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Genistein inhibits phorbol ester-induced NF-κB transcriptional activity and COX-2 expression by blocking the phosphorylation of p65/RelA in human mammary epithelial cells

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ABSTRACT

Genistein, an isoflavone present in soy products, has chemopreventive effects on mammary carcinogenesis. In the present study, we have investigated the effects of genistein on phorbol ester-induced expression of cyclooxygenase-2 (COX-2) that plays an important role in the pathophysiology of inflammation-associated carcinogenesis. Pretreatment of cultured human breast epithelial (MCF10A) cells with genistein reduced COX-2 expression induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). There are multiple lines of evidence supporting that the induction of COX-2 is regulated by the eukaryotic transcription factor NF-κB. Genistein failed to inhibit TPA-induced nuclear translocation and DNA binding of NF-κB as well as degradation of IκB. However, genistein abrogated the TPA-induced transcriptional activity of NF-κB as determined by the luciferase reporter gene assay. Genistein inhibited phosphorylation of the p65 subunit of NF-κB and its interaction with cAMP regulatory element-binding protein-binding protein (CBP)/p300 and TATA-binding protein (TBP). TPA-induced NF-κB phosphorylation was abolished by pharmacological inhibition of extracellular signal-regulated kinase (ERK). Likewise, pharmacologic inhibition or dominant negative mutation of ERK suppressed phosphorylation of p65. The above findings, taken together, suggest that genistein inhibits TPA-induced COX-2 expression in MCF10A cells by blocking ERK-mediated phosphorylation of p65 and its subsequent interaction with CBP and TBP.

1. Introduction

According to epidemiological studies, Asian populations who consume a diet rich in soy products have a relatively low incidence of breast, colon and prostate cancers [1]. However, Asian' immigrants in the United States who adopt a Western diet become at a higher risk of breast cancer [2]. Genistein, a soy derived isoflavone, has been reported to have substantial chemopreventive effects [3]. It has been shown that genistein has numerous intracellular targets in exerting its cancer chemopreventive effects, including suppression of protein tyrosine kinases [3]. Genistein also causes inhibition of angiogenesis and metastasis, cell cycle arrest, attenuation of oxidative stress, and induction of apoptosis and differentiation of cancer cells [3]. Additionally, it has been reported that genistein has an anti-inflammatory activity, which can also account for its chemopreventive potential. Thus, genistein inhibits 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammatory responses in mouse skin [4] and TPA- or transforming growth factor β (TGF-β)-induced cyclooxygenase-2 (COX-2) expression [5]. Genistein inhibited COX-2 expression in bacterial lipopolysaccharide (LPS)-stimulated human chondrocytes [6] and release and expression of tumor necrosis factor-α (TNF-α) and interleukin-8 in human peripheral blood leukocytes challenged with LPS [7]. This isoflavone also inhibited COX-2 expression in head and neck cancer cells [8] and TPA-stimulated MCF-7 cells [9]. Genistein exerted complementary action with n-3 polyunsaturated fatty acids in

Abbreviations: CBP, cAMP regulatory element-binding protein-binding protein; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated protein kinase; IKK, IκB kinase; PGE_2, prostaglandin E_2; TBP, TATA-binding protein; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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down-regulating COX-2 expression in MDA-MB-231 breast cancer cells [10].

COX-2, a key enzyme involved in inflammatory signaling, is an immediate early response gene product that is highly inducible by mitogenic and pro-inflammatory stimuli, including tumor promoters, growth factors, cytokines, and reactive oxygen species. Elevated levels of prostaglandins and COX-2 expression/activities have been frequently observed in various cancers of epithelial origin [11]. It becomes increasingly evident that abnormally upregulated COX-2 is implicated in the pathogenesis of cancer because it affects mitogenesis, cellular adhesion, angiogenesis, metastasis, apoptosis, etc. Overexpression of COX-2 blunted apoptosis and potentiated the invasiveness of malignant cells [12]. Based on these findings, targeted inhibition of COX-2 has been considered to be a promising approach toward chemoprevention as well as treatment of cancer [13].

Nuclear factor κB (NF-κB) regulates the expression of many genes involved in immune and inflammatory responses [14]. NF-κB is a ubiquitous, pleiotropic, multi-subunit eukaryotic transcription factor consisting of either homo- or heterodimer of various subunits of Rel family proteins referred to as p50, p52, p65 (RelA), c-Rel, and RelB. The conventional active form of NF-κB is a heterodimer, which usually consists of p65 (RelA) and p50 subunits. NF-κB is activated by antigens, viruses, bacteria, inflammatory cytokines, phorbol ester, etc., leading to transcriptional initiation of a diverse set of genes the products of which are important in mediating inflammatory responses [14].

In most cells, NF-κB is sequestered in the cytoplasm as an inactive complex with an inhibitory protein, IκB. NF-κB activation is achieved through the signal-induced proteolytic degradation of IκB in the cytoplasm. The free NF-κB complex then migrates to the nucleus, binds to the DNA consensus sequence and transactivates target genes. In contrast to phosphorylation of IκB, phosphorylation of p65 has not been clearly defined and seems to vary depending on the nature of the stimulus and the cell type [15]. In response to TNF-α treatment, p65 was phosphorylated at Ser536 by IκB kinases (IKK) [16]. p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated protein kinase (ERK) are also known to regulate the NF-κB transcriptional activity [17].

In the present study, we attempted to examine whether genistein could inhibit COX-2 expression and prostaglandin E_2 (PGE_2) production induced by TPA in the cultured human mammary epithelial cell line (MCF10A), preferentially by blocking NF-κB signaling.

2. Materials and methods

2.1. Materials

Genistein and TPA were purchased from Alexis Biochemicals (San Diego, CA, USA). Cholera toxin, hydrocortisone, anti-actin, and recombinant human epithelial growth factor (hEGF) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium/F-12nutrient (DMEM/F12), L-glutamine, horse serum, penicillin/streptomycin/fungi zone mixture were purchased from Gibco BRL (Grand Island, NY, USA). NF-κB consensus oligonucleotide and the luciferase assay system with reporter lysis buffer were supplied by Promega (Madison, WI, USA). [γ-^{32}P]ATP was the product of NEN Life Science (Boston, MA, USA). pELAM-Luc construct containing the NF-κB site was a generous gift from Dr. Youngmi K. Pak of the University of Ulsan, College of Medicine, Seoul, Korea. ERK dominant negative (pCEP4-ERK DN) vector was a generous gift from Dr. Yun-Sil Lee of Ewha Woman's University. PD98059 and U0126 were products of Calbiochem (La Jolla, CA, USA). Anti-COX-2, anti-phospho-ERK, and anti-ERK antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-phospho-p65 was obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against cAMP regulatory element-binding protein-binding protein (CBP) and TATA-binding protein (TBP) were the products of Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). BCA reagent was a product of Pierce (Rockford, IL, USA). Polyvinylidene difluoride (PVDF) membranes were from Gelman Laboratory (Ann Arbor, MI, USA). Transfection reagents and protease inhibitor cocktail tablet were provided from Roche Molecular Biochemicals (Mannheim, Germany). ECL reagent was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.2. Cell culture

The MCF10A cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air in DMEM/F12 medium supplemented with 5% heat-inactivated horse serum, 10 μg/ml insulin, and 100 ng/ml Cholera toxin, 0.5 μg/ml hydrocortisone, 20 ng/ml recombinant EGF, 2 mM L-glutamine, 100 ng/ml penicillin/streptomycin/fungizone mixture. Cells were grown to 60–80% confluence and trypsinized with 0.05% trypsin containing 2 mM EDTA.

