tract. In the present work we examine some aspects of the PI3K-Akt-FOXO1 pathway in rat uterus as an in vivo model and in tissue samples derived from cycling and menopausal women.

The aim of our study

In this work we aimed to answer the following questions:

Is Akt expressed and phosphorylated in rat uterus? If so, then does acute E2 treatment have any effect on its expression or phosphorylation?

How does expression, phosphorylation an E2 responsiveness change during development?

Is it estrogen receptor α or some other, ”non-classical” estrogen receptor that mediates the effect of E2 on Akt phosphorylation?

Is Akt activated through a PI3K dependent, or a PI3K independent way in the uterus?

Is FOXO1 expressed in rat uterus? Is it phosphorylated on its two Akt sensitive sites (Thr^{24} és Ser^{256})? How do accute E2 treatment and inhibition if PI3K by Wortmannin affect phosphorylation of these residues?

How does the level of Cyclin D1 and Fas-ligand mRNA change in response to E2 treatment and inhibition if PI3K?

What are the characteristics of Akt expression and activation in human uterus during the menstrual cycle and menopause?

Methods

Animals

Sexually immature (7, 11, 14, 21, 28 and 35-day old) and adult female Wistar rats were used for our experiments. Mature rats were ovariectomized five days before treatment under light ether narcosis. The experimental protocol was approved by our Institutional Ethics Committee for Animal Care and Use.

Treatments

The antiestrogen ICI 182,780 (2 mg/ml, in 96% ethanol) was injected intraperitoneally (0.1 mg/100g tt.). Wortmannin was dissolved in DMSO (1 mg/ml) and 5 µl of this solution (or pure DMSO) was injected in the right uterine horn of anesthetized animals. Estradiol (E2) was dissolved in 25% ethanol and injected i.p. at a dose of 10µg/100g bw unless stated otherwise. At the end of the experiments animals were decapitated, their uteri were removed, weighed and the two horns were separated and stored at −80 ◦C.

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Human uterus

Examination of human tissue samples was carried out in collaboration with the Department of Obstetrics and Gynecology of our University. The Institutional Human Studies Committee approved the use of the tissues and informed consent was obtained by hysterectomy from the patients. Normal and pathological (leiomyoma) myometrial specimens were obtained from cyclic and menopausal women (aged 38–55 year) undergoing hysterectomy for benign indications with no history of hormonal treatment for at least 3 mo. before hospitalization. The stage of menstrual cycle or the presence of menopause was assessed by hystological examination of the endometriums and determination of FSH, estrogen and progesterone levels of the blood taken on the morning of the operation.

After the operation myoma was dissected from the uterus. Myometrial samples, for use as paired controls, were obtained from surrounding normal myometrium**.** Samples were frozen in liquid nitrogen and stored at −80 ◦C until use.

Western blot

Frozen uteri were homogenized at 4 ◦C, homogenates were electrophoretised on polyacrylamide gel and blotted on nitrocellulose membrane. Membranes were treated with primary and secondary antibodies. We used anti-rabbit, HRP-conjugated antibody as a secondary antibody. Blots were visualized by peroxidase reaction.

Isolation of subcellular fractions

Tissue samples were partially homogenized by ulta-turax homogenizer in slightly hypotonic isolation buffer, further homogenized using a Dounce Tissue Grinder and centrifuged at 750g. Supernatant was collected and centrifuged at 10 000g, the resulting

supernatant (cytoplasm-rich homogenate) was collected and trichloro-acetic acid was added to denatured proteins. The pellet of the first (low-speed) centrifugation was washed in the presence of a slight detergent and centrifuged again at 750g. The pellet (nucleus-rich homogenate) was dissolved in isolation buffer, and trichloro-acetic acid was added. Denaturized proteins from both fractions were washed three times with acetone and after complete drying they were dissolved in the homogenization buffer used for electrophoresis.

Quantitative RT PCR

Total RNA was isolated using TRIzol reagent, and reverse transcription was performed. The quality of the cDNA was checked by conventional PCR. Quantitative PCR reaction was performed using SyBr Green Supermix, as described by the manufacturer. The program settings were: 3` 95^{o}C, then 45 amplification cycles (10`` 92 $^{o}$C, 10'' 55 $^{o}$C, plate read, 12`` 72 ^{o}C), and then a final elongation step 1` 72 ^{o}C. After the reaction melting curve analysis was performed. Results were analised using the $\Delta\Delta C_T$ Livak method. Primers used for our work are listed in tabe I.

