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Dose-dependent Differential Activation of AKT Induced by Progesterone in Human Breast Cancer Cells

Deuk Hee Oh, Hyun Woo Kim, Young Chai Lim*

Department of Pharmacology, Chonnam National University Medical School, Hwasun 58128, Korea
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ABSTRACT

The dose-dependent effect of progesterone on PI3K/AKT signaling pathway, which plays a vital role in regulating various biological processes in human breast cancer, is not well defined. Herein, we analyzed the dose-associated influence of progesterone on AKT activation in human breast cancer cells. MCF-7 cells were treated with progesterone at a concentration ranging from 10^{-10} M through 10^{-6} M for 30 minutes and the extent of AKT phosphorylation was assessed using western blotting. Treatment with progesterone at various concentrations led to AKT phosphorylation in these cells. Notably, AKT activation, when treated with lower doses of progesterone was higher than that observed upon treatment with higher doses. Pre-treatment with PI3K inhibitor (LY294002) attenuated progesterone-induced AKT activation in a parallel manner over the range of progesterone doses administered. RU486, which is a progesterone receptor antagonist, induced AKT phosphorylation. These results suggest that progesterone seems to induce dose-dependent differential activation of PI3K/AKT pathway in MCF-7 cells.

Key words : Progesterone, Human breast cancer cells, AKT activation, PI3K

1. Introduction

Progesterone, together with estrogen or alone, is essential for the regulation of the pathophysiological process of human breast cancers, as well as the normal breast growth and development [1-3]. Breast cancer is the most common cancer in women worldwide and also the principal cause of death from cancers [4].

The effect of progesterone on many biological processes is very variable in human breast cancer cell models [2,3]. For example, many authors reported very heterogeneous results regarding the responses of progesterone on the growth of human breast cancer cell lines [1,2]. The progestational agents have been reported to inhibit [5,6], stimulate [7,8], or have no effect [9] on the proliferation of breast cancer cells. The effect of progestational agents on apoptosis in breast cancer cells is also contradictory as it was reported that they can either induce [10] or inhibit cell death [11]. These diverse controversial effects of progesterone in human breast cancer cells could be elicited depending on the laboratories, the experimental model systems, the cell types, the duration of treatment, the dose of agents, and etc.

Activation of AKT is a phosphorylation process mediated by a PI3K dependent kinase in response to different extracellular stimuli [12]. This PI3K/AKT signaling pathway acts as a key regulator of various cancer cell functions such as the proliferation, apoptosis, cell cycle progression, glucose usage, angiogenesis, resistance, and etc [13-15]. Enhanced PI3K/AKT signaling in breast cancer cells has been shown to be related with the increased growth of cancer cells. In recent, AKT protein is

* Correspondence should be addressed to Dr. Young Chai Lim, Professor, Department of Pharmacology, Chonnam National University Medical School, Hwasun 58128, Korea. Tel: +82-61-379-2833, Fax: +82-61-373-6974, E-mail: limyc@jnu.ac.kr
DH Oh and HW Kim are graduates, Department of Pharmacology, Chonnam National University Medical School, Hwasun 58128, Korea.

regarded to be a rational target and inhibitors of the PI3K/AKT signaling pathway have been investigated for the treatment of human breast cancer [16-18].

In spite of the significance of PI3K/AKT signaling in breast cancer, the effect of progesterone on AKT phosphorylation over the wide range of doses was not so far clearly defined in human breast cancer cells. We attempted therefore to investigate the effect of various doses of progesterone on the activation of AKT protein in human breast cancer cell models.

2. Materials and Methods

2.1 Cell lines and reagents

MCF-7 and HCC-38 human breast cancer cell lines were obtained from the Korean Cell Line Bank. Progesterone and RU486 were purchased from Sigma-Aldrich (St Louis, MO, USA) and R&D Systems, Inc (Minneapolis, MN, USA), respectively. We used cell culture media and sera from Invitrogen Life Technologies (San Diego, CA, USA). The polyclonal rabbit antibodies against AKT and against phosphorylated AKT were from Cell Signaling Technology, Inc (Beverly, MA, USA). Secondary antibodies (anti-rabbit IgG) were from Zymed Laboratories (Carlsbad, CA, USA). The Super Signal chemiluminescent substrate from Pierce Biotechnology Inc (Rockford, IL, USA) was used for detection of antibodies.