2.3. Immunoprecipitation and Western blotting

MCF10A cells were treated with RIPA lysis buffer [150 mM NaCl, 0.5% Triton X-100, 50 mM Tris–HCl (pH 7.4), 25 mM NaF, 20 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4], protease inhibitor cocktail tablet for 15 min on ice followed by centrifugation at 12,000 × g for 20 min. The protein concentration of the supernatant was measured by using the BCA reagents. Protein was loaded to 7 to 12% SDS-PAGE gel and transferred to the PVDF membrane. PVDF membrane was then incubated with the primary antibodies diluted in blocking buffer. Antibodies used were a goat polyclonal anti-COX-2 antibody (diluted 1:1000), a rabbit polyclonal anti-phospho-p65 antibody (diluted 1:100), a rabbit polyclonal anti-phospho-p38 antibody (diluted 1:100), a rabbit polyclonal p38 antibody (diluted 1:1000), a mouse monoclonal phospho-ERK antibody (diluted 1:1000), a goat polyclonal ERK1 antibody (diluted 1:1000), a rabbit polyclonal anti-TBP antibody (diluted 1:500) and a rabbit monoclonal anti-actin antibody (diluted 1:5000). Afterwards, membranes were washed and incubated with anti-goat or anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Zymed Laboratories Inc., San Francisco, CA, USA) was used as a secondary antibody. The transferred proteins were visualized with the ECL Western blot detection system according to the manufacturer's instructions. Interactions of CBP with p65 or TBP were determined by immunoprecipitation of cell extracts with anti-p65 or anti-CBP, respectively. Cell extracts (500 μg) were rotated for 2 h at 4 °C with anti-p65 or anti-CBP and then protein G plus agarose (Santa Cruz product, Santa Cruz, CA, USA) beads for overnight at 4 °C according to the manufacturer's protocol. The beads were washed four times with 1 ml of lysis buffer and boiled for 5 min in SDS-denaturing sample buffer. Proteins were resolved through SDS-12% polyacrylamide gels.

2.4. Measurement of PGE_2 production

MCF10A cells (6 × 105) were plated in 6-well dishes and grown to 80% confluence in 2 ml growth medium. The cells were then pretreated with various concentrations of genistein for 1 h, followed by 20 nM TPA. The amounts of PGE_2 released into media were measured by the enzyme-linked immunoassay using the commercially

available kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.5. Preparation of cytosolic and nuclear extracts from MCF10A cells

Confluent cells in 100 mm dishes were treated with genistein for 1 h before addition of 20 nM TPA. Cells were gently washed twice with ice-cold phosphate-buffered saline (PBS), scraped in 1 ml PBS and centrifuged at 12,000 × *g* for 30 sec at 4 °C. Pellets were suspended in 200 μl of hypotonic buffer A (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF) for 15 min on ice, and 12.5 μl of 10% Nonidet P-40 solution was added for 5 min. The mixture was then centrifuged for 6 min at 12000 × *g*. Supernatant was kept for Western blot analysis. The nuclei were washed once with 400 μl of PBS, and suspended in 70 μl of buffer C (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol) for 20 min on ice and centrifuged for 6 min at 12000 × *g*. The supernatant containing nuclear proteins was collected and stored at −70 °C after determination of the protein concentration.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a DNA-protein binding detection kit according to the manufacturer's protocol (GIBCO BRL, Grand Island, NY, USA). This assay measures the binding of the nuclear extract and the radio-labeled oligonucleotide harboring the NF-κB consensus sequence on the premise that substantial amounts of cytoplasmic NF-κB protein are migrated to the nucleus of stimulated cells. Briefly, the NF-κB oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech, Buckinghamshire, UK). The binding reaction was carried out in 25 μl of the mixture containing 5 μl of incubation buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (v/v) glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 10 μg of nuclear extracts of MCF10A cells, and 100,000 cpm of the labeled probe. After 1-h incubation at room temperature, 2 μl of 0.1% bromophenol blue was added and samples were electrophoresed on a 6% non-denaturing polyacrylamide gel at 150 V in a cold room. Finally, the gel was dried and exposed to an X-ray film (Agfa-Gevaert, NV, USA) to detect the NF-κB-DNA complex by autoradiography. The intensity of specific bands is a measure for the amount of NF-κB in the nuclear fraction. The specificity of NF-κB band was assessed by adding the fifty-fold excess of unlabeled NF-κB oligonucleotide as a competitor to the reaction mixture [18].

2.7. Transient transfection and the luciferase reporter gene assay

MCF-10A cells were plated at a density of 2.5×10^5 cells in 6-well dishes followed by 12 h-incubation for stabilization. The cells were then transiently transfected with the 2.5 μg of the plasmid pELAM-Luc harboring the NF-κB binding site ligated to the luciferase reporter gene and 1 μg of pCDNA3.1/hisB/lacZ (encoding galactosidase for normalization of luciferase activity) using the transfection reagent DOTAP (Roche Molecular Biochemicals, Mannheim, Germany) according to the instructions supplied by the manufacturer. After 24 h, the medium was changed and 20 nM TPA was added to each well in the absence or presence of genistein. Cells were then washed twice with cold PBS and digested with reporter lysis buffer (Promega Co., Madison, USA). After vortex-mixing and centrifugation for 1 min at 12,000 × *g* at 4 °C, the supernatant was stored at −70 °C for the luciferase assay. Twenty μl of cell extract and 100 μl of the luciferase assay reagent were mixed at room temperature and placed in a luminometer (AutoLumat LB 953, EG&G Berthold, Bad Widbad, Germany) for detection of the light produced. In dominant-negative mutant experiments, cells were co-transfected with the pCDNA3.1/hisB/lacZ and ERK dominant negative (pCEP4-ERK2 DN) vector or an empty vector.

2.8. Kinase assay

Cell lysates (200 μg) were immunoprecipitated with anti-IKKα or anti-IKKβ (for IKK activity assay) antibody by using protein-A Sepharose beads (Zymed Laboratories, San Francisco, CA, USA) and incubated overnight at 4 °C. The activities of IKKα and IKKβ were determined by the radioactive kinase assay protocol.

2.9. Immunofluorescence staining

MCF10A cells grown on coverslips were pretreated with genistein for 1 h and then treated with 20 nM TPA for an additional 2 h. Cells were then rapidly washed with PBS and fixed for 10 min at 4 °C with 4% formaldehyde. After being washed with PBS, the cells were blocked by PBS containing 10% BSA and 0.5% Tween-20 for

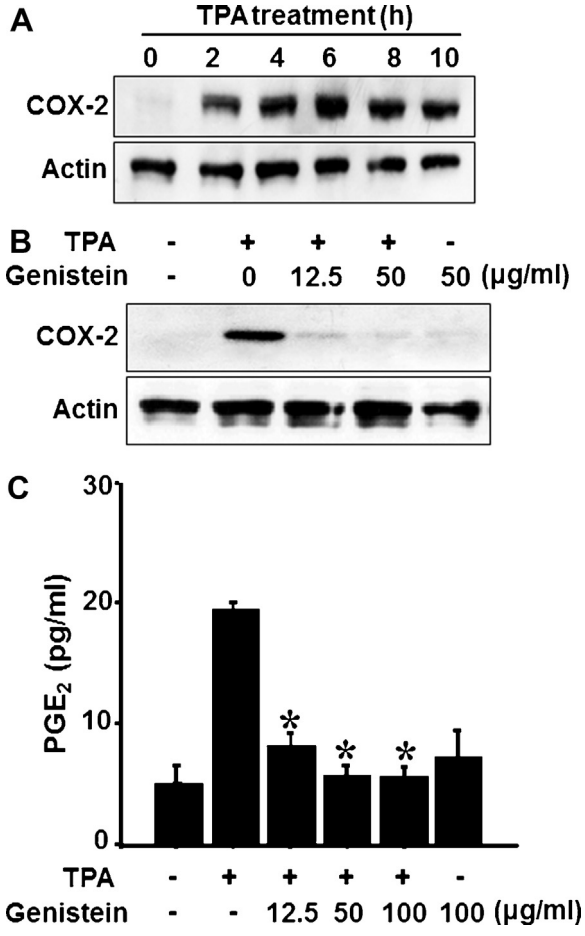

Fig. 1. TPA-induced COX-2 expression and its suppression by genistein in MCF10A cells. (A) MCF10A cells were stimulated with 20 nM of TPA. Whole cell lysates were prepared at various time intervals and subjected to Western blot analysis using an antibody specific for COX-2. (B) Cells were pretreated with indicated concentrations of genistein for 1 h and then stimulated for 4 h with TPA (20 nM). Whole cell lysates were prepared and subjected to Western blotting, as described in Section 2. Each blot is representative of three different experiments. (C) Cells were pretreated with or without genistein for 1 h, followed by treatment with 20 nM TPA or vehicle alone. After 6 h, the medium was collected to determine the amounts of PGE_2 released. Production of PGE_2 was measured by the enzyme-immunoassay. Results are expressed as the mean ± SD of three independent experiments performed in triplicate. *Significantly low ($p < 0.05$) as compared with TPA alone.

