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Progesterone induces NFκB DNA binding activity through a PI3K/Akt-dependent pathway in MCF-7 breast cancer cells

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Abstract: Progesterone (PG) is a steroid hormone that regulates normal reproductive functions including uterine and mammary gland development. The PG receptor belongs to the nuclear receptor superfamily of ligand dependent transcription factors that mediates gene expression, however, it also promotes cell signaling pathways through a non-genomic pathway including the activation of PI3K/Akt and Src/ERK1/2 pathways. However the role of PG in NFκB-DNA complex formation remains to be studied. We demonstrate here that PG induces Akt2 activation and an increase of NFκB DNA binding activity in MCF-7 breast cancer cells. Akt2 activation requires PI3K activity, whereas increase of NFκB DNA binding activity requires PI3K, mTOR, Akt, Src, and G-proteins activity, as well as the integrity of cytoskeleton. In summary, our findings demonstrate that PG induces an increase of NFκB DNA binding activity in MCF-7 cells.

Keywords: Breast Cancer, Progesterone, PI3K/Akt, NFκB, MCF-7

1. Introduction

Epidemiological and clinical studies demonstrate that administration of progesterone (PG) is associated with an increased risk of developing postmenopausal breast cancer [1, 2]. In addition, clinical studies demonstrate that ~10% of estrogen-receptor-negative (ER-) breast cancers are PG-receptor-positive (PR+), and these tumors mainly affect women under 50 years of age [3, 4].

PG is a steroid hormone that regulates normal reproductive functions, including uterine and mammary gland development, as well as the signals required for sexually responsive behavior in the brain. The PG receptor (PR) belongs to the nuclear receptor superfamily of ligand dependent transcription factors that mediates gene expression (genomic pathway). However, PG also mediates non-genomic pathways including the activation of phosphatidylinositol 3-kinase (PI3K)/Akt and the c-Src/extracellular signal regulated kinase 1/2 (ERK1/2) pathways [5, 6].

The nuclear factor-kappa B (NFκB) is a family of transcription factors, its common form is p50/Rel A and regulates the expression of a wide range of genes that are critical for survival and proliferation. NFκB is required for normal lobuloalveolar development of mammary gland; however it is overexpressed in different stages of breast cancer progression. Particularly, a high activity of NFκB has been reported in ~86% of Her-2 positive breast tumors [7, 8].

In the present study, we demonstrate that PG induces Akt2 activation and an increase of NFκB DNA binding activity in MCF-7 cells. Akt2 activation requires PI3K activity, whereas increase of NFκB DNA binding activity requires PI3K, mTOR, Akt, Src and G-proteins activity, as well as the integrity of cytoskeleton.

2. Materials and Methods

2.1. Chemicals

PG, Akt 1/2 kinase inhibitor A6730, *pertussis toxin* (PTX) and *cholera toxin* (CTX) were from Sigma (St, Louis, MO). Wortmaninn, rapamycin, PP2 and PP3 were from Calbiochem (San Diego, CA). Akt2 antibody (Ab) F-7 was

obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific Ab to Thr-308 of Akt 244F9 (anti-p-Akt) was obtained from Cell Signaling (Danvers, MA). [γ-^{32}P] ATP was obtained from Perkin-Elmer (Boston, MA).

2.2. Cell culture

The human MCF-7 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.7 g/l sodium bicarbonate, 5% fetal bovine serum (FBS), in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. For experimental purposes, cells were starved in DMEM without FBS for 12 h before treatment with inhibitors and/or PG.

2.3. Cell stimulation

After starvation, MCF-7 cells were washed twice with DMEM without FBS, equilibrated in the same medium at 37 °C for at least 30 min, and then treated with inhibitors and/or PG for the times and concentrations indicated. We used confluent cultures of cells grown in 60-mm dishes containing 3 ml of DMEM for each experimental condition. The stimulation was terminated by aspirating the medium and nuclear extracts were obtained or cells were solubilized in 0.5 ml of ice-cold radioimmune precipitation assay (RIPA) buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 100 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 1.5 mM MgCl2, 0.1% SDS and 1 mM phenyl methylsulfonyl fluoride (PMSF).

