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Original article

Crosstalk between Wnt signaling and Phorbol ester-mediated PKC signaling in MCF-7 human breast cancer cells

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ABSTRACT

Although many studies have implicated the crosstalk between the Wnt and PKC signaling pathways in tumor initiation and progression, the molecular roles of PKC isoforms in the Wnt signaling pathway remain poorly understood. In this study, we explored the contribution of PKC isoforms to canonical and noncanonical Wnt signaling pathway in mediating cell migration and an epithelial-mesenchymal transition (EMT). When MCF-7 cells were treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for up to 3 weeks, the effect of TPA on Wnt signaling pathway was dramatically different depending on the exposure time. The short term exposure (3 days) of MCF-7 cells to TPA exhibited significant induction of Wnt5a expression, along with the enhanced expression of PKC-α, to promote cell migration, which suggested that activation of noncanonical Wnt signaling pathway is associated with PKC-α. However, the chronic exposure (3 weeks) of cells to TPA completely suppressed Wnt5a expression and the expression of PKC-η and PKC-δ, whereas the expression of Wnt3a and PKC-θ were up-regulated to activate the canonical Wnt signaling pathway. Moreover, the loss of epithelial markers, including E-cadherin and GATA-3, suggested that chronic exposure of TPA stimulates EMT. Taken together, our data suggest that PKC-θ positively regulates the canonical Wnt signaling pathway, and that PKC-η and PKC-δ negatively modulate this signaling pathway.

1. Introduction

Protein kinase C (PKC) is a multigene family of phospholipid-dependent serine-threonine kinases that plays central roles in signal transduction, regulating a wide range of physiological or abnormal cellular functions, such as cell growth, transformation, differentiation, and cell motility [1]. Since PKC isoforms are often overexpressed in disease states, including cancer, they have been evaluated as potential pharmaceutical targets [2]. The conventional PKCs (cPKCs; α, β1, β2, and γ) require Ca^{2+} and diacylglycerol (DAG) for full activation, whereas the novel PKCs (nPKCs; δ, ε, η, θ, ν, and μ) do not require Ca^{2+} for activation but are DAG dependent. On the other hand, the atypical PKCs (aPKCs; ζ and ι/λ) are insensitive to both Ca^{2+} and DAG [1–3]. It is known that conventional and novel PKC isoforms are activated by the tumor-promoting phorbol esters such as the tumor-promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), while atypical isoforms are not [3,4]. PKC isoform distributions are tissue and cell type specific, which suggests that their functions differ from one cell type to another. However, the specific function of each isoform has not been fully understood.

The Wnt signaling pathway plays an important role in a variety of cellular processes including cellular proliferation, differentiation, and migration during embryogenesis and carcinogenesis [5,6]. There are two distinct Wnt signaling pathways, that is, the well-established canonical Wnt/β-catenin pathway and β-catenin-independent noncanonical Wnt pathway. Canonical Wnt signaling is transmitted by the binding of Wnt ligands, such as, Wnt1 and Wnt3, to Frizzled (FZD) and low-density lipoprotein receptors 5/6 (LRP5/6) to promote stabilization and nuclear translocation of β-catenin and the subsequent activations of target genes. On the other hand, noncanonical Wnt signaling is diverse and poorly characterized. However, it is known binding of Wnt5a to FZD mediates the activation of phospholipase C (PLC) via G proteins, leading to an increase in intracellular calcium levels and to the activation of PKC, referred to as the Wnt/Ca^{2+} pathway.

Abbreviation: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EMT, epithelial-mesenchymal transition; DAG, diacylglycerol; FZD, frizzled; LRP5/6, lipoprotein receptors 5/6; PLC, phospholipase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3β, glycogen synthase kinase-3β.

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Although the exact roles of PKC isoforms in this pathway have not been well established, increasing evidences indicate that PKC is a critical component of noncanonical Wnt signaling pathway. Several studies showed that Wnt5a activates PKC to promote the invasiveness and malignant progression in cancer cells [7]. Moreover, the activation of PKC can stabilize Wnt5a mRNA, thus leading to an increase in Wnt5a levels. In contrast to the positive role played by PKC in the noncanonical Wnt pathway, several studies have pointed out that PKC conferred either a positive or negative regulation on canonical Wnt signaling pathway depending on the PKC isoforms that involved [8–11].

Although it is known that the Wnt and PKC signaling pathways are both involved in tumor initiation and progression, little is known of crosstalk between them or the molecular role of PKC isoforms in the Wnt signaling pathway. In the present study, we investigated the contribution of PKC isoforms to either canonical or noncanonical Wnt signaling pathway involved in the regulation of cell motility and epithelial-mesenchymal transition (EMT) in TPA-treated MCF-7 human breast cancer cells.