1 h at room temperature. The translocation of p65 NF-κB subunit was visualized using a mouse monoclonal antibody (BD Bioscience Pharmingen, San Diego, CA, USA) that recognizes an epitope overlapping the nuclear location signal of the p65 NF-κB subunit. The phosphorylated p65 NF-κB subunit was visualized using a rabbit monoclonal antibody (Cell Signaling Technology Inc., Beverly, MA, USA). The antibodies were diluted 1:100 in blocking buffer and incubated overnight at 4 °C. Afterwards, cells were washed with PBS and labeled with FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG for 1 h at room temperature. After washing, a coverslip was mounted with Gel/Mount (BD Bioscience Pharmingen, San Diego, CA, USA). The translocated p65 or phosphorylated p65 was scanned using the Leica TCS confocal systems (Leica Microsystems Heidelberg GmbH, Germany).

3. Results

3.1. Genistein inhibits the TPA-induced expression of COX-2 and production of PGE_2 in MCF10A cells

When MCF10A cells were treated with 20 nM of TPA for various times, COX-2 expression was evident from 2 h (Fig. 1A). When pretreated to MCF10A cells for 1 h, genistein strongly inhibited the TPA-induced COX-2 expression (Fig. 1B). In contrast, treatment with vehicle (0.1% DMSO) or genistein (50 μg/ml) alone did not affect constitutive expression of COX-2. Because the major prostaglandin synthesized by COX-2 is PGE_2, we next examined the effect of genistein on TPA-induced PGE_2 production in MCF10A cells. TPA induced a significant increase in the amounts of PGE_2

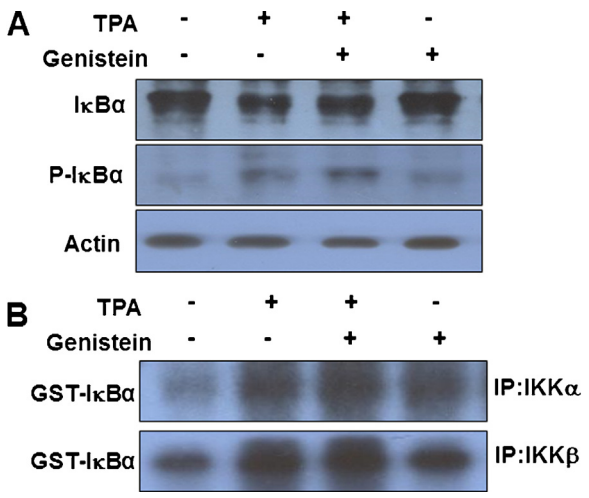

Fig. 3. Effects of genistein on TPA-induced phosphorylation and degradation of IκBα and IKKα/β activity. (A) Cells pretreated with 50 mg/ml of genistein for 1 h and then incubated with 20 nM of TPA for additional 2 h. Cytosolic levels of IκBα were immunodetected using a specific antibody. (B) The IKK activity was measured as described in Section 2.

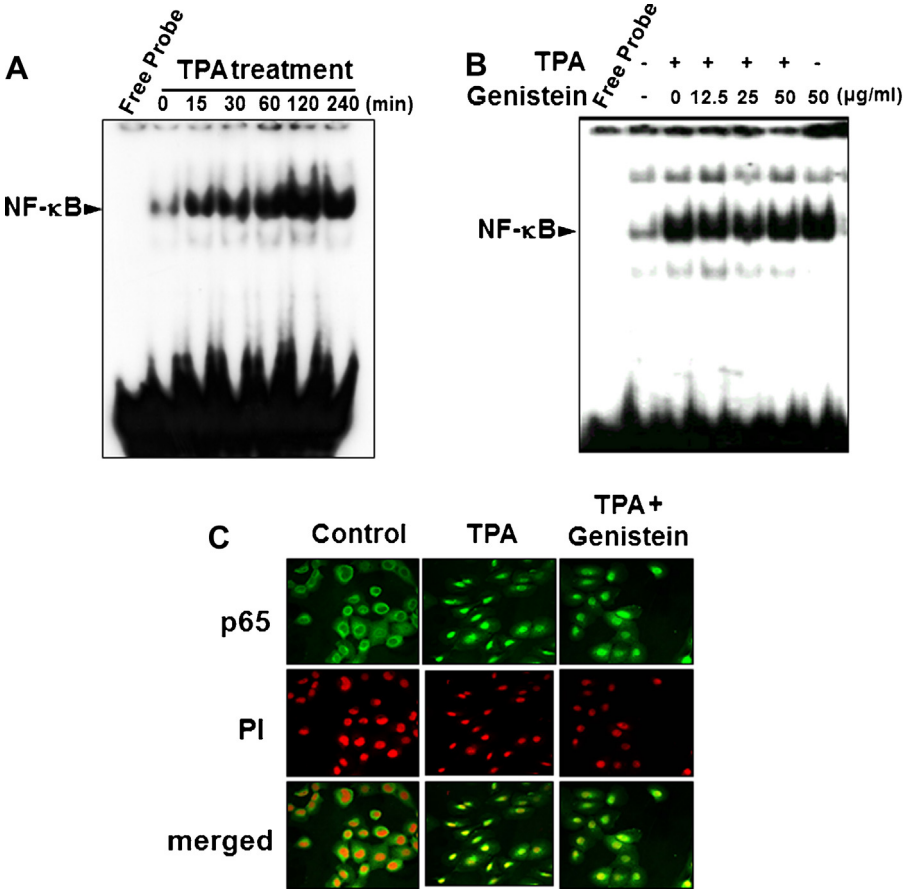

Fig. 2. Effects of genistein on TPA-induced DNA-binding and nuclear translocation of NF-κB and IκBα degradation in MCF10A cells. (A) Cells were treated with 20 nM TPA for the indicated time intervals, then nuclear extracts were prepared. The NF-κB-specific DNA binding activity in nuclear extracts was determined by EMSA. (B) Cells were cultured for 1 h in the presence or absence of genistein at the indicated concentrations and then stimulated with 20 nM TPA for 2 h. Nuclear protein was isolated, and binding reactions were performed using the consensus NF-κB oligonucleotide labeled with [γ-^{32}P]ATP. (C) MCF10A cells were incubated with genistein (0 or 50 μg/ml) for 1 h followed by stimulation with TPA (20 nM) for 2 h. Immunofluorescence staining of the NF-κB subunit RelA with affinity-purified anti-p65 antibody (1:100) in MCF10A cells. Cells were fixed and stained as described in Section 2.

released into media, which was attenuated by genistein pretreatment (Fig. 1C).

3.2. Effects of genistein on nuclear translocation and DNA binding of NF-κB and IκBα phosphorylation

Because NF-κB is considered to play a crucial role in the inducible expression of many genes involved in inflammatory responses including COX-2, we determined whether genistein could suppress the activation of this transcription factor in MCF10A cells treated with TPA.

In a preliminary study, the time course of NF-κB-DNA binding after treatment with TPA was examined. TPA-induced NF-κB-DNA binding, as assessed by the gel-shift assay, was detectable in as early as 15 min after the TPA treatment with maximal activation observed at 2 h (Fig. 2A). However, TPA-induced binding of the NF-κB-specific oligonucleotide to nuclear extracts from TPA-stimulated MCF10A cells was not inhibited by pre-incubation with genistein (Fig. 2B). p65 is the major functionally active subunit of NF-κB which is predominantly located in the cytoplasm in unstimulated MCF10A cells. TPA treatment for 2 h led to substantial accumulation of p65 in nucleus, but this was also not affected by genistein (Fig. 2C).