2.4. Western blotting

Equal amounts of protein were separated by SDS-PAGE using 10% separating gels followed by transfer to nitrocellulose membranes. After transfer, membranes were blocked using 5% non-fat dried milk in phosphate buffered saline (PBS), pH 7.2, and incubated overnight at 4 °C with the primary Ab. The membranes were washed three times with PBS/0.1% Tween 20 and then incubated with secondary Ab (1:5000) for 2 h at 22°C. After washing three times with PBS/0.1% Tween 20, the immunoreactive bands were visualized using ECL detection reagents. Autoradiograms were scanned and the labeled bands were quantified using the Image J software (Jandel Scientific).

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared as described previously [9]. Briefly, 1.5 x 10^6 cells were lysed with 0.1% nonionic detergent Nonidet P40 in Buffer A (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF). Lysates were pelleted at 2 600 rpm for 15 min and resuspended in Buffer B (20 mM HEPES, pH 7.9, 420 mM NaCl, 20% glycerol 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 1 mM DTT, 0.2 mM PMSF). Nuclear extracts were recovered by centrifugation at 14 000 rpm for 15 min at 4 °C and the protein level of each sample was determined by the micro Bradford protein assay.

2.5. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotide containing specific binding sites for NFκB 5'-AGCTAAGGGACTTTCCGCTGGGGACTTTCCAGG-3', was used as probe [10]. A total amount of 20 pmol of annealed NFκB oligonucleotide was labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. The ^{32}P-labeled oligonucleotide probe (~1 ng) was incubated with 5 µg of nuclear extract in a reaction mixture containing 13 µg of poly (dI-dC), 0.25 M HEPES, pH 7.5, 0.6 M KCl, 50 mM $MgCl_2$, 1 mM EDTA, 7.5 mM DTT, and 9% glycerol for 20 min at 4 °C. One hundred-fold excess of unlabeled NFκB probe was used as specific competitor. The samples were fractionated on a 6% polyacrylamide gel in 0.5X Tris borate-EDTA buffer. Gels were dried and they were analyzed by autoradiography.

2.6. Statistical analysis

Results are expressed as mean ± S.D. Data were statistically analyzed using one-way ANOVA and the pairwaise comparisons were performed using Newman-Keuls multiple comparisons test. Statistical probability of $P<0.05$ was considered significant.

3. Results

3.1. PG induces Akt2 activation in MCF-7 Cells

First, we examined whether PG promotes Akt2 activation in MCF-7 cells. Akt2 activation was analyzed by using its phosphorylation at Thr-309 [11]. Cultures of MCF-7 cells were treated with 100 nM PG for various times and lysed. Cell lysates were immunoprecipitated with anti-Akt2 Ab and the immune complexes were analyzed by SDS-PAGE followed by Western blotting with anti-p-Akt Ab, which recognizes the phosphorylation on Thr-308 and Thr-309 of Akt1 and Akt2 respectively. As shown in Fig. 1A (upper panel), treatment of cells with PG induced an increase on Akt2 phosphorylation at Thr-309 (p-Akt2) that reached a maximum between 15 and 30 min of treatment. Western blotting with anti-Akt2 Ab of the same membrane was used as loading control (Fig. 1A, lower panel).

