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COUP-TFII inhibits NFkappaB activation in endocrine-resistant breast cancer cells

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ABSTRACT

Reduced COUP-TFII expression contributes to endocrine resistance in breast cancer cells. Endocrine-resistant breast cancer cells have higher NFkappa B (NFκB) activity and target gene expression. The goal of this study was to determine if COUP-TFII modulates NFκB activity. Endocrine-resistant LCC9 cells with low endogenous COUP-TFII displayed ~5-fold higher basal NFκB activity than parental endocrine-sensitive MCF-7 breast cancer cells. Transient transfection of LCC9 cells with COUP-TFII inhibited NFκB activation and reduced NFκB target gene expression. COUP-TFII and NFκB were inversely correlated in breast cancer patient samples. Endogenous COUP-TFII coimmunoprecipitated with NFκB subunits RelB and NFκB1 in MCF-7 cells. COUP-TFII inhibited NFκB-DNA binding *in vitro* and impaired coactivator induced NFκB transactivation. LCC9 cells were growth-inhibited by an NFκB inhibitor and 4-hydroxytamoxifen compared to MCF-7 cells. Together these data indicate a novel role for COUP-TFII in suppression of NFκB activity and explain, in part, why decreased COUP-TFII expression results in an endocrine-resistant phenotype.

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1. Introduction

Selective estrogen receptor modulators (SERMs) such as tamoxifen (TAM) exert antiproliferative effects in breast cancer by competing with estrogens for binding to estrogen receptor α (ERα), leading to an inhibition of downstream genes, including those involved in breast cancer proliferation. Fulvestrant is a selective ERα downregulator (SERD) because it causes proteasomal degradation of ERα in addition to its SERM-like ability to antagonize estrogen-activated ERα transactivation (Wijayaratne et al., 1999). TAM inhibits cell cycle progression and induces apoptosis (Mandlekar and Kong, 2001; Salami and Karami-Tehrani, 2003). TAM has greatly increased the survival rate of breast cancer patients since its initial FDA approval in 1977, resulting in a 31% reduction in annual death rate (Early Breast Cancer Trialists' Collaborative, 2005; Jordan, 2009). Despite the initial efficacy of SERM and aromatase inhibitor treatment, approximately 40% of patients relapse and die from metastatic disease because the cancer cells become refractory to endocrine therapies (Ring and Dowsett, 2004).

The mechanisms for acquired endocrine resistance are complex and a better understanding of maintenance of endocrine sensitivity is clearly needed. Reduced expression of the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) has a demonstrated role in acquired endocrine resistance in a breast cancer cell line model of human disease progression (Riggs et al., 2006). Restoration of COUP-TFII expression, which is reduced in endocrine-resistant breast cancer cell lines, inhibited cell proliferation and motility and increased apoptosis in TAM and fulvestrant-treated endocrine-resistant LCC9 and LY2 breast cancer cells (Riggs et al., 2006). Conversely, inhibition of COUP-TFII expression caused MCF-7 cells to become resistant to SERM and fulvestrant inhibition of proliferation (Riggs et al., 2006). A mechanism for the involvement of COUP-TFII in maintenance of endocrine sensitivity has not yet been determined. Since COUP-TFII acts as either a transcriptional activator or repressor in a gene- and cell-specific manner (Litchfield and Klinge, 2012), the mechanism of action may involve modulation of specific target genes including tumor suppressors and oncogenes.

The NFκB family of dimeric transcription factors have important functions related to immune and inflammatory responses, and recent studies have demonstrated NFκB activation plays a role in acquired endocrine-resistant breast cancer (Gu et al., 2002;

Abbreviations: COUP-TFII, chicken ovalbumin upstream promoter transcription factor II; NFκB/NFkappa B, nuclear factor κB; SERM, selective estrogen receptor modulator; TAM, tamoxifen; ERα, estrogen receptor; SERD, selective estrogen receptor downregulator; 4-OHT, 4-hydroxytamoxifen; TNFα, tumor necrosis factor; RE, response element; qPCR, quantitative real-time PCR; IL6, interleukin 6 (IL6); ICAM1, intercellular adhesion molecule 1; A20/TNFAIP3, TNFα-induced protein 3.

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Nehra et al., 2010; Riggins et al., 2005). Inactive NFκB is located in the cytoplasm bound to IκB, the inhibitor of NFκB. Also present in the cytoplasm is the IKK complex containing the regulatory subunit NEMO (nuclear factor-κB essential modulator or IKKγ) and the catalytic subunits IKKα and IKKβ. Upon activation by a variety of stimuli, the IKK complex phosphorylates the inhibitor IκB leading to its degradation, freeing the now activated NFκB to translocate to the nucleus to regulate target gene transcription. Because NFκB activation results in expression of genes that lead to anti-apoptotic and pro-proliferative activities, increased NFκB signaling promotes cancer cell survival (Perkins, 2007).

Dysregulation of the NFκB subunits p65 (*RELA*), RelB (*RELB*), p50 (*NFKB1*), p52 (*NFKB2*), and c-Rel (*REL*) results in altered activation of the NFκB pathway in breast cancer. Increased expression of p50/NFκB1, p52/NFκB2, and c-Rel was detected in breast tumors compared to adjacent normal tissue (Cogswell et al., 2000). Elevated p50/NFκB1-DNA binding correlated with metastatic relapse and reduced disease-free survival in patients with ERα-positive tumors (Zhou et al., 2005a,b). An increase in NFκB activity has been reported in endocrine-resistant breast cancer cells (Gu et al., 2002; Nehra et al., 2010; Zhou et al., 2007). p65/RelA level was also elevated (Nehra et al., 2010). The expression and DNA binding of p50 and p65 are also enhanced in LCC1 estrogen-independent, TAM-sensitive breast cancer cells compared to MCF-7 estrogen-dependent cells, further demonstrating a role for NFκB in the pathway to endocrine resistance (Pratt et al., 2003).