2. Materials and methods

2.1. Cell culture and TPA treatment

The MCF-7 human breast cancer cell line was purchased from the Korean Cell Line Bank (Seoul, Korea) and routinely maintained in DMEM (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 10 μg/mL insulin (Welgene), and 1% antibiotic-antimycotic solution (Welgene). For chronic exposure of MCF-7 cells to 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma, St. Louis, USA), cells were cultured in presence of 100 nM TPA and passaged several times when cells reached 80% confluence.

2.2. RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated using easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology. Inc., Sungnam, Korea) and cDNA synthesis and RT-PCR were performed as previously described [12]. The primer sequences used for the RT-PCR are presented in Table 1. All experiments were conducted in triplicate and results are presented as representative images of gel electrophoresis from of RT-PCR analysis.

2.3. Wound healing assay

Cells were seeded on 6-well plates coated with collagen IV (Corning, Bedford, MA) and grown to reach confluence. Monolayers of cells were scratched with a 200 μl pipette tip to create wounds and cells were then cultured in media with or without TPA. Pictures were taken at time 0 and after 3 days of culture.

2.4. Immunofluorescence analysis

Cells grown in 4-well chamber slides (Thermo Fisher Scientific, Rochester, NY) were fixed with ice-cold methanol and acetone for 4 and 2 min, respectively. After washing three times with PBS, cell preparations were blocked in 10% fetal bovine serum for 1 h at room temperature. Cells were then stained with anti-rabbit E-cadherin (Cell Signaling, Beverly, MA) and anti-mouse CD44 (BD Biosciences, California, USA) antibodies overnight. Samples were incubated with anti-rabbit IgG- Alexa 546 and anti-mouse IgG 488 secondary antibodies (Life Technologies, Carlsbad, CA) for 1 h at room temperature. Finally, cells were mounted in Prolong Gold Antifade reagent with DAPI (Life Technologies) and visualized using a Zeiss Axio Observer A1 microscope (Radiance 2100, Biorad, Hercules, CA).

2.5. Flow cytometry analysis

Cells were trypsinized and washed with PBS containing 2% FBS. Cells were incubated with CD24-PE (BD Biosciences) and CD44-FITC (BD Biosciences) for 30 min on ice and washed with PBS containing 2% FBS. They were then resuspended in a final volume of 500 μl PBS for analysis. Flow cytometry analysis was performed on a FACS Calibur II (BD Biosciences). Unstained samples and single color-labeled samples were used to calibrate the analyzer prior to each experiment.

3. Results

3.1. The TPA-induced morphological changes were associated with enhanced migratory ability rather than induction of EMT in MCF-7 cells

We treated MCF-7 cells with 100 nM TPA for 3 days and inspected cellular change daily. Similar to other studies reporting morphological changes of breast cancer cell lines on treatment with TPA [4], TPA treatment caused marked morphological changes depending on the exposure time. The morphological changes appeared gradually afterwards, with cell shape change becoming spindle-like shape and dissociating from each other within first 24 h (Fig. 1A) . However, further treatment with TPA up to 3 days led to cells becoming enlarged and more flattened in shape (Fig. 1A) and these morphological changes were sustained until cells began to detach from the dish. Since morphological

Table 1
Primer sequences for RT-PCR.

Primers	Forward	Reverse
Snail-1	5′-CCGGACCCACACTGGCGAGA-3′	5′-CTCGAGGGTCAGCGGGGACA-3′
Slug	5′-GCTGCTCCATTCCACGCCCA-3′	5′-AGGCTTCTCCCCCGTGTGAGTT-3′
E-cadherin	5′-CGACCCAACCCAAGAATCTA-3′	5′-AGGCTGTGCCTTCCTACAGA-3′
Fibronectin	5′-CAGTGGGAGACCTCGAGAAG-3′	5′-TCCCTCGGAACATCAGAAAC-3′
Wnt5a	5′-GGGAGGTTGGCTTGAACATG-3′	5′-GAATGGCACGCAATTACCTT-3′
Wnt3a	5′-TGAACAAGCACAACAACGAG-3′	5′-CAGTGGCATTTTTCCTTCC-3′
Axin2	5′-TTATGCTTTGCACTACGTCCCTCCA-3′	5′-CGCAACATGGTCAACCCTCAAGAC-3′
GATA3	5′-CAGCACAGAAGGCAGGGAGT-3′	5′-TCCTCCAGAGTGTGGTTGTG-3′
αPKC	5′-CGACTGTCTGTAGAAATCTGG-3′	5′-CTACCATGGTGCACTCCACGTC-3′
θPKC	5′-CTATCAATAGCCGAGAAACCATG-3′	5′-CTCATCCAACGGAGACTCCC-3′
ηPKC	5′-CCATGAAGATGCCACAGGGATC-3′	5′-CATCCTTCACAGAGTTCTTCACTTGC-3′
δPKC	5′-CACTATATTCCAGAAAGAACG-3′	5′-CTTGCCGTAGGTCCCACTGTTG-3′
GAPDH	5′-ATCCCATCACCATCTTCCAG-3′	5′-TTCTAGACGGCAGGTCAGGT-3′