Akt is the primary downstream mediator of PI3K signaling, whereas mTOR activates Akt and then promotes proliferation and survival [12, 13]. We determined whether Akt2 activation requires PI3K and mTOR activity. MCF-7 cells were treated for 1 h with 60 nM wortmannin or 100 nM rapamycin, which are selective inhibitors of PI3K and mTOR respectively [14], and then stimulated with 100 nM PG for another 15 min and cells were lysed. Cell lysates were immunoprecipitated with anti-Akt2 Ab, followed by Western blotting with anti-p-Akt Ab. Our results showed that p-Akt induced by PG required PI3K activity, however it did not require mTOR activity (Fig. 1B)

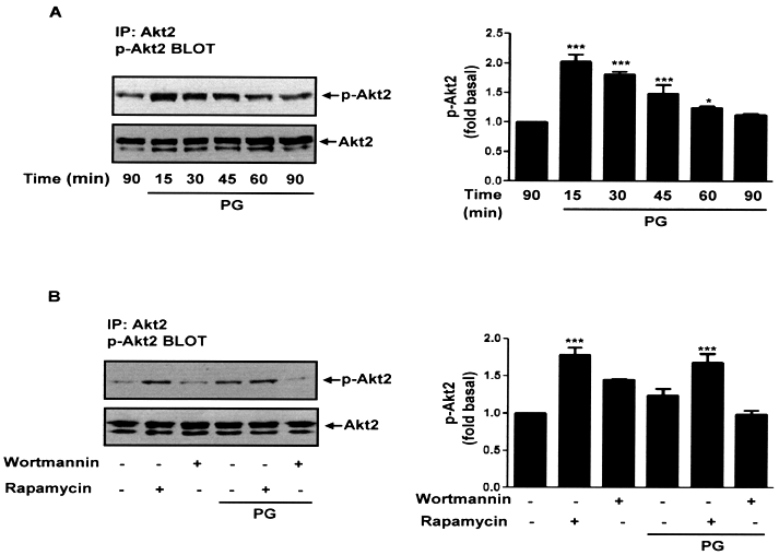

Figure 1. *Progesterone induces Akt2 activation trough a PI3K-dependent pathway in MCF-7 cells. Panel A. MCF-7 cells were treated with 100 nM progesterone (PG) for various times and lysed. Panel B. MCF-7 cells were treated for 1 h without (-) or with (+) 60 nM wortmannin or 100 nM rapamycin, stimulated with 100 nM PG for 15 min and lysed. Cell lysates were analyzed by immunoprecipitation with anti-Akt2 Ab followed by Western blotting with anti-p-Akt Ab. The graphs represent the mean ± S.D. and are expressed as fold of p-Akt2 above unstimulated cells. Asterisks denote comparisons made to unstimulated cells. ***P<0.001.*