I. táblázat

	Primer name	Sequence	NCBI accession number of the cDNA used for the design of the primer
conventional PCR	Beta actin forward primer	5' AGCCATGTACGTAGCCATCC 3'	NM_031144
	Beta actin reverse primer	5' AAGGGTGTAAAACGCAGCTC 3'	
QT PCR	Beta actin forward primer	5' AGCCATGTACGTAGCCATCC 3'	
	Beta actin reverse primer	5'AGCGCGTAACCCTCATAGAT 3'	
	Cyclin D1 forward primer	5'TAGGGCTGGTAGCATGAGGT 3'	NM_171992
	Cyclin D1 reverse primer	5' CACGGTCCCTACTTCCAAAC 3'	
	Fas-ligand forward primer	5' TCTGGTTGGAATGGGGTTAG 3'	NM_012908
	Fas-ligand reverse primer	5' TTGGTTTCAGAGGGTGTGC 3'	

Statistics

The program Image Tool (Roswell, GA, USA) was used for the densitometric analysis of Western blots. For statistical analysis of the results of animal experiments we used Student's t test, and for the results of human experiments ANOVA test followed by Student-Newman-Keul's multiple range test.

Results

Akt is expressed in the uterus of developing and adult rats, and it is phosphorylated in an age dependent manner.

Akt protein was expressed in all animals examined (7, 14, 21, 28 and 35 days old) to the same extent, but the rate of its phosphorylation increased during development.

Estradiol administration increased the rate of Akt phosphorylation in 28 and 60 days old (ovariectomized) rats, but not in 11 days old ones.

Akt is expressed and phosphorylated in an E2 dependent manner in adult rat uterus.

Adult, ovariectomized rats were treated with different doses of E2 (1, 10 or 100μg/100g bw.). A two hour E2 treatment (10μg/100g bw) increased the level of $pSer^{473}$-Akt significantly, while it had no effect on the expression of the protein.

Using higher E2 concentration (100μg/100g bw) did not increase effectivity, but lowering the dose to 1μg/100g bw resulted in a delayed response.

Akt is phosphorylated in an ICI 182,780 and Wortmannin sensitive way in the uterus

Adult rats were treated with ICI 182,780 (a selective antagonist of estrogen receptor) and one hour later with E2. Estradiol failed to increase phosphorylation of Akt in the uteri pretreated with ICI 182,780. Injecting ICI 182,780 alone had no effect.

Intrauterine injection of Wortmannin (5μg/animal) reduced the level of $pSer^{473}$-Akt, suggesting a role of PI3K in Akt phosphorylation in the uterus.

FOXO1 is expressed in rat uterus and it is phosphorylated in response to E2 in a PI3K dependent way

Akt phosphorylates FOXO1 at Thr^{24} and Ser^{256} residues in vivo. We analyzed changes in FOXO1 phosphorylation in response to E2 treatment and inhibition of PI3K.

The level of $pSer^{256}$-FOXO1 was low in control animals, increased at 2 and 6 hours and returned to control level by 12 hours after E2 injection. Phosphorylation rate of Thr^{24} residue of FOXO1 did not respond to a two hour E2 treatment but it was increased 12 and 24 hours after E2 administration. Wortmannin efficiently inhibited phosphorylation of Thr^{24}-FOXO1 and reduced phosphorylation of Ser^{256} to about 50%.

Estradiol influences the intracellular distribution of phosphorylated forms of FOXO1

Next we isolated nucleus rich and cytoplasm rich fractions from uteri of animals treated with E2 and/or Wortmannin. In the uteri treated with E2 alone a strong pSer^{256}-FOXO1 signal was detected in the nuclear fraction. In the uteri treated with Wortmannin or both E2 and Wortmannin this signal was weak. The strength of the signal was not affected by DMSO. Cytoplasm rich fractions contained low kevels of pSer^{256}-FOXO1 in all treatments. PhosphoThr^{24}-FOXO1 was detected in cytoplasm rich fractions only.

Cyclin D1 expression changes in response to E2 and Wortmannin treatment

Cyclin D1 plays a central role in cell cycle regulation by facilitating G1-S transition, E2 was shown to increase its expression in several tissues and cell culture systems. We treated ovariectomized rats with E2 and Wortmannin and analyzed the expression of Cyclin D1 mRNA by quantitative RT PCR. Six hours after E2 administration the level of Cyclin D1 mRNA increased with about 80%, and by 12 hours after the injection it decreased to the contol level. Wortmannin not only inhibited this effect of E2, but reduced the expression of Cyclin D1 either used alone or in combination with E2.