2.2 Cell culture and drug treatment

Monolayer cultures of MCF-7 and HCC-38 cells were grown and maintained in improved minimal essential medium (IMEM), supplemented with 10% fetal bovine serum (FBS). Cells (2×10^6) were first seeded into 100 cm^2 Petri dishes of phenol red-free IMEM containing 10% charcoal-stripped FBS. The media of these cells were replaced daily with phenol-red free IMEM containing 10% charcoal-stripped FBS for 3 days to remove endogenous steroids [19]. Various doses of progesterone or RU486 were administered into the cells for 30 min. These cells treated with drugs were then harvested. For the treatment with PI3K inhibitors, cells were pretreated with LY294002 for 20 minutes prior to the administration of progesterone.

2.3 Western blot analysis

Cells (2×10^5) were plated in 100 cm^2 Petri dishes, cultured for 3 days, and then incubated with progesterone for 30 min. Cultured cells were washed in ice cold Tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5), and lysed for 20 min on ice-chilled lysis buffer (50 mM Tris, 0.5% NP-40, 120 mM NaCl, 200 μM Na_3VO_4, 100 mM NaF, 1 mM PMSF, pH 8.0) added with 1 mM protease inhibitor cocktail tablet. Protein extracts were clarified by centrifugation (14,000 × g, 4°C 15 min), and protein contents were measured by Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of total proteins (100 μg) were subjected to 10% SDS-PAGE, which was followed by transfer onto the nitrocellulose membranes. Membranes were incubated with primary antibodies against AKT or phosphorylated AKT proteins and the respective species-specific secondary HRP-conjugated antibodies. The bands were visualized with the Super Signal detection system.

2.4 Statistical analysis

Experimental data were presented with mean ± SD. All statistical analyses were performed using Student's t-test and ANOVA. $P<0.05$ was considered as statistically significant.

3. Results

3.1 Dose-differential effect of progesterone on AKT phosphorylation in MCF-7 cells

In order to observe the effect of progesterone on the AKT phosphorylation in human breast cancer cells, a wide range of progesterone ranging from 10^{-10} M through 10^{-6} M was administered into MCF-7 cells for 30 minutes. This range of progesterone encompasses the concentration seen during the follicular and luteal phases of menstrual cycle (10^{-10} and 10^{-9} M, respectively), and at pregnancy levels (10^{-7} M) [20]. Progesterone induced the phosphorylation of AKT protein in these cells (Fig. 1). Remarkably, AKT activation at the lower doses of progesterone treatment was more relatively increased than that of the higher doses of progesterone. These results might suggest dose-differential responses of progesterone in breast cancer cells. That is, the lower doses of progesterone seem to exert more stimulatory influences on AKT activation while the higher doses of progesterone less stimulatory or rather inhibitory influences on AKT activation.

3.2 Effect of LY294002, PI3K inhibitor, on the progesterone-induced AKT phosphorylation

Activation of AKT is a phosphorylation dependent event

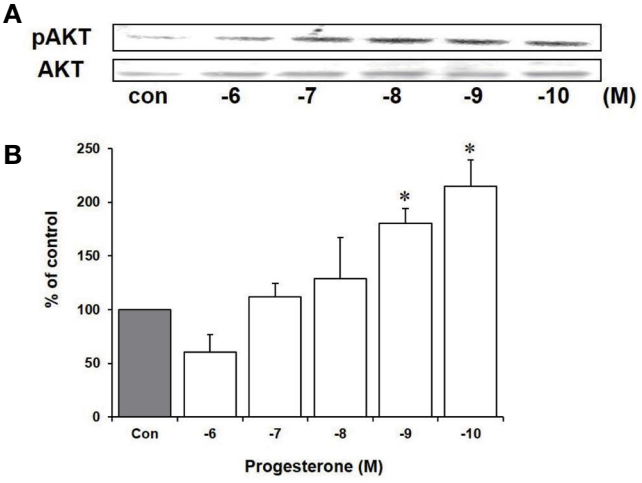

Fig. 1. Effect of various doses of progesterone on the activation of AKT. MCF-7 cells were treated with a wide range of concentrations (from 10^{-10} M through 10^{-6} M) of progesterone for 30 min. AKT and phosphoprylated AKT (pAKT) were measured using western blotting probing with antibodies on 10% SDS-polyacrylamide gels. A. Expression images of pAKT and AKT in MCF-7 cells treated with various doses of progesterone. B. The bar diagram on the ratio of pAKT over total AKT in MCF-7 cells treated with progesterone are shown. Each experiment was separately performed in triplicate. Data are presented with mean ± SD.