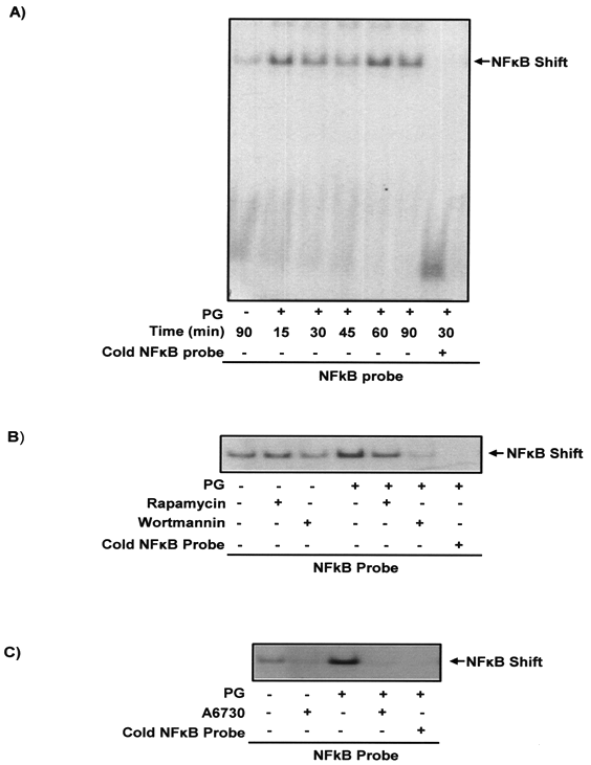

Figure 2. *PG induces NFκB DNA binding activity through a PI3K/Akt-dependent pathway. Panel A. MCF-7 cells were treated with 100 nM PG for various times and nuclear extracts were obtained. Panel B. MCF-7 cells were treated for 1 h without (-) or with (+) 60 nM wortammin or 100 nM rapamycin, stimulated with 100 nM PG for 15 min and nuclear extracts were obtained. Panel C. MCF-7 cells were treated for 1 h without (-) or with (+) 2 μM A6730, stimulated with 100 nM PG for 15 min and nuclear extracts were obtained. NFκB DNA binding activity was analyzed by EMSA. One control included EMSA reactions with 100 fold-excess of cold NFκB as competitor. The autoradiograms shown are representative of at least three independent experiments.*

3.2. PG induces NFκB-DNA complex formation in MCF-7 cells

NFκB is a transcription factor that plays an important role in oncogenesis [15]. We determined whether PG induces NFκB-DNA complex formation. MCF-7 cells were treated with 100 nM PG for various times and nuclear extracts were obtained. EMSAs were performed using nuclear extracts and a radiolabeled oligonucleotide probe representing a canonical NFκB binding site. As illustrated in Fig. 2A, a weak specific NFκB-DNA complex was observed in untreated cells, however treatment with PG significantly increased the DNA binding activity of NFκB in a time-dependent manner, which reached a maximum at 60 min of treatment. The specificity of these complexes was demonstrated by inhibition of binding in the presence of a cold competitor.

Since PG induced Akt2 activation, we determined the role of PI3K, Akt and mTOR in NFκB DNA binding activity induced by PG. MCF-7 cells were treated for 1 h with 60 nM wortmannin or 100 nM rapamycin, stimulated with 100 nM PG for 15 min, nuclear extracts were obtained and EMSAs were performed. Our results showed that treatment with PI3K and mTOR inhibitors inhibited NFκB DNA binding activity induced by PG (Fig. 2B).

Next, we determined whether NFκB DNA binding activity induced by PG requires Akt activity. Cultures of MCF-7 cells were treated for 1 h with 2 μM A6730, which is a selective inhibitor of Akt1/2 and has been used previously in breast cancer cells [16], and then cells were stimulated with 100 nM PG for 15 min, nuclear extracts were obtained and EMSAs were performed. As illustrated in Fig. 2C, treatment with Akt inhibitor completely inhibited NFκB DNA binding activity induced by PG.

3.3. Role of Src, G proteins activity and the cytoskeleton integrity on the increase of NFκB DNA binding activity induced by PG

Since PG induces Src activation [17]. We determined the role of Src activity on NFκB DNA binding activity induced by PG. Cultures of MCF-7 cells were treated for 1 h with 10 μM PP2, which is a selective inhibitor of Src family members, or with PP3, an inactive analog of PP2, and then stimulated with 100 nM PG for 15 min. Nuclear extracts were obtained and EMSAs were performed. As illustrated in Fig. 3A, treatment with PP2 inhibited NFκB DNA binding activity induced by PG, whereas treatment with PP3 did not inhibit the NFκB DNA binding activity.

Next, we determined whether NFκB DNA binding activity requires the activity of G proteins. We studied the effect of PTX, and inhibitor of Gi/Go proteins, and CTX, a toxin that catalyzes the ADP-ribosylation of the α-subunit of the Gαs, reducing its GTPase activity and activating the α-subunit [18, 19]. Cultures of MCF-7 cells were treated for 12 h with 100 ng/ml PTX or 100 ng/ml CTX, and stimulated with 100 nM PG for 15 min. Nuclear extracts were obtained and EMSAs were performed. As illustrated in Fig. 3B, treatment with PTX partly inhibited NFκB-DNA complex formation, whereas treatment with CTX completely inhibited NFκB DNA binding activity induced by PG.