Estradiol and Wortmannin treatment inhibits Fas-ligand expression

Next, from the cDNA samples we determined Fas-ligand expression, and found that it was decreased in Wortmannin treated uteri six hours after E2 treatment. A slight decrease was observed in E2 and E2+Wortmannin treated animals too. By twelve hours after the treatment the inhibitory effect on E2 on Fas-ligand expression grew stronger while that of Wortmannin did not change. The strongest inhibition of Fas-ligand expression was detected in the uteri treated with both, E2 and Wortmannin.

PI3K-Akt pathway is required for E2 induced water imbibition

Water imbibition is one of the E2 induced uterine responses occurring shortly after the administration of E2. Young, 21 days old rats were given intraperitoneal Wortmannin (1,4 μg/g bw.) and one hour later E2 (10μg /100g bw.) injections. Some animals were treated with E2 alone. Estradiol caused an increase of the uterine weigh but failed to do it in the presence of Wortmannin.

Expression and phosphorylation of Akt is increased in the myoma compared to the matched myometrium of human uterus

Next we analyzed the expression and phosphorylation of Akt protein in myoma and in matched myometrium of human uterus.

The level of Akt expression was higher in myoma then in samples from the control myometrium. No changes were observed during the menstrual cycle but after menopause the level of Akt protein decreased significantly.

The level of pSer^{473}-Akt was low in all myometrium samples examined as well as in menopausal myomas. However, in myomas from cycling uteri the phosphorylation rate of Akt was higher.

Differences in the levels of the antiapoptotic Bcl-2 and the proapoptotic Bax between the myoma and the surrounding myometrium

Abundant expression of Bcl-2 was observed in myomas during the menstrual cycle, with significantly higher levels in secretion than in proliferation phase. Its level was low in myometrial samples and in menopausal myoma.

The highest level of Bax protein was detected in myoma samples from menopausal uteri, in other samples a slight expression was observed only.

Discussion

In this study we aimed to characterize in vivo the PI3K-Akt signal transduction pathway, one of the rapid effects of E2. We established a model which allowed us to examine the role of PI3K in rat uterus.

We found, that Akt was expressed and phosphorylated in all studied (7, 14, 21, 28, 35 days old and adult) rats. In response to E2 treatment phosphorylation rate of Akt increased in the uterus in a dose dependent manner. Estradiol-induced Akt phosphorylation was inhibited with the E2 antagonist ICI 182,780, and with the PI3K inhibitor Wortmannin, indicating the role of ER and PI3K in this effect of E2.

The FOXO1 transcription factor is expressed in rat uterus. The two Akt sensitive residues (Ser^{256} and Thr^{24}) of FOXO1 differs in their responses to either E2 treatment or inhibition of PI3K. Phosphorylation of Ser^{256} residue can be detected earlier than that of Thr^{24}. On the other hand, the Thr^{24} residue is more sensitive to inhibition of PI3K.

Wortmannin treatment decreases the level of pThr^{24}-FOXO1 more efficiently than that of pSer^{256}-FOXO1.

Phosphorylated form of FOXO1 (pSer^{256}) was detected mainly in the nucleus in the uterus of both, control and E2 treated animals, while pThr^{24}-FOXO1 was localized to the cytoplasm.

Phosphorylated forms of both, Akt and FOXO1 can be detected in control, non-treated animals. Therefore, it is reasonable to propose that E2 independent mechanisms are also involved in the regulation of PI3K/Akt/FOXO1 signaling cascade and related survival mechanisms in uterus.

Estradiol increases the expression of Cyclin D1 in a Wortmannin sensitive manner, so a PI3K dependent pathway is involved in this effect of E2. This effect of E2 must play a central role in proliferation of uterine cells. Expression of Fas-ligand mRNA is reduced by both, E2 treatment and inhibition of PI3K.

Analysis of human tissue samples revealed that the level of the survival factors examined (Akt, pSer^{473}-Akt and Bcl-2) is higher in the myoma than in the matched myometrium, and it attenuates after menopause. Expression of the proapoptotic protein Bax increases in myoma after menopause. The results of our present data suggest that Akt/PI3K signaling seems to be involved in the tightly regulated hormonal hierarchy in human uterine smooth muscles. At present stage of investigation the causative links between the mentioned events are not known. The are many questions: whether changes of E2-ERalpha signaling or the changes of PI3K/Akt signaling are initiative events in the leiomyoma pathogenesis; how are influenced by different growth factors initiated signaling mechanisms; does in situ estrogen production contribute in the activation of different signaling pathways?

We conclude that the PI3K-Akt-FOXO1 signal transduction pathway functions in rat and human uterus and it has a central role in proliferative effect of E2 and in development of different pathological conditions.

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