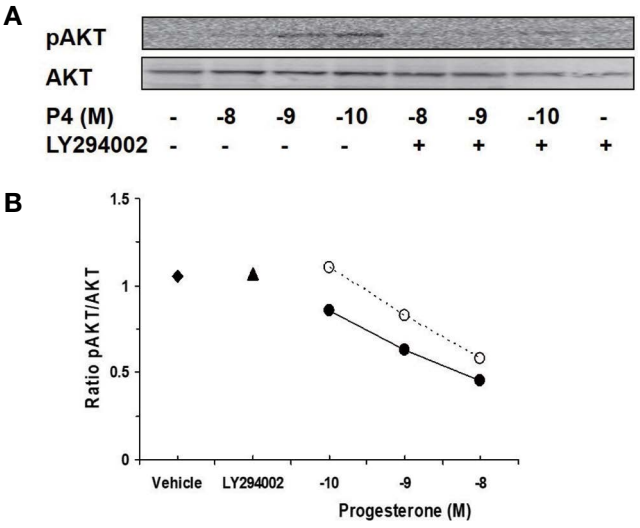

Fig. 2. Effect of LY294002 on the progesterone-induced activation of AKT. MCF-7 cells were pretreated with 50 μM of LY294002 20 min prior to the treatment of progesterone for 30 min. A. Expression images of pAKT and AKT in MCF-7 cells treated with vehicle, various doses (from 10^{-10} M through 10^{-8} M) of progesterone alone, combination of LY294002 and various doses of progesterone, and LY294002 alone. B. The diagram on the ratio of pAKT over total AKT in MCF-7 cells treated with various drugs. The line with open circles represents the group treated with various doses of progesterone alone. The line with filled circles represents the group treated with combination of LY294002 and various doses of progesterone.

mediated by a PI3K dependent kinase [24,26]. To identify the involvement of PI3K/AKT signaling pathways, we observed the effect of LY294002, PI3K inhibitor, on the progesterone-induced AKT activation (Fig. 2). Dose-differential effect of progesterone (10^{-10} M through 10^{-8} M) alone on AKT phosphorylation was identified again. 50 μM of LY294002 alone did not cause any influence on AKT activation. The progesterone-induced AKT activation was however inhibited when cells were treated together with 50 μM of LY294002. Notably, dose-differential effect of progesterone on AKT activation was in a parallel manner inhibited with LY294002 pre-treatment over the range of doses of progesterone given.

3.3 Influence of RU486 on AKT phosphorylation

RU486, which has progesterone receptor-antagonizing activities [21], treatment for 30 minutes induced overall increase the phosphorylation of AKT over the wide range of doses from 10^{-10} M through 10^{-6} M (Fig. 3).

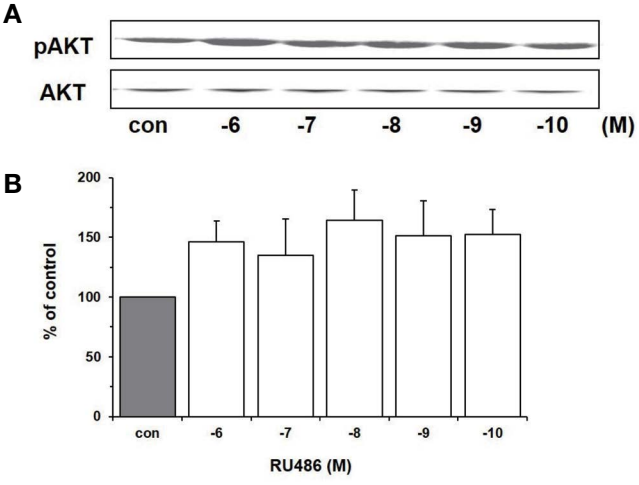

Fig. 3. Effect of RU486 on the phosphorylation of AKT. MCF-7 cells were treated with a wide range of concentrations (from 10^{-10} M through 10^{-6} M) of RU486 for 30 min. A. Expression images of pAKT and AKT in MCF-7 cells treated with various doses of RU486. B. The bar diagram on the ratio of pAKT over total AKT in MCF-7 cells treated with RU486 are shown. Data are presented with mean ± SD.

3.4 Effect of progesterone on AKT phosphorylation in progesterone receptor-negative HCC-38 breast cancer cells

Progesterone receptor (PR)-negative HCC-38 cells were treated with various doses of progesterone for 30 minutes. AKT activation was not observed (data not shown).

