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Genistein Inhibits NF-κB Activation in Prostate Cancer Cells

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Abstract: *Prostate cancer is the second leading cause of cancer-related deaths in men in the United States. Epidemiological studies indicate that susceptibility to prostate cancer may be partly due to environmental influences, especially diet. An association has been shown between decreased prostate cancer risk and mortality with increased consumption of soy products, resulting in increased levels of isoflavones. We previously demonstrated that the soy isoflavone genistein inhibits cell growth and induces apoptosis in prostate cancer cells. To further elucidate the molecular mechanism by which genistein elicits its apoptotic effect, we investigated the role of a transcription factor, nuclear factor-κB (NF-κB), in the androgen-sensitive cell line LNCaP and the androgen-insensitive cell line PC3. Here we show that genistein decreases NF-κB DNA binding and abrogates NF-κB activation by DNA-damaging agents, H_2O_2 and tumor necrosis factor-α, in prostate cancer cells regardless of androgen sensitivity. Additionally, we have demonstrated that genistein reduces phosphorylation of the inhibitory protein IκBα and blocks the nuclear translocation of NF-κB, prohibiting DNA binding and preventing NF-κB activation. These results provide a mechanism by which genistein induces apoptosis in prostate cancer cells: the inactivation of NF-κB. Furthermore, genistein's ability to abrogate NF-κB activation by DNA-damaging agents strongly supports genistein's role as a chemopreventive agent.*

Introduction

Genistein, an isoflavone found primarily in soy, has gained much attention recently as a potential chemopreventive agent. Epidemiological and migrant studies have correlated increased isoflavone levels in the serum and urine of Asian women and men with decreased levels of breast and prostate cancer (1,2). Recently, a cross-national study involving 59 countries identified soy products as having a highly significant protective effect against prostate cancer (3). Although a number of genistein's effects have been documented *in vitro*, its mechanism of action *in vivo* is unknown. Genistein has been shown to inhibit cell growth in prostate, breast, and lung cancer cell lines (4–7). Genistein is also a known inhibitor of tyrosine kinases (8), toposiomerase II (9), 5α-reductase (10), and angiogenesis (11). We previously demonstrated that genistein induces G_2/M cell cycle arrest and modulates at least two cell cycle regulatory proteins: downregulation of cyclin B and upregulation of $p21^{WAF1}$ protein expression levels (4). Additionally, we demonstrated that genistein induces apoptosis in prostate cancer cells independent of androgen sensitivity. However, the mechanism by which genistein elicits its proapoptotic effect remains to be determined. Here we investigated whether genistein was able to modulate nuclear factor-κB (NF-κB), a transcription factor known to protect cells from apoptosis.

NF-κB was originally discovered as a κ-immunoglobulin enhancer DNA-binding protein that correlated with κ-gene transcription (12). NF-κB is now known to induce and control a broad spectrum of genes, including inflammatory cytokines, chemokines, cell adhesion molecules, growth factors, interferons, major histocompatibility complex proteins, and viruses (for review see References 13 and 14). NF-κB belongs to a family of proteins including Rel A, c-Rel, Rel B, Bcl-3, p100, and p105. Under nonstimulated conditions, NF-κB consists primarily of the Rel A (p65) and p50 heterodimers associated with cytosolic IκB inhibitory protein. The p50 and Rel A proteins share a 300-amino acid residue region of homology minimally required for DNA binding and dimerization. In addition, Rel A has a 250-amino acid COOH-terminal extension containing two or three transactivating domains. The activation of NF-κB is primarily a posttranslational event involving the release of NF-κB complex from the IκB inhibitory protein. After stimulation with an inducer such as tumor necrosis factor-α (TNF-α), lipopolysaccharide, phorbol myristate acetate, or H_2O_2, IκB becomes phosphorylated by activated IκB kinase (IKK), ubiquitinated, and then degraded by the 26S proteosome. The free NF-κB complex is then translocated into the nucleus, binds to the DNA consensus sequence (5′-GGGPuNNPyPyCC-3′), and transactivates genes (for review see References 13 and 14).

There is growing evidence to support the role of NF-κB in the protection against programmed cell death. Inhibition of NF-κB/Rel proteins induces apoptosis of murine B cells, whereas overexpression of c-Rel protein makes cells resistant to apoptosis (15). An antiapoptotic role has been suggested

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from *in vivo* studies where mice lacking NF-κB p65/ Rel A die embryonically from extensive apoptosis in the liver (16). Several studies have shown the absence or inhibition of NF-κB subunits in cultured cells, resulting in potentiation of apoptosis in response to TNF-α, ionizing radiation, and anticancer agents (17,18). Concurrently, increasing evidence indicates that resistance to apoptosis mediates resistance to anticancer therapies. Wang and co-workers (17) demonstrated that anticancer agents may be less effective at cell killing because of their activation of NF-κB. Therefore, agents that inhibit NF-κB activity may be beneficial in the prevention or treatment of cancer.