Because the amount of NF-κB protein migrating to the nucleus is thought to be proportional to the degradation of IκBα through phosphorylation, the levels of IκBα and its phosphorylated form, were examined. Genistein failed to inhibit TPA-induced degradation of IκBα as well as phosphorylation (Fig. 3A) and activity of IKKα/β responsible for IκBα phosphorylation (Fig. 3B). While nuclear translocation of NF-κB and its subsequent DNA binding were apparently not inhibited by genistein, this isoflavone (50 μg/ml) suppressed TPA-induced NF-κB transcriptional activity as assessed by the luciferase reporter gene assay (Fig. 4A).

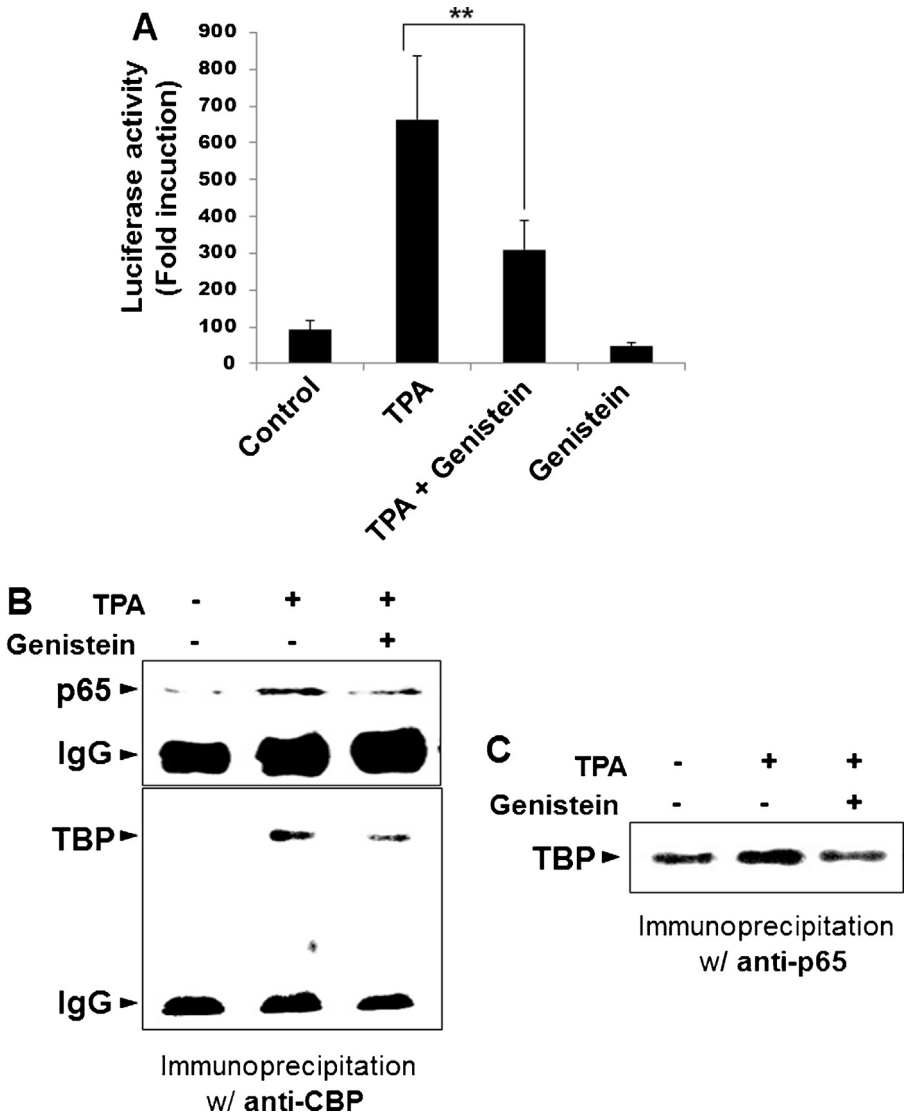

Fig. 4. Effects of genistein on TPA-stimulated transcriptional activity and p65 interaction with components of the transcription machinery in MCF10A cells. (A) Cells were transfected with the pELM-Luc (NF-κB construct ligated to the luciferase gene) expression vector as described in Section 2, then pretreated with genistein (50 μg/ml) for 1 h before incubation with 20 nM of TPA for 6 h. Luciferase activity was assayed as described in Section 2. The data are normalized using the β-galactosidase activity and expressed as the mean ± S.D. of three independent experiments performed in triplicate. *Significantly lower ($p < 0.05$) than the value obtained with TPA alone. (B, C) Cells pretreated with genistein (50 μg/ml) for 1 h and then incubated with TPA (20 nM) for 2 h. Whole cell lysates were subjected to immunoprecipitation with anti-CBP antibody, followed by Western blotting with an antibody directed against p65 or TBP in MCF10A cells (B). Whole cell lysates were immunoprecipitated with anti-p65 followed by Western blotting with an antibody directed against TBP (C).

3.3. Inhibitory effects of genistein on TPA-induced interaction of p65 with CBP or TBP

It appears that there exists an IκBα-independent mechanism responsible for regulation of the transcriptional activity of NF-κB. In this context, it is noticeable that phosphorylation of p65 subunit of NF-κB is essential for its transcriptional activation [19]. The CBP is a ubiquitously expressed nuclear coactivator [20]. CBP functions as an integrator linking various transcription factors to the basal transcription factor IIB (TFIIB), which in turn contacts the TBP of the TFIID complex in the basal apparatus [21]. While CBP is an important coactivator of NF-κB-dependent transcription, TBP is an essential component of the transcription initiation complex. Because genistein inhibits the transcriptional activity of NF-κB without interfering with DNA binding of the transcription factor, the possibility of its inhibition of phosphorylation of NF-κB and interaction between NF-κB and CBP or TBP was further investigated. For this purpose, lysates from MCF10A cells were immunoprecipitated with anti-CBP, and then immunoblotting was performed with anti-p65 or anti-TBP. In unstimulated MCF10A cells, association between CBP and TBP or between CBP and p65 was weakly detectable. However, TPA stimulation resulted in the appearance of a band of p65 and another of 36 kDa representing TBP, indicative of association between CBP and p65 or between CBP and TBP, and this was greatly attenuated by genistein pretreatment (Fig. 4B). In another experiment, lysates from MCF10A cells were immunoprecipitated with anti-p65, and then immunoblotting was performed with anti-TBP. TPA-induced interaction of p65 with TBP was inhibited by genistein pretreatment (Fig. 4C). These results, taken together, demonstrated the ability of genistein to inhibit the TPA-stimulated interaction between the NF-κB subunit p65 and the basal transcription factor TBP or the coactivator p300/CBP.

3.4. Inhibitory effects of genistein on phosphorylation of the p65 subunit of NF-κB

Several studies have shown that the phosphorylation of p65 facilitates its nuclear translocation and subsequent interaction with coactivators that bridge various transcriptional activators and recruitment of components of the basal transcriptional machinery. The interaction of p65 with CBP is hence essential for NF-κB transcriptional activity [21–23], and can be facilitated by p65 phosphorylation [21,24]. We therefore analyzed the effect of genistein on phosphorylation of p65 by immunocytochemical analysis. Preincubation with genistein resulted in a significant reduction in the nuclear accumulation of P-p65, preferentially phosphorylated at serine 536 (Fig. 5A).