Since COUP-TFII expression is decreased in endocrine-resistant breast cancer cells, we hypothesized COUP-TFII may play a part in the normal suppression of NFκB activity in endocrine-sensitive breast cancer cells by regulating the transcription of components of the NFκB pathway. In this study, we observed that COUP-TFII overexpression inhibited NFκB activity in LCC9 endocrine-resistant breast cancer cells. This suppression of the NFκB pathway resulted in reduced expression of downstream NFκB target genes as well as NFκB subunits and increased sensitivity to 4-hydroxytamoxifen (4-OHT) treatment. Our results provide a mechanism by which COUP-TFII maintains endocrine sensitivity by suppressing NFκB expression and activity in breast cancer cells.

2. Materials and methods

2.1. Chemicals

4-hydroxytamoxifen (4-OHT) and BMS-345541 were purchased from Sigma–Aldrich (St. Louis, MO). Tumor necrosis factor α (TNFα was purchased from PeproTech (Rocky Hill, NJ).

2.2. Antibodies

The following antibodies were purchased: monoclonal (mAb) anti-human COUP-TFII (R&D systems, Minneapolis, MN); polyclonal RelA/p65, polyclonal and mAb RelB, mAb NFκB1 p105/p50, and polyclonal p-NFκB p65/RelA (Ser 529) (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal c-Rel, polyclonal NFκB1 p105/p50, and polyclonal NFκB2 p100/p52 (Cell Signaling, Danvers, MA), β-actin (Sigma–Aldrich). HRP-conjugated secondary antibodies were from GE Healthcare (Piscataway, NJ).

2.3. Cell culture and transient transfection

MCF-7 breast cancer cells were purchased from ATCC and used at passage <10. LCC9 are ERα/progesterone receptor (PR)+, multi-antiestrogen (tamoxifen and fulvestrant)- resistant breast cells derived from MCF-7 cells and were kindly provided by Dr. Robert Clarke (Brunner et al., 1997). MCF-7 and LCC9 cells were maintained as in (Riggs et al., 2006). Cells were transiently transfected as indicated in figure legends using FuGENE HD (Roche, Indianapolis, IN) as per the manufacturer's protocol.

2.4. Luciferase assay

To analyze NFκB activity, MCF-7 and LCC9 cells were transiently transfected with pGL4.32[luc2P/NF-κB-RE/Hygro] (Promega, Madison, WI) containing five copies of a NFκB response element, pGL4-hRluc-TK (*Renilla*, Promega), and pcDNA3.1 or pcDNA3.1-mCOUP-TFII (kindly provided by Drs. Sophia and Ming-Jer Tsai (Qiu et al., 1995)) for 48 h before performing dual luciferase assay (Promega). Where indicated, cotransfection was performed with plasmids for CBP (kindly provided by Dr. Margarita Hadzopoulou-Cladaras (Dell and Hadzopoulou-Cladaras, 1999)), SRC-1 (kindly provided by Dr. Bert W. O'Malley (Lanz et al., 1999)), GRIP1/SRC-2, or ACTR/SRC-3 (kindly provided by Dr. Michael Stallcup (Koh et al., 2001)).

2.5. NFκB pathway PCR array

LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-mCOUP-TFII for 24 h before treating with 10 ng/ml TNFα for 6 h. RNA was isolated using RNeasy (Qiagen, Valencia, CA) and cDNA was prepared using RT^2 First Strand Kit (SABiosciences/Qiagen, Valencia, CA). Human NFκB Signaling Pathway PCR Array (SABiosciences/Qiagen) was run according to manufacturer's instructions.

2.6. NFκB family DNA-binding assays

MCF-7 or LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNFα for 6 h before preparing nuclear extract (NE). In brief, following centrifugation, cells were resuspended in 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT, Sigma–Aldrich), 1× protease and phosphatase inhibitors (Roche). The cells were lysed with a 27 gauge syringe and centrifuged. The cytosolic extract was removed and the resulting nuclear pellet was resuspended in NE buffer (10 mM Tris HCl, 150 mM KCl, 0.5 mM EDTA, 0.1% Triton-X 100, 12.5% glycerol, pH 7.9), 1 mM DTT, 1X protease and phosphatase inhibitor (Roche)), sonicated, and then sedimented by centrifugation with the resulting supernatant being the NE. Following protein concentration determination (as above), 15 μg NE was used for TransAM NFκB DNA binding ELISA assays (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Briefly, NE was added to 8 well strips containing immobilized NFκB RE oligonucleotide. Primary antibodies specific for NFκB subunits RelB, RelA, p50, p52, and c-Rel were used to identify subunit-specific NFκB activation. Secondary HRP-conjugated antibody and provided buffers provided colorimetric quantitation via spectrophotometric analysis at 450 nm.

2.7. Co-immunoprecipitation

Nuclear extracts were prepared from MCF-7 cells as in (Litchfield et al., 2012). Dynabeads protein A (Life Technologies, Carlsbad, CA) were incubated with COUP-TFII antibody (Abcam, Cambridge, MA) or negative control IgG (Santa Cruz) for 30 min prior to the addition of 400 μg MCF-7 NE for 4 h at 4 °C with rotation. Following washes, protein was eluted with Elution Buffer (Life Technologies, Carlsbad, CA) and pH was adjusted with 1 M TrisHCl.

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