In this report, we have demonstrated that administration of genistein reduces NF-κB DNA binding in prostate cancer cells. In addition, genistein is able to block NF-κB activation by two known inducers, TNF-α and H_2O_2. The results of our studies, therefore, suggest that cells exposed to genistein decrease NF-κB activity, which leads to induction of apoptosis. Second, genistein may protect cells from DNA-damaging agents, as indicated by the abrogation of NF-κB DNA binding by H_2O_2 and TNF-α in genistein-pretreated cells, providing evidence for its chemopreventive role.

Experimental Procedures

Cell Culture

Human prostate cancer cell lines, PC3 and LNCaP cells (American Type Culture Collection, Rockville, MD), were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C. Genistein (Toronto Chemicals, Toronto, ON, Canada) was dissolved in 0.1 M Na_2CO_3 to make a 10 mM stock solution and was added directly to the culture medium at a final concentration of 50 μM. Where indicated, the proteosome inhibitor carbobenzoxyl-leucinyl-leucinyl-leucinal-H (MG- 132, Biomol, Plymouth Meeting, PA) was dissolved in dimethyl sulfoxide and added directly to the culture medium at a final concentration of 25 μM. The same concentration of Na_2CO_3 and/or dimethyl sulfoxide was added to the control cultures.

Preparation of Nuclear and Cytoplasmic Proteins

PC3 and LNCaP cells were plated at a density of 1×10^5 cells in 100-mm dishes and cultured for 24 hours; subsequently, the cultures were treated with or without 50 μM genistein for an additional 48 hours. After the 48-hour pretreatment, respective dishes were treated with 20 ng/ml of TNF-α for 10 minutes or 250 μM H_2O_2 for 70 minutes or kept untreated as the control. After treatment, cells were scraped off the plates with a cell scraper, collected by centrifugation, and washed in phosphate-buffered saline (PBS). The cell pellet was resuspended in 0.5 ml of 10 mM tris(hydroxymethyl)aminomethane (Tris)·HCl (pH 7.5)-5 mM $MgCl_2$-0.05% (vol/vol) Triton X-100 and lysed with 20 strokes in a 1-ml Dounce homogenizer. The homogenate was centrifuged at 10,000 *g* for 15 minutes at 4°C. The supernatant (cytosolic extract) was collected and frozen at −70°C until future use. The pellet was further treated for nuclear protein extractions. The nucleus pellet volume was estimated, the pellet was resuspended in an equal volume of 10 mM Tris·HCl (pH 7.4)-5 mM $MgCl_2$, and one nucleus pellet volume of 1 M NaCl-10 mM Tris·HCl (pH 7.4)-4 mM $MgCl_2$ was added. The lysing nucleus was left on ice for 30 minutes and then centrifuged at 10,000 *g* for 15 minutes at 4°C. The supernatant (nuclear extract) was removed, and 80% glycerol was added to a final glycerol concentration of 20% (vol/vol). Bicinchoninic acid protein assay (Pierce, Rockford, IL) was conducted to determine protein concentration of each sample.

Labeling NF-κB Oligo With [γ-^{32}P]ATP

The following reagents were assembled for the phosphorylation reaction: 3 pmol of consenus oligonucleodtide 5′-AGTTGAGGGGACTTTCCCAGG-3′ (NF-κB) DNA binding sequence (Promega, Madison, WI), 5× T4 polynucleotide kinase buffer, T4 polynucleotide kinase, nuclease-free water, and 10 μCi of [γ-^{32}P]ATP. The reaction was incubated for 10 minutes at 37°C. The reaction was stopped by the addition of 0.5 M EDTA followed by the addition of sodium-Tris-EDTA buffer. Adding the reaction to a chromatography column (Stratagene, La Jolla, CA) removed unincorporated nucleotides. The probe was diluted with H_2O to 20,000 cpm/μl.

Gel Mobility Shift Assay/Electrophoretic Mobility Shift Assay

Protein (10 μg) was assembled with the following reagents: 5× gel shift binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris·HCl, pH 7.5), 0.25 mg/ml poly(dI):poly(dC), and double-distilled H_2O. The reactions were incubated at room temperature for 10 minutes, and then 1 μl (20,000 cpm) of ^{32}P-labeled NF-κB oligo was added to each reaction followed by 1 μl of sample loading dye (250 mM Tris·HCl, pH 7.4, 40% glycerol, 0.2% bromphenol blue). The samples were loaded on a prerun 8% polyacrylamide gel and run at 30 mA for 45 minutes. The gel was dried, exposed to X-ray film overnight at −70°C, and then developed.