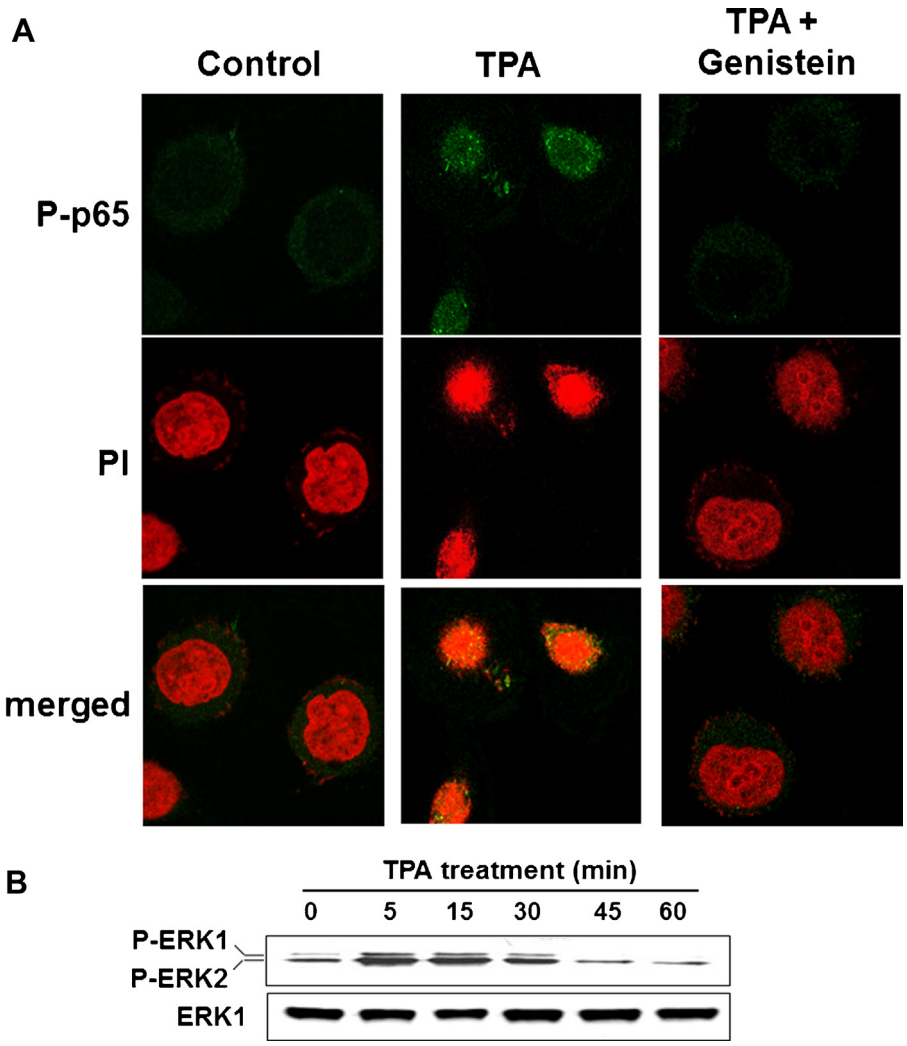

Fig. 5. Effects of genistein on TPA-stimulated phosphorylation of the p65 subunit of NF-κB. (A) Immunofluorescence staining of the phosphorylated p65 with affinity-purified anti-P-p65 antibody in MCF10A cells. Cells were fixed and stained as described in Section 2. (B) Cells were treated with 20 nM of TPA, and cell lysates were prepared at indicated time points and subjected to Western blot analysis using a phospho-specific ERK1/2 antibody.

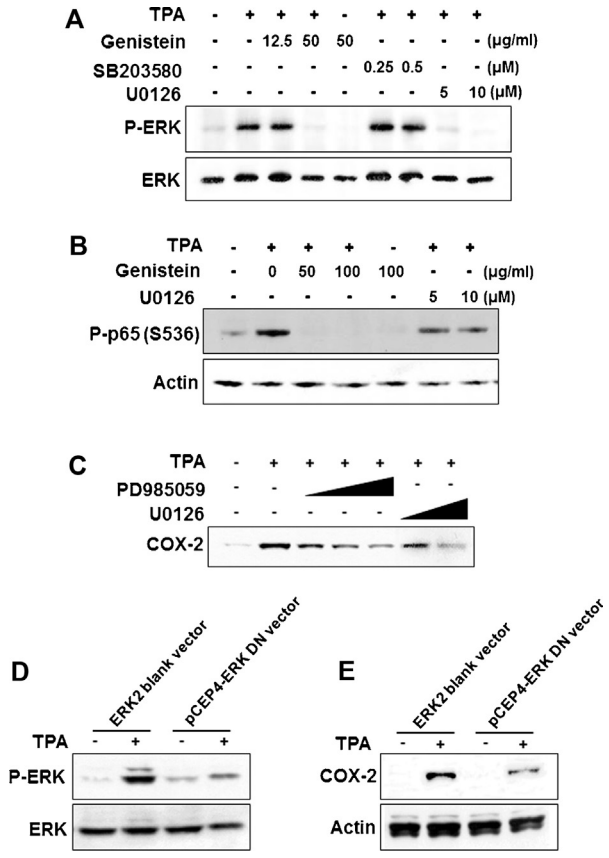

Fig. 6. Effects of genistein and ERK inhibition on TPA-induced p65 phosphorylation and/or COX-2 expression in MCF10A cells. (A) Cells were pretreated with the indicated concentrations of genistein, U0129 (MEK inhibitor) or SB203580 (p38 inhibitor) for 1 h before incubation with 20 nM of TPA for 5 min. Whole cell lysates were prepared and subjected to Western blotting using antibodies specific for phosphorylated ERK1/2 or parental ERK, as described in Section 2. (B) Cells pretreated with the indicated concentrations of genistein or U0126 for 1 h and then incubated with 20 nM of TPA for 2 h. Phosphorylated p65 subunit of NF-κB was immunodetected using a specific antibody. Western blot analysis for actin was performed to confirm equal loading of proteins. (C) MCF10A Cells were pretreated with the indicated concentrations of PD985059 (0.125, 0.25, or 0.5 μM) or U0126 (5 or 10 μM) for 1 h before incubation with 20 nM TPA for 4 h. Whole cell lysates were prepared and subjected to immunoblot analysis using an antibody specific for COX-2. (D) Cells were transfected with pCEP4-ERK2 dominant-negative (DN) mutant vector or pCEP4 blank vector. After transfection, cells were treated with 20 nM TPA for 5 min. Whole cell lysates were prepared and subjected to Western blotting, as described in Section 2. (E) Cells were transfected with pCEP4-ERK2 dominant negative (DN) mutant vector or pCEP4 blank vector. After transfection, cells were treated with 20 nM TPA for 4 h. Whole cell lysates were prepared and subjected to Western blot analysis of COX-2.

3.5. *Inhibition of TPA-induced ERK phosphorylation by genistein in MCF10A cells*

ERK1/2 is involved in mediating many cellular signal transduction pathways. In the present work, a lower concentration (i.e., 20 μM) of TPA resulted in rapid activation of ERK1/2 through phosphorylation (Fig. 5B), which was inhibited by genistein (Fig. 6A). Activation of ERK was also blunted by the MAPK kinase (MEK) inhibitor U0126, but not affected by the p38 MAPK inhibitor SB 203580 (Fig. 6B). TPA-induced COX-2 expression in MCF10A cells was inhibited when the MEK-ERK signaling was blocked by U0126 or PD985059 (Fig. 6C). Transient transfection of MCF10A cells with an ERK dominant negative vector attenuated not only TPA-induced ERK activation (Fig. 6D), but also COX-2 expression (Fig. 6E). These findings suggest that activation of ERK is an important event in TPA-induced NF-κB transcriptional activity and that this MAPK is a target of genistein in its suppression of p65 phosphorylation and COX-2 expression in TPA-stimulated MCF10A cells.

4. Discussion

An expanding body of evidence supports that COX-2 inhibitors are useful for not only treating inflammation but also preventing cancer [13,25]. Abnormal up-regulation of COX-2 has been observed in a number of human tumors including those of colorectal, pancreatic, breast and gastric origin [11]. Numerous epidemiological studies have revealed that the use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) with COX-2 inhibitory activity may protect against formation of gastrointestinal tumors [26,27] and other malignancies [28]. The anti-tumor effect of NSAIDs may be attributable to their inhibition of COX-2 activity/expression and PGE_2 production, while other mechanisms can also be involved. Therefore, the search for selective COX-2 inhibitors is an important approach to chemoprevention of cancer.