In order to determine the contribution of cytoskeleton in the NFκB DNA binding activity induced by PG, we determined the effect of colchicine and cytochalasin D, which promote the disappearance of microtubules and disrupt the organization of actin fibers respectively. Cultures of MCF-7 cells were treated for 1 h with 10 μM colchicine or 2.4 μM cytochalasin D, and then stimulated with 100 nM PG for 15 min, nuclear extracts were obtained and EMSAs were performed. Our results showed that treatment with colchine inhibited the NFκB DNA binding activity induced by PG, whereas treatment with cytochalasin D did not inhibit the NFκB-DNA complex formation (Fig. 3C).

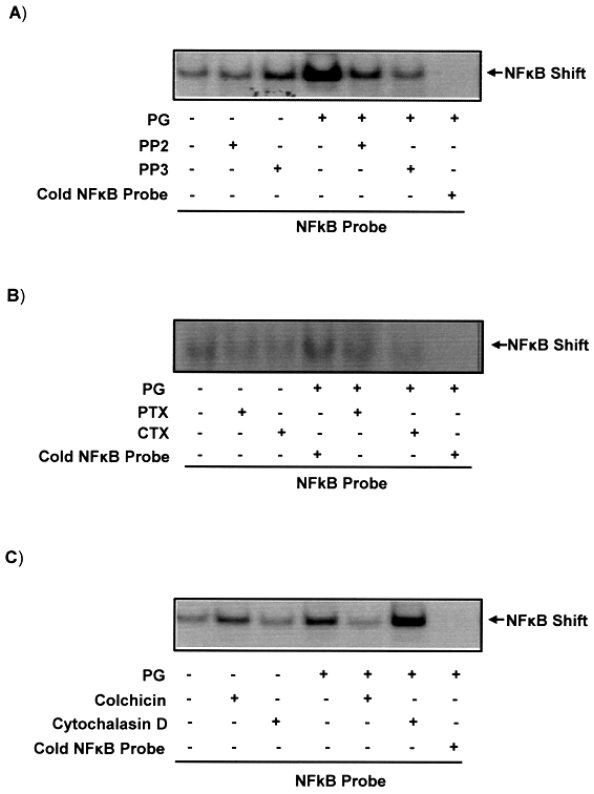

Figure 3. *Role of Src, cytoskeleton integrity and G-proteins on NFκB DNA binding activity induced by PG. Panel A. MCF-7 cells were treated for 1 h without (-) or with (+) 10 μM PP2 or 10 μM PP3, stimulated with 100 nM PG for 15 min and nuclear extracts were obtained. Panel B. MCF-7 cells were treated for 12 h without (-) or with (+) 100 ng/ml PTX or 100 ng/ml CTX, stimulated with 100 nM PG for 15 min and nuclear extracts were obtained. Panel C. MCF-7 cells were treated for 1 h without (-) or with (+) 10 μM colchicine or 2.4 μM cytochalasin D, stimulated with 100 nM PG for 15 min and nuclear extracts were obtained. NFκB DNA binding activity was analyzed by EMSA. One control included EMSA reactions with 100 fold-excess of cold NFκB as competitor. The autoradiograms shown are representative of at least three independent experiments.*

4. Discussion

PG regulates breast development, function and differentiation, because it is able to stimulate proliferation or inhibit growth and promote differentiation [20, 21]. Moreover, it has been suggested that PG plays an important role in development and progression of breast cancer because postmenopausal women under treatment with estrogens and PG present an increased incidence of breast cancer development [1, 22]. However, the signal transduction pathways mediated by PG have been mainly studied by using progestin, which is a synthetic progestogen with progestinic effects similar to PG, whereas the signal transduction pathways mediated by PG have been poorly studied.

