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Expression and activation of Akt/protein kinase B in sexually immature and mature rat uterus

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Abstract

This study investigated the expression and activation of Akt/PKB in developing and adult rat uterus. Expression of Akt was observed in uteri from adult ovariectomized and 7–35-day-old rats and no changes were observed in response to in vivo estradiol treatment (1–100 μg/100 g b.w.). To examine the mechanisms of PKB/Akt activation, phosphorylation at Thr^{308} and Ser^{473} regulatory sites were studied in uteri. Akt was constitutively phosphorylated on Ser^{473} residue in the untreated, control uteri, while phosphorylation of Thr^{308} was observed only after estradiol 17β (E2) treatment. The effects of E2 treatment were age dependent, no response was induced in 11-day-old uteri, while in 28 days and older rats the activation of Akt at both regulatory sites, Ser^{473} and Thr^{308}, increased, the first response was detected 2 h after treatment, reaching the highest rate at 6 h. The rate of phosphorylation was stronger at Ser^{473} residue. The results suggest that the regulation of Akt activation at two regulatory sites in rat uteri are different, phosphorylation of Thr^{308} seems to be entirely estrogen dependent, while the phosphorylation of Ser^{473} is regulated by other factors as well as estrogen.

Keywords: Rat uterus; Akt; Estradiol; In vivo; Development

1. Introduction

Akt/PKB is involved in the regulation of widely divergent cellular processes including apoptosis, proliferation, differentiation and metabolism [for review see [1]]. Expression of Akt and its activation by estrogen was shown in several estrogen sensitive cells [2–4]. Recent data demonstrate that ERalpha in several types of cells interacts with the PI3K (phosphatidylinositol 3-Kinase)/Akt pathway [5]. Estrogen bound ERalpha activates the PI3K/Akt pathway and the PI3K/Akt pathway regulates ERalpha transcriptional activity by its phosphorylation on Ser^{167} [6]. Phosphorylation of Akt mediates the estrogenic action of EGF and IGF in breast cancer cells [2]. Most experiments were carried out using in vitro cell culture systems, only few of them dealt with the in vivo effects of estradiol on lipid kinase signalling. The aim of our present experiments was to investigate the effect of in vivo administered estradiol on expression and activation of Akt in adult and developing rat uterus.

2. Materials and methods

2.1. Chemicals

All antibodies and chemicals, unless stated otherwise, were purchased from SIGMA (St. Louis, MO, USA).

2.2. Animals and treatments

Mature (200–250 g b.w.) and 7, 11, 14, 21, 28 and 35-day-old (the day of birth was considered day 1) sexu-

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ally immature female Wistar rats were used. Mature rats were ovariectomized 8 days before treatment under light ether narcosis. Animals were treated intraperitoneally with 1–100 μg 17β-estradiol (E_2)/100 g b.w. Control animals were treated with vehicle only. The animals were killed under light ether anesthesia by decapitation, their uteri were removed, cleaned and weighed, then put into liquid nitrogen and stored until use. The experimental protocol was approved by our Institutional Ethics Committee for Animal Care and Use.

2.3. Western blotting

Frozen uteri were homogenized at 4 °C in 1 ml/100 mg tissue of ice cold Buffer I (50 mM Tris-Cl pH 8.0, 1mM Na-orthovanadate) for 30–40 sec. We then added 1 ml/100 mg tissue of ice cold Buffer II (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 1% SDS, 5% mercaptoethanol, 40% glycerol) and further homogenized the samples for 10–15 sec. Homogenates were heated in boiling water for 5 min and centrifuged. Supernatants were stored at −20°C. After electrophoresis and blotting membranes with transferred proteins were treated with primary antibodies (anti-Akt 1:1200, anti-Akt (pSer^{473}) 1:1000, anti-Akt (pThr^{308}) 1:800 and anti-actin 1:250). As secondary antibody we used anti-rabbit antibody conjugated with HRP peroxidase. Blots were developed and visualized by chemiluminescence (ECL). Expression of actin was used as loading control. The Western blot analyses were made at least three times in three independent tissue preparations with comparable results.

3. Results

3.1. E_2 enhances phosphorylation of Akt in mature rat uterus

Western blot analysis using antibodies raised against Akt revealed that it was expressed in untreated animals and its expression was not altered by E_2 (10 μg/100 g b.w., i.p.) treatment for up to 24 h (Fig. 1). Uterine samples analysed with antibody specific to Akt (pSer^{473}) showed that a portion of Akt was phosphorylated in untreated animals and this basal phosphorylation was significantly increased in response to E_2, reaching the highest rate at 6 h after the injection (Fig. 1).