It has been reported that COX-2 expression can be regulated through distinct MAPK signaling pathways, depending on the types of inducers and cells. It has been reported that activation of MAPK is also associated with LPS-induced COX-2 expression in human neutrophils [29], COX-2 expression and PGE_2 production by epidermal growth factor in articular chondrocytes [30] and phorbol ester-induced expression of COX-2 in mouse skin [31]. In our present study, TPA rapidly induced activation of ERK1/2. Pharmacologic inhibition of ERK1/2 by pretreatment with PD98059 and U0126 (MEK1/2 inhibitors) attenuated TPA-induced COX-2 expression in MCF10A cells. Furthermore, transfection of MCF10A cells with dominant negative ERK2 also abrogated COX-2 expression stimulated by TPA, corroborating that activation of ERK is required for TPA-induced COX-2 expression in these cells. The suppression by genistein of TPA-induced activation of ERK may account for its attenuation of TPA-induced COX-2 expression and PGE_2 production. In other studies with various stimuli, activation of ERK was found to be inhibited by genistein. Thus, phosphorylation of ERK by 5-hydroxytryptamine (2A) receptors in rat pheochromocytoma PC12 cells was inhibited by genistein [32]. The 12-lipoxygenase metabolite 12*S*-HETE stimulated human pancreatic cancer cell proliferation via ERK activation which was also attenuated by genistein [33]. Genistein inhibited eotaxin-induced cytokine production in bronchial epithelial cells and caused growth inhibition in human breast cell lines by suppressing MAPK [34,35].

NF-κB is essential for controlling the inducible expression of COX-2. Inhibitors of NF-κB, such as pyrrolidine dithiocarbamate, abrogated TPA-induced COX-2 expression in MCF10A cells [36]. These data support the notion that activation of NF-κB is linked to the TPA-induced COX-2 expression in MCF10A cells. As an initial approach toward determining whether genistein could inhibit COX-2 induction by blocking NF-κB activation, we examined the effect of genistein on TPA-induced NF-κB-DNA binding. Although TPA clearly elevated NF-κB-DNA binding as well as nuclear translocation of p65, we noted that genistein failed to block TPA-induced nuclear accumulation and DNA binding of NF-κB as well as IκBα degradation.

Like COX-2, inducible nitric oxide synthase (iNOS) is often overexpressed in inflammation-associated carcinogenesis. Pretreatment of genistein attenuated the hemolysate-induced increase of iNOS expression by blocking NF-κB activation in astrocytes [37]. In another study, genistein inhibited expression of iNOS as well as COX-2 induced by IL-1β/IFN-γ in human islets [38]. We also found that TPA treatment induced the iNOS expression in MCF10A cells, and this was inhibited by genistein treatment (Supplementary Fig. 1).

The regulation of NF-κB-dependent gene expression can occur at multiple levels after cell stimulation. Early regulation occurs in the cytoplasm with the activation of IKK and subsequent phosphorylation, ubiquitination, and proteolytic degradation of IκB, liberating p65 for translocation into the nucleus [39,40]. However, some studies have shown that transactivation of NF-κB can be regulated independently of its nuclear translocation and DNA binding [41–43]. Although genistein did not suppress NF-κB-DNA binding activity, it did inhibit TPA-induced NF-κB transcriptional activation at a step after binding to DNA. The transcriptional activation of NF-κB is regulated at least in part through phosphorylation of p65 at several distinct sites [44]. In the present work, genistein inhibited TPA-induced phosphorylation of p65 at Ser 536 located in the transactivation 1 (TA1) domain, which is highly conserved among humans, mice, and *Xenopus*. Within TA1, there are 7 serine residues including Ser 536, which are predicted to align on one side of a helix and form a polar region believed to act similar to acidic transcriptional activators [44]. These serine residues are known to be potential phosphorylation sites of p65 [45,46]. Upstream kinases that have been shown to be involved in the regulation of NF-κB transcriptional activation include protein kinase A [24], p38 MAPK [15], and casein kinase II [47]. It is possible that ERK also contributes to the transmission of extracellular signals that can finally cause indirect phosphorylation of p65. It has been reported that p38 MAPK and ERK are indispensable for NF-κB-driven gene transcription in response to TNF-α [17]. MSK1, a nuclear kinase that is activated by both ERK and p38 kinases, phosphorylates p65 in response to TNF-α stimulation [48]. We found that the pharmacologic MEK1/2 inhibitor, U0126, attenuated phosphorylation of p65 at Ser 536. Therefore, it is speculated that genistein inhibits phosphorylation of p65 at Ser 536 by targeting the ERK-MSK1 pathway in MCF10A cells.

An additional regulatory element in the NF-κB transcriptional activity is the nuclear coactivator protein CBP. CBP has been shown to interact with the TA1 + TA2 domains of p65 [22], which contributes to NF-κB transcriptional activation [22,49]. CBP plays an important role in the integration of diverse signaling pathways by linking p65 with components of the basal transcriptional machinery, such as TFIIB, TBP and RNA polymerase II [50]. Cotransfection with Rel A and CBP expression plasmids in COS cells increased the NF-κB-dependent promoter activity by 3–5 fold [22]. In addition, p65 is known to interact with TFIIB and TBP of the basal transcriptional complex, and these interactions appear to be essential for optimal NF-κB-driven transcription [51]. TBP, an essential component of transcription initiation [52,53], is one of the subunits of TFIID [53]. The p65 subunit interacts directly with these components of the basal transcription machinery in a way that activates gene expression in COS7 cells [51]. Based on these findings, we hypothesized that genistein may inhibit the formation of p65-CBP and p65-TBP complexes, thereby inhibiting the NF-κB transcriptional activity. In support of this supposition, genistein significantly inhibited NF-κB interaction with TBP and CBP in MCF10A cells treated with TPA, which appears to be attributable to its suppression of p65 phosphorylation.

Numerous phytochemicals have been tested for their chemopreventive and anticarcinogenic activities, mostly in cell culture systems. Although *in vitro* studies have contributed significantly to identification of distinct molecular targets and signaling pathways affected by given phytochemicals, concentrations employed in mechanistic studies exceed those physiologically achievable in humans [54]. In our study, genistein was added to the culture medium at concentrations up to 100 μg/ml, which did not affect the viability of these cells. We noticed that genistein at a concentration of even 12.5 μg/ml (46 nmol/ml) almost completely inhibited

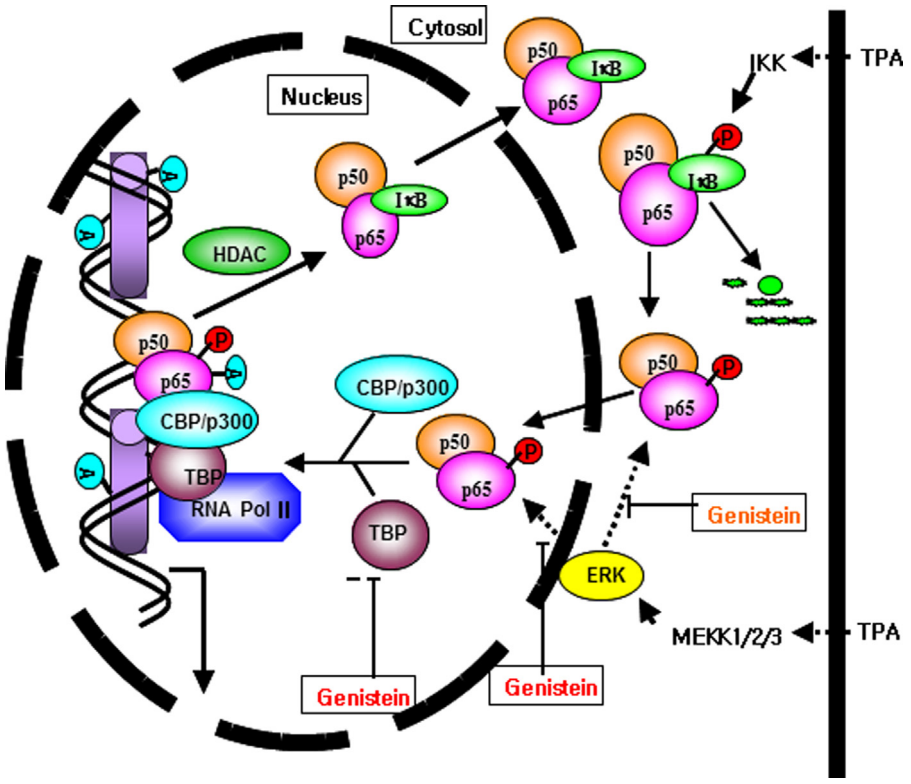

Fig. 7. Schematic representation of postulated mechanisms of genistein inhibition of transcriptional activation of NF-κB. TPA stimulation initiates intracellular signal transduction that leads to transcriptional activation of NF-κB. The phosphorylated p65 regulates transactivation of NF-κB which is a potential target for genistein inhibition. Other potential sites of genistein inhibition of transcriptional activation in the nucleus include recruitment of transcriptional coactivators CBP/p300 to p65 or interactions between p65 and TBP. Symbols: A, acetylated; P, phosphorylated. HDAC denotes histone deacetylase.