Immunohistochemistry and Confocal Microscopy

PC3 and LNCaP cells were seeded onto 0.5-in.-diameter round coverslips and treated with 0 or 50 μM genistein for 48 hours. After 48 hours of pretreatment, respective coverslips were treated with 20 ng/ml TNF-α for 10 minutes or 250 μM H_2O_2 for 70 minutes. The cells were fixed in 100% cold methanol for 10 minutes and then washed three times with PBS. Nonspecific binding was blocked by incubating coverslips in 0.2% bovine serum albumin in PBS for 45 minutes. For intracellular binding, all subsequent solutions contained PBS plus 0.1% saponin (PBS-S). Cells were incu-

bated with primary antibodies to NF-κB p65 or NF-κB p50 (Rockland, Gilbertsville, PA) subunit at 1 μg/ml in PBS-S for two hours at 37°C, washed three times with PBS-S to remove unbound primary antibody, and then incubated with 10 μg/ml of Texas red-conjugated goat anti-rabbit immunoglobulin G for one hour at room temperature. The coverslips were washed three times with PBS-S, fixed in 100% cold methanol for two minutes, and subsequently washed three times with PBS. Slides were allowed to dry almost completely before the addition of a drop of antifade, and then the coverslip was mounted onto a slide. The slides were then viewed under a confocal microscope.

Western Blot Analysis

Cytoplasmic and nuclear extracts were prepared as stated above. Protein concentration was measured using bicinchoninic acid protein assay reagents (Pierce). Cell extracts were boiled for 10 minutes and chilled on ice, subjected to 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, and electrophoretically transferred to nitrocellulose membranes. Each membrane was incubated with antibodies to NF-κB p65, NF-κB p50 (Rockland), IκBα (Santa Cruz, Santa Cruz, CA), or β-actin (used as a control for protein loading) antibodies. The membranes were washed and incubated with secondary antibody conjugated with peroxidase, and the signal was detected using a chemiluminescent detection system (Pierce).

Results

Genistein Decreases NF-κB DNA Binding in Prostate Cancer Cells

We investigated whether genistein modulates NF-κB DNA binding in prostate cancer cells. PC3 and LNCaP cells were treated with 50 μM genistein, a concentration that we previously had shown to induce a 30% apoptotic index (4). Nuclear extracts were harvested from control and treated cells, incubated with a radiolabeled NF-κB oligonucleotide probe, and subjected to electrophoretic mobility shift assay. Autoradiography revealed that 50 μM genistein significantly inhibited NF-κB DNA binding at 24, 48, and 72 hours in both cell lines compared with the control (Figure 1). The specificity of NF-κB binding to the DNA consensus sequence was confirmed by competition with excess unlabeled oligonucleotide. However, noncompeting oligonucleotides, such as AP-1 and SP-1 DNA-binding sequences, did not replace the specific binding (data not shown). As previously reported (18), the slowest-migrating DNA protein complex represents the p65 and p50 heterodimers and the faster-migrating complex is the p50 homodimer. These results indicate that genistein decreases NF-κB DNA binding in prostate cancer cell lines.

Genistein Abrogates NF-κB Activation by TNF-α and H_2O_2

We further investigated whether genistein could block NF-κB induction by a known inducer. H_2O_2, a reactive oxygen species, and TNF-α, a DNA-damaging agent, have been previously shown to induce NF-κB binding (19,20). On treatment with either inducing agent, we observed an increase in NF-κB DNA binding in PC3 and LNCaP cell lines, as expected. However, when cells were pretreated with 50 μM genistein for 48 hours before stimulation with the inducing agent, genistein abrogated the induction of NF-κB binding elicited by H_2O_2 or TNF-α (Figure 2). Western blot analysis of nuclear extracts confirmed these results by revealing decreased protein expression of the p65 subunit in cells pretreated with genistein before treatment with TNF-α (Figure