4. Discussion

It has been known that progesterone induces phosphorylation of AKT in breast cancer cells [22,23]. However, definite effect of progesterone on AKT phosphorylation over the wide range of doses was not yet determined in these cells. Various changes in progesterone concentration over the extensive range (e.g. during the puberty, menopause, each phase of menstrual cycle, pregnancy, inflammation, cancer, or etc.) are closely related with lots of physiological and pathological responses in human breast. We presented first the dose-differential AKT activation within a certain wide range of progesterone treatment in human breast cancer cell models. Inhibitory role of higher doses of progesterone in AKT activation was interestingly suggested in this study, because the higher doses of progesterone exert less stimulatory or rather inhibitory influences on AKT activation while the lower doses of progesterone more stimulatory influences on AKT activation. Similar dose-differential effects were also noted in other report [23], in which greater AKT activation was observed rather in lower doses of progestational agent treatment over the specific range than in higher doses, though the cells used were from endometrial cancer tissue which is also female sex hormone dependent like breast cancer cells.

Regarding the influence of progesterone dose on the breast cancer cell growth, proliferation of these cells has been reported to be more inhibited by the administration of relatively higher concentration of progestational agents than by the lower ones, which seems to rather induce proliferative responses [5-7]. Enhanced signaling of the PI3K/AKT pathway in breast cancer cells is also related with the cellular proliferation [16,17]. Reduced AKT activation in higher doses of progesterone observed in this study is therefore compatible with one of the mechanisms responsible for the inhibition of proliferation of breast cancer cells to be treated with higher doses of progestational agents.

The activation of PI3K results in PIP_3-mediated activation of AKT protein by phosphorylation [13,24]. The PI3K/AKT signaling is in turn involved in the regulation of various cell functions such as proliferation, apoptosis, cell cycle progression, and etc. [13-15]. The activated AKT protein in turn modulates the function of numerous substrates involved in the regulation of cell functions. AKT phosphorylation by progesterone was inhibited by the pretreatment of LY294002, PI3K inhibitor, indicating the involvement of PI3K/AKT signaling pathway in progesterone-induced AKT activation. The notable finding in the inhibiting effect of PI3K inhibitor on AKT activation was its parallel inhibitory pattern over the range of progesterone treatment (Fig. 2).

AKT phosphorylation by the treatment of RU486 alone, which has progesterone receptor-antagonizing activities [21], was also observed over the wide range of doses of RU486 in this study. This suggests the inhibitory role of progesterone receptor (PR) for the AKT activation in breast cancer cells. Three PR isoforms are the full length PR-B (116 kDa), N-terminally truncated PR-A (94 kDa), and PR-C isoform (60 kDa) [19,25]. PR-positive cancer cells usually co-express PR-A and PR-B isoforms [26]. PR-B is essential for the mammary gland development [27]. We also noted that progesterone did not induce AKT phosphorylation in PR-negative HCC-38 cells, suggesting the involvement of PRs for PI3K/AKT signaling activation. The action mechanism through the specific PR isoform for the progesterone-induced AKT activation is yet to be determined further.

Concerning clinical significance of the PI3K/AKT pathway, many cellular processes in breast cancer are closely linked with the enhanced signaling of the PI3K/AKT pathway. AKT protein is therefore regarded to be a rational target and inhibitors of the PI3K/AKT pathway have been investigated for cancer treatment [16-18]. Early clinical trials to test the safety, pharmacokinetics, and pharmacodynamics of novel PI3K inhibitor analogs were recently undertaken in patients with solid tumors [28].

Clinical benefits and risks for the patients could be very variable, depending on the dose of progestational agents used in clinical circumstances. High-dose progestational agents such as medroxyprogesterone acetate or megestrol acetate were used for the treatment of metastatic breast cancer [29-31]. The dose range of medroxyprogesterone acetate for the high-dose breast cancer therapy was approximately 400 mg through 1,600 mg a day. However, adverse drug reactions such as the weight gain, edema, Cushing-like symptoms, and etc. limit its use [31]. Its anti-tumor action mechanism was not fully understood, but suppression of estrogen, suppression of adrenal steroid synthesis, activation of androgen receptors, and direct killing of tumor cells have been suggested [32]. Our results that relatively higher dose of progesterone inhibit the phosphorylation of AKT could be one of the possible mechanisms responsible for the anti-tumor activity of high-dose progestin therapy for breast cancer.

Relating to the association of apoptosis with the dose of progesterone in human breast cancer cells, progesterone in relatively high doses ranging from 10^{-6} M through 10^{-5} M induced apoptosis in breast cancer cells [10], while other authors observed that 10^{-7} M of progesterone or other progestin inhibited cell

death [11].

Based upon the findings of others and our results, we suggest that the higher doses of progestational agents seem to be related with anti-tumor activities, while the lower doses with tumorigenic effects in human breast cancer. In this regard, it is inferred that these progestational agents could be clinically utilized for the therapeutic purpose and also for preventing adverse drug reactions in the treatment of breast cancer, depending on their doses.

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