the TPA-induced COX-2 expression and PGE_2 production. As this is the concentration of genistein added to the medium, it does not necessarily reflect the intracellular concentration, which must be much lower. Though physiologic concentrations of genistein are considered to rarely exceed the nanomolar range, some Japanese who intake relatively large amounts of soya products on regular basis exhibit submicro-molar concentrations [55,56].

In conclusion, genistein inhibits COX-2 expression through inhibition of the ERK-mediated phosphorylation of p65 and interference with subsequent interaction between NF-κB and the coactivator protein CBP and recruitment of basal transcription factor TBP (Fig. 7). Such inhibition of COX-2 expression and PGE_2 secretion probably contributes to the anti-inflammatory and chemopreventive properties of genistein.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrfmmm.2014.04.003.

References

[1] D.B. Fournier, J.W. Erdman Jr., G.B. Gordon, Soy, its components, and cancer prevention: a review of the *in vitro*, animal, and human data, Cancer Epidemiol. Biomarkers Prev. 7 (1998) 1055–1065.
[2] H. Adlercreutz, H. Honjo, A. Higashi, T. Fotsis, E. Hamalainen, T. Hasegawa, H. Okada, Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet, Am. J. Clin. Nutr. 54 (1991) 1093–1100.
[3] O.J. Park, Y.J. Surh, Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies, Toxicol. Lett. 150 (2004) 43–56.
[4] Y. Nakamura, A. Murakami, Y. Ohto, K. Torikai, T. Tanaka, H. Ohigashi, Suppression of tumor promoter-induced oxidative stress and inflammatory responses in mouse skin by a superoxide generation inhibitor 1′-acetoxychavicol acetate, Cancer Res. 58 (1998) 4832–4839.
[5] Y.C. Liang, Y.T. Huang, S.H. Tsai, S.Y. Lin-Shiau, C.F. Chen, J.K. Lin, Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages, Carcinogenesis 20 (1999) 1945–1952.
[6] S. Hooshmand, Y. Soung do, E.A. Lucas, S.V. Madihally, C.W. Levenson, B.H. Arjmandi, Genistein reduces the production of proinflammatory molecules in human chondrocytes, J. Nutr. Biochem. 18 (2007) 609–614.
[7] N. Richard, D. Porath, A. Radspieler, J. Schwager, Effects of resveratrol, piceatannol, tri-acetoxystilbene, and genistein on the inflammatory response of human peripheral blood leukocytes, Mol. Nutr. Food Res. 49 (2005) 431–442.
[8] F. Ye, J. Wu, T. Dunn, J. Yi, X. Tong, D. Zhang, Inhibition of cyclooxygenase-2 activity in head and neck cancer cells by genistein, Cancer Lett. 211 (2004) 39–46.
[9] T.Y. Lau, L.K. Leung, Soya isoflavones suppress phorbol 12-myristate 13-acetate-induced COX-2 expression in MCF-7 cells, Br. J. Nutr. 96 (2006) 169–176.
[10] E. Horia, B.A. Watkins, Complementary actions of docosahexaenoic acid and genistein on COX-2, PGE_2 and invasiveness in MDA-MB-231 breast cancer cells, Carcinogenesis 28 (2007) 809–815.
[11] K. Muller-Decker, G. Furstenberger, The cyclooxygenase-2-mediated prostaglandin signaling is causally related to epithelial carcinogenesis, Mol. Carcinog. 46 (2007) 705–710.
[12] M. Tsujii, R.N. DuBois, Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2, Cell 83 (1995) 493–501.
[13] K.S. Chun, Y.J. Surh, Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention, Biochem. Pharmacol. 68 (2004) 1089–1100.
[14] M. Karin, F.R. Greten, NF-κB: linking inflammation and immunity to cancer development and progression, Nat. Rev. Immunol. 5 (2005) 749–759.
[15] W. Vanden Berghe, L. Vermeulen, G. De Wilde, K. De Bosscher, E. Boone, G. Haegeman, Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6, Biochem. Pharmacol. 60 (2000) 1185–1195.
[16] H. Sakurai, H. Chiba, H. Miyoshi, T. Sugita, W. Toriumi, IκB kinases phosphorylate NF-κB p65 subunit on serine 536 in the transactivation domain, J. Biol. Chem. 274 (1999) 30353–30356.
[17] W. Vanden Berghe, S. Plaisance, E. Boone, K. De Bosscher, M.L. Schmitz, W. Fiers, G. Haegeman, p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-κB p65 transactivation mediated by tumor necrosis factor, J. Biol. Chem. 273 (1998) 3285–3290.
[18] S.A. Park, H.K. Na, E.H. Kim, Y.N. Cha, Y.J. Surh, 4-Hydroxyestradiol induces anchorage-independent growth of human mammary epithelial cells via activation of IκB kinase: potential role of reactive oxygen species, Cancer Res. 69 (2009) 2416–2424.
[19] L. Vermeulen, G. De Wilde, S. Notebaert, W. Vanden Berghe, G. Haegeman, Regulation of the transcriptional activity of the nuclear factor-κB p65 subunit, Biochem. Pharmacol. 64 (2002) 963–970.
[20] P.S. Goldman, V.K. Tran, R.H. Goodman, The multifunctional role of the co-activator CBP in transcriptional regulation, Recent Prog. Horm. Res. 52 (1997) 103–119, discussion 119–120.
[21] R.P. Kwok, J.R. Lundblad, J.C. Chrivia, J.P. Richards, H.P. Bachinger, R.G. Brennan, S.G. Roberts, M.R. Green, R.H. Goodman, Nuclear protein CBP is a coactivator for the transcription factor CREB, Nature 370 (1994) 223–226.
[22] M.E. Gerritsen, A.J. Williams, A.S. Neish, S. Moore, Y. Shi, T. Collins, CREB-binding protein/p300 are transcriptional coactivators of p65, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 2927–2932.
[23] G.C. Parry, N. Mackman, Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-κB-mediated transcription, J. Immunol. 159 (1997) 5450–5456.
[24] H. Zhong, H. SuYang, H. Erdjument-Bromage, P. Tempst, S. Ghosh, The transcriptional activity of NF-κB is regulated by the IκB-associated PKAc subunit through a cyclic AMP-independent mechanism, Cell 89 (1997) 413–424.
[25] F.H. Sarkar, S. Adsule, Y. Li, S. Padhye, Back to the future: COX-2 inhibitors for chemoprevention and cancer therapy, Mini Rev. Med. Chem. 7 (2007) 599–608.
[26] S. Futagami, K. Suzuki, T. Hiratsuka, T. Shindo, T. Hamamoto, N. Ueki, M. Kusunoki, K. Miyake, K. Gudis, T. Tsukui, C. Sakamoto, Chemopreventive effect of celecoxib in gastric cancer, Inflammopharmacology 15 (2007) 1–4.
[27] R.F. Jacoby, K. Seibert, C.E. Cole, G. Kelloff, R.A. Lubet, The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis, Cancer Res. 60 (2000) 5040–5044.
[28] N.L. Barnes, F. Warnberg, G. Farnie, D. White, W. Jiang, E. Anderson, N.J. Bundred, Cyclooxygenase-2 inhibition: effects on tumour growth, cell cycling and lymphangiogenesis in a xenograft model of breast cancer, Br. J. Cancer 96 (2007) 575–582.
[29] S. Nagano, T. Otsuka, H. Niiro, K. Yamaoka, Y. Arinobu, E. Ogami, M. Akahoshi, Y. Inoue, K. Miyake, H. Nakashima, Y. Niho, M. Harada, Molecular mechanisms of lipopolysaccharide-induced cyclooxygenase-2 expression in human neutrophils: involvement of the mitogen-activated protein kinase pathway and regulation by anti-inflammatory cytokines, Int. Immunol. 14 (2002) 733–740.
[30] Y.H. Huh, S.H. Kim, S.J. Kim, J.S. Chun, Differentiation status-dependent regulation of cyclooxygenase-2 expression and prostaglandin E_2 production by epidermal growth factor via mitogen-activated protein kinase in articular chondrocytes, J. Biol. Chem. 278 (2003) 9691–9697.
[31] Y.J. Surh, J.Y. Lee, K.J. Choi, S.R. Ko, Effects of selected ginsenosides on phorbol ester-induced expression of cyclooxygenase-2 and activation of NF-κB and ERK1/2 in mouse skin, Ann. N. Y. Acad. Sci. 973 (2002) 396–401.
[32] J.C. Quinn, N.N. Johnson-Farley, J. Yoon, D.S. Cowen, Activation of extracellular-regulated kinase by 5-hydroxytryptamine(2A) receptors in PC12 cells is protein kinase C-independent and requires calmodulin and tyrosine kinases, J. Pharmacol. Exp. Ther. 303 (2002) 746–752.
[33] X.Z. Ding, W.G. Tong, T.E. Adrian, 12-Lipoxygenase metabolite 12(S)-HETE stimulates human pancreatic cancer cell proliferation via protein tyrosine phosphorylation and ERK activation, Int. J. Cancer 94 (2001) 630–636.
[34] C.H. Cui, T. Adachi, H. Oyamada, Y. Kamada, T. Kuwasaki, Y. Yamada, N. Saito, H. Kayaba, J. Chihara, The role of mitogen-activated protein kinases in eotaxin-induced cytokine production from bronchial epithelial cells, Am. J. Respir. Cell Mol. Biol. 27 (2002) 329–335.
[35] K. Dampier, E.A. Hudson, L.M. Howells, M.M. Manson, R.A. Walker, A. Gescher, Differences between human breast cell lines in susceptibility towards growth inhibition by genistein, Br. J. Cancer 85 (2001) 618–624.
[36] J.H. Kim, H.K. Na, Y.K. Pak, Y.S. Lee, S.J. Lee, A. Moon, Y.J. Surh, Roles of ERK and p38 mitogen-activated protein kinases in phorbol ester-induced NF-κB activation and COX-2 expression in human breast epithelial cells, Chem. Biol. Interact. 171 (2008) 133–141.
[37] H. Lu, J.X. Shi, D.M. Zhang, H.D. Wang, C.H. Hang, H.L. Chen, H.X. Yin, Inhibition of hemolysate-induced iNOS and COX-2 expression by genistein through suppression of NF-κB activation in primary astrocytes, J. Neurol. Sci. 278 (2009) 91–95.
[38] J.A. Corbett, G. Kwon, M.H. Marino, C.P. Rodi, P.M. Sullivan, J. Turk, M.L. McDaniel, Tyrosine kinase inhibitors prevent cytokine-induced expression of iNOS and COX-2 by human islets, Am. J. Physiol. 270 (1996) C1581–C1587.