3.2. E_2 is effective in activating Akt in a wide range of concentrations

Different dosages of E_2 were found to be effective in activating Akt (Fig. 1). At 1 μg/100 g b.w. Akt phosphorylation began to increase later than in rats treated with 10 μg/100 g b.w.; the highest increase in Akt activation was observed only at 6 h after the injection. The pattern of Akt phosphorylation after injection of E_2 at a dose of 100 μg/100 g b.w. was the same as in the animals treated with 10 μg/100 g b.w. (data not shown).

3.3. Akt is expressed in the untreated uterus of rats of different ages

During postnatal development (Fig. 2), expression of Akt was observed in all age groups. The level of the protein was

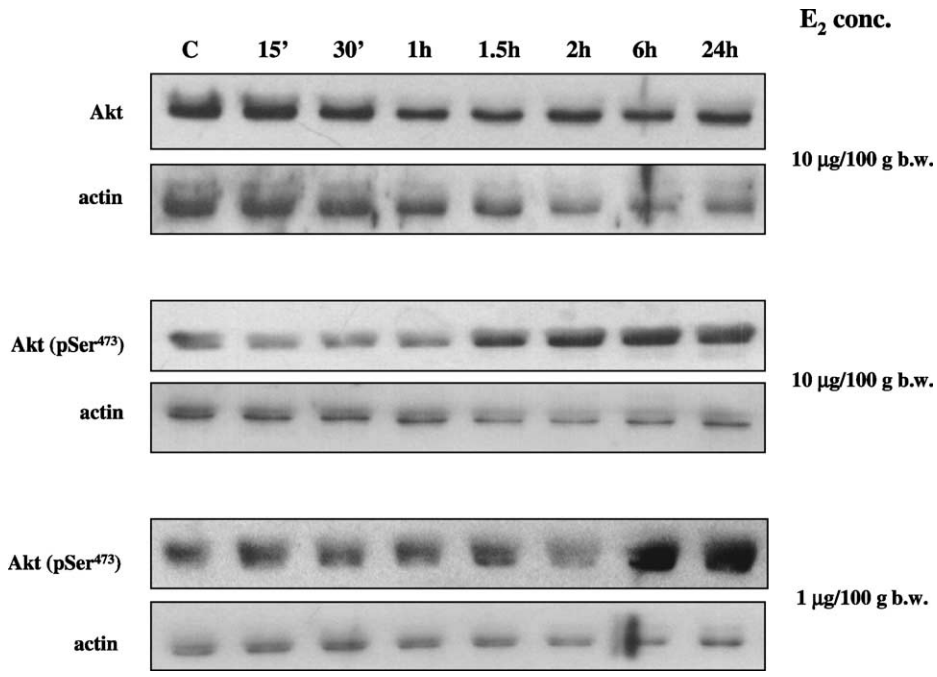

Fig. 1. Effect of E_2 treatment on the level of Akt and Akt (pSer^{473}) in uteri of ovariectomized adult rats. The animals were treated 8 days after ovariectomy with E_2 at a dose of 1 or 10 μg/100 g b.w. and were killed by decapitation at the indicated times. Controls (C) received only vehicle. The expression of Akt and pAkt were determined by Western blots. The experiments were repeated at least three times with similar results.

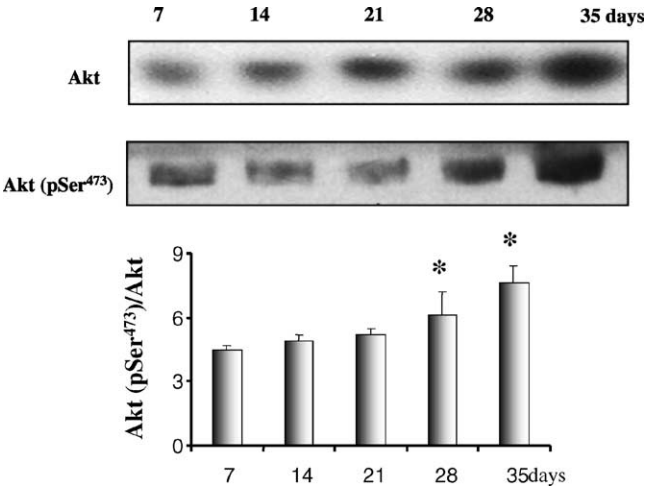

Fig. 2. Expression of Akt and Akt (pSer^{473}) in uteri of 7, 14, 21, 28 and 35-day-old untreated rats. The expression of Akt and pAkt were determined by Western blots. The experiments were repeated at least three times with similar results. Data are presented in the graph. (*$P < 0.05$, different than 7 days values).

similar until 28 days, but it was higher in the uterus of 35-day-old rats. Samples analysed with Akt (pSer^{473}) showed basal phosphorylation of the protein from the age of 7–21 days; its rate began to increase from the age of 28 days and reached its highest rate in 35-day-old rats.