The PI3K/Akt pathway regulates a wide variety of biological processes including migration, proliferation, growth, survival, angiogenesis and cancer [23, 24]. The PI3Ks are a family of kinases that generate a number of phosphoinositol lipids, which act as second messengers in several signaling pathways, including the activation of PDK and Akt. The Akt family of serine-threonine kinases is the primary downstream mediator of PI3K, and through the phosphorylation of target substrates mediates a number of cell processes [24, 25]. Here we demonstrate that PG induces Akt2 activation and that it requires the activity of PI3K and mTOR in MCF-7 cells. We propose that PG plays an important role in the invasion process. Supporting our proposal, it has been demonstrated that in a mouse model of oncogene-induced mammary tumorigenesis, Akt2 participates in tumor invasion and metastasis [26, 27]. Moreover, we previously demonstrated that AA induces migration and invasion through a PI3K/Akt-dependent pathway in MDA-MB-231 breast cancer cells [28].

NFκB is a transcription factor that plays an important

role in inflammation, survival, transformation and oncogenesis [29]. Particularly, mammary carcinoma cell lines and human breast cancer tissue present an elevated NFκB DNA-binding activity, whereas rat mammary tumors have high levels of nuclear NFκB DNA binding activity. In addition, transformation of the rat mammary cell line RM22-F5 from an ER positive to an ER negative state is accompanied by a constitutive activation of NFκB [30, 31]. NFκB also mediates the expression of cyclin D1 gene, because its promoter contains an NFκB binding site, whereas the overexpression of cyclin D1 in the mammary gland increases the incidence of mammary carcinomas [32, 33]. We demonstrate here that PG induces NFκB DNA-binding activity in a time-dependent manner in MCF-7 cells. In addition, the increase on NFκB DNA binding activity requires the activity of PI3K, Akt and mTOR, as well as the cytoskeleton integrity. We propose that PG mediates the oncogenic processes through the activation of PI3K/Akt pathway and then NFκB mediates gene expression. Supporting our proposal, it has been demonstrated that NFκB signaling pathway is commonly constitutively activated and that p65 and p50 subunits are frequently overexpressed in breast cancer compared to normal breast tissue [30, 31]. Moreover, cancer cells remodel the cytoskeleton to develop membrane protrusions including filopodia and lamellipodia, whereas PG mediates the remodeling of actin cytoskeleton and the formation of membrane ruffles through an extra-nuclear PG receptor in T-47D breast cancer cells [34-36].

Recently, three progestin receptors unrelated to classical PG receptors and localized in membrane (mPRs), namely mPRα, mPRβ and mPRγ have been described in humans [37]. The mPRs are 7-membrane 40 kDa proteins that are unrelated to nuclear steroid receptor and G protein coupled receptor (GPCR) superfamilies, however they belong to the progestin and adiponectin Q receptor family and mediate signal transduction pathways through G protein activation [38, 39]. The mPRs have been identified in human breast cancer biopsies and breast cancer cell lines, including MCF-7, SKBR3 and MDA-MB-468 cells. Particularly, mPRα mediates epithelial to mesenchymal transition through a PI3K/Akt-dependent pathway [40-42].

The mPRα activation is coupled with Gi proteins in MDA-MB-231 and SKBR3 breast cancer cells [41, 43]. CTX is an ADP-ribosyltransferase that modifies the Gsα subunit in the heterotrimeric G proteins, and this ADP ribosylation inhibits GTP hydrolysis by the G protein and maintains the activation of adenylyl cyclase with an increase of cAMP levels, whereas PTX is an inhibitor of Gi/Go proteins [18, 44]. Since, our findings demonstrate that PTX and CTX treatment inhibit the increase of NFκB DNA binding activity induced by PG, we propose that NFκB-DNA complex formation is mediated by Gi and/or Go proteins and that it is inhibited by high levels of cAMP. The cAMP production is mediated by Gsα. Moreover, our findings strongly suggest NFκB DNA binding activity is mediated by mPRs.

In conclusion, PG induces Akt2 activation through a PI3K-dependent pathway in MCF-7 breast cancer cells. Moreover, PG also promotes an increase of NFκB DNA binding activity through a PI3K, mTOR, Akt, Src and G-proteins-dependent pathway.

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Conflict of interest

The authors have no potential conflicts of interest.

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