[39] J. DiDonato, F. Mercurio, C. Rosette, J. Wu-Li, H. Suyang, S. Ghosh, M. Karin, Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation, Mol. Cell. Biol. 16 (1996) 1295–1304.
[40] E.B. Traenckner, H.L. Pahl, T. Henkel, K.N. Schmidt, S. Wilk, P.A. Baeuerle, Phosphorylation of human IκBα on serines 32 and 36 controls IκBα proteolysis and NF-κB activation in response to diverse stimuli, EMBO J. 14 (1995) 2876–2883.
[41] X. Jiang, N. Takahashi, N. Matsui, T. Tetsuka, T. Okamoto, The NF-κB activation in lymphotoxin beta receptor signaling depends on the phosphorylation of p65 at serine 536, J. Biol. Chem. 278 (2003) 919–926.
[42] K. Muraoka, K. Shimizu, X. Sun, T. Tani, R. Izumi, K. Miwa, K. Yamamoto, Flavonoids exert diverse inhibitory effects on the activation of NF-κB, Transpl. Proc. 34 (2002) 1335–1340.
[43] D. Qiu, G. Zhao, Y. Aoki, L. Shi, A. Uyei, S. Nazarian, J.C. Ng, P.N. Kao, Immunosuppressant PG490 (triptolide) inhibits T-cell interleukin-2 expression at the level of purine-box/nuclear factor of activated T-cells and NF-κB transcriptional activation, J. Biol. Chem. 274 (1999) 13443–13450.
[44] M.L. Schmitz, S. Bacher, M. Kracht, IκB-independent control of NF-κB activity by modulatory phosphorylations, Trends Biochem. Sci. 26 (2001) 186–190.
[45] J. Ostrowski, J.E. Sims, C.H. Sibley, M.A. Valentine, S.K. Dower, K.E. Meier, K. Bomsztyk, A serine/threonine kinase activity is closely associated with a 65-kDa phosphoprotein specifically recognized by the κB enhancer element, J. Biol. Chem. 266 (1991) 12722–12733.
[46] D. Wang, S.D. Westerheide, J.L. Hanson, A.S. Baldwin Jr., Tumor necrosis factor α-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II, J. Biol. Chem. 275 (2000) 32592–32597.
[47] T.A. Bird, K. Schooley, S.K. Dower, H. Hagen, G.D. Virca, Activation of nuclear transcription factor NF-κB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit, J. Biol. Chem. 272 (1997) 32606–32612.
[48] L. Vermeulen, G. De Wilde, P. Van Damme, W. Vanden Berghe, G. Haegeman, Transcriptional activation of the NF-κB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1), EMBO J. 22 (2003) 1313–1324.
[49] N.D. Perkins, L.K. Felzien, J.C. Betts, K. Leung, D.H. Beach, G.J. Nabel, Regulation of NF-κB by cyclin-dependent kinases associated with the p300 coactivator, Science 275 (1997) 523–527.
[50] X.J. Yang, V.V. Ogryzko, J. Nishikawa, B.H. Howard, Y. Nakatani, A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A, Nature 382 (1996) 319–324.
[51] M.L. Schmitz, G. Stelzer, H. Altmann, M. Meisterernst, P.A. Baeuerle, Interaction of the COOH-terminal transactivation domain of p65 NF-κB with TATA-binding protein, transcription factor IIB, and coactivators, J. Biol. Chem. 270 (1995) 7219–7226.
[52] E. Martinez, C.M. Chiang, H. Ge, R.G. Roeder, TATA-binding protein-associated factor(s) in TFIID function through the initiator to direct basal transcription from a TATA-less class II promoter, EMBO J. 13 (1994) 3115–3126.
[53] H.T. Timmers, R.E. Meyers, P.A. Sharp, Composition of transcription factor B-TFIID, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 8140–8144.
[54] E.P. Moiseeva, M.M. Manson, Dietary chemopreventive phytochemicals: too little or too much? Cancer Prev. Res. (Phila.) 2 (2009) 611–616.
[55] M. Iwasaki, M. Inoue, T. Otani, S. Sasazuki, N. Kurahashi, T. Miura, S. Yamamoto, S. Tsugane, Japan Public Health Center-based prospective study group, Plasma isoflavone level and subsequent risk of breast cancer among Japanese women: a nested case-control study from the Japan Public Health Center-based prospective study group, J. Clin. Oncol. 26 (2008) 1677–1683.
[56] M.S. Morton, O. Arisaka, N. Miyake, L.D. Morgan, B.A. Evans, Phytoestrogen concentrations in serum from Japanese men and women over 40 years of age, J. Nutr. 132 (2002) 3168–3171.