3.4. Phosphorylation of the Thr^{308} residue seems to be specific for E_2

When comparing the two sites that need to be phosphorylated for activation of Akt (Ser^{473} and Thr^{308}), we found that, the phosphorylation of the two sites is different from each other. In contrast to the basal phosphorylation pattern of the Ser^{473} residue, no phosphorylation on Thr^{308} was observed in 11, 28 and 60-day-old control animals. After E_2 treatment, besides phosphorylation on Ser^{473}, phosphorylation of Thr^{308} residue was also observed, but only in the 28 and 60-day-old animals. In 11-day-old animals, no E_2 induced changes in Akt activation were detected (Fig. 3).

4. Discussion

The present study demonstrates age dependent expression and in vivo activation by estradiol of Akt/PKB in rat uterus. In vivo, Akt/PKB is activated by E_2 in uteri of rats older than 28 days of age. This effect of E_2 is time dependent. Six hours after E_2 treatment, the phosphorylation of Akt increased transiently. E_2 induced Akt activation seems to be parallel with the development of estrogen sensitivity of the uterus. The development of the complete system for E_2 stimulation of cell growth and proliferation takes place during the first weeks of life. In rats, 21 days of age is the earliest time when increased DNA synthesis can be observed after E_2 stimulation [7]. At younger ages, the regulation of uterine growth seems to be dependent on growth factors other than estrogen [8]. Full activation of PKB/Akt requires its phosphorylation on Ser^{473} and Thr^{308} [1]. In most situations, the phosphorylation of these two sites are parallel, but can also occur independently [9], as seen in our experiments. Phosphorylation of Akt on Ser^{473} was detected in untreated uteri of all age groups studied; however, Akt (pThr^{308}) was seen only in response to E_2. The time and age dependencies of Akt/PKB activation by E_2 on both activation sites were sim-

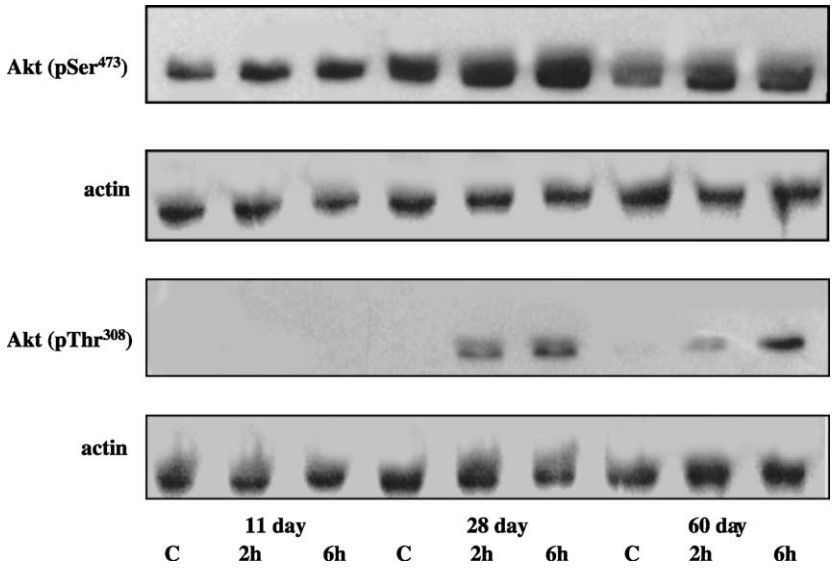

Fig. 3. Effect of E_2 treatment on the level of Akt (pSer^{473}) and Akt (pThr^{308}) in uteri of 11, 28 and ovariectomized 60-day-old rats. The animals were treated with E_2 at a dose of 10 μg/100 g b.w. and were killed by decapitation at the indicated times. Controls (C) received only vehicle. The rate of formation of Akt (pSer^{473}) or Akt (pThr^{308}) was determined by Western blots. The experiments were repeated at least three times with similar results.

ilar. In vitro, PDK-1 (3-phosphoinositide-dependentkinase-1) catalyzes phosphorylation of Akt exclusively on Thr^{308} [10]. Inhibition of that enzyme activity causes a reduction in cell proliferation; defective PDK-1 results in impaired development in mice and decreased cell size independently of cell number and proliferation [11]. E_2 induces the transcription of the cell cycle regulator cyclin D1 [12]. In addition, a rapid (1–6 h) E_2 induced cyclin D1 expression is also reported in several cell lines, suggesting that E_2 induced non genomic mechanisms are also sufficient to induce cyclin D1 expression or activation [13,14]. Recent data in MCF7 cells indicate the contribution of PI3K/Akt signalling in that mechanism [14]. In adult uteri, only the luminal epithelium proliferates in response to E_2, but expression of ER can be detected in all layers of the uterus, suggesting the contribution of Akt signalling in other uterine events as well. To clarify the molecular events of Akt signalling and their roles in the age dependent effects of E_2 in rat uteri further experiments are required.

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