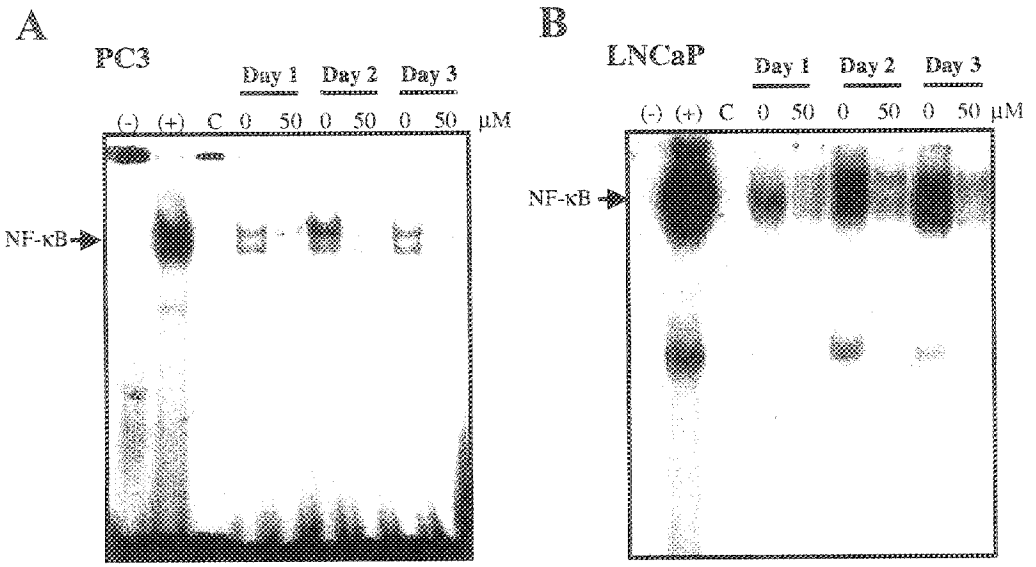

Figure 1. Genistein decreases nuclear factor-κB (NF-κB) binding in PC3 (A) and LNCaP (B) prostate cancer cells. Cells were treated with 0 or 50 μM genistein over 3 days. Nuclear extracts were prepared and analyzed for NF-κB binding by electrophoretic mobility shift assay. A negative control (–) was prepared without cell extracts, and HUT 78 cells incubated with 10 μM phorbol myristate acetate and Zn^{2+} were used as a positive control (+). C, binding with NF-κB competitive inhibitor.

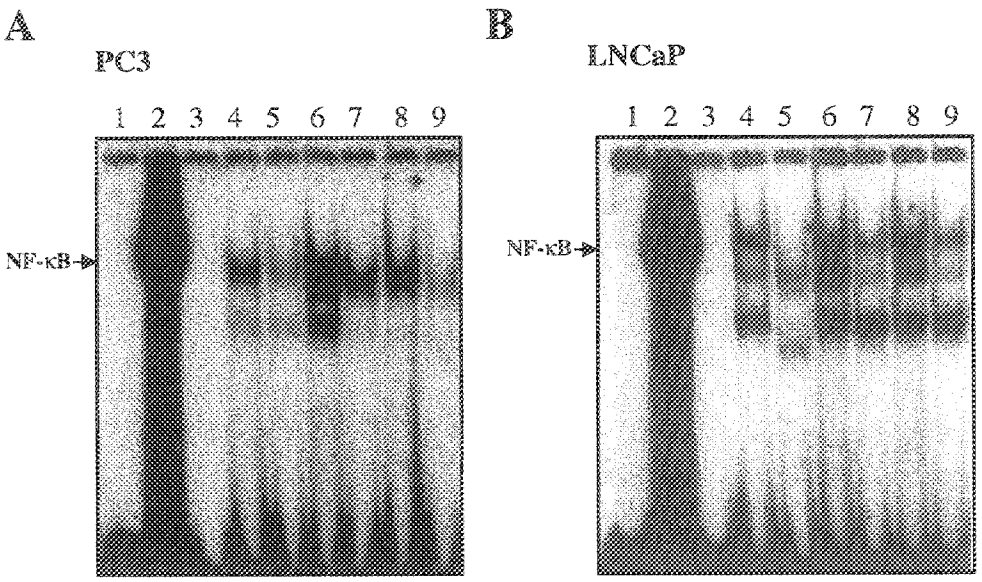

Figure 2. Genistein abrogates tumor necrosis factor-α (TNF-α)- and H_2O_2-induced NF-κB activation in PC3 (A) and LNCaP (B) prostate cancer cells. Cells were pretreated in absence (Lanes 4, 6, and 8) or presence of 50 μM genistein (Lanes 5, 7, and 9) for 48 h. Lanes 6 and 7 were subsequently treated with 20 ng/ml TNF-α for 15 min, and Lanes 8 and 9 were treated with 250 μM H_2O_2 for 70 min. Nuclear extracts were prepared and analyzed for NF-κB binding by electrophoretic mobility shift assay. Lane 1, negative control without protein extract; Lane 2, positive control (HUT 78 cells incubated with 10 μM phorbol myristate acetate and Zn^{2+}); Lane 3, competitive inhibition with excessive unlabeled NF-κB oligonucleotide.

3). These results demonstrate that genistein not only reduces NF-κB DNA binding in unstimulated conditions but inhibits H_2O_2- and TNF-α-induced NF-κB activation.

To determine whether the reduction of NF-κB DNA binding by genistein was due to decreased protein translation, we investigated whether genistein could affect the protein expression levels of the p65 subunit by Western blot analysis. As indicated in Figure 4, genistein pretreatment did not affect protein expression levels of the p65 subunit. These results were confirmed by densiometric analysis, in which the data for the p65 subunit were normalized to β-actin. These results suggest that the reduction of NF-κB DNA binding by genistein is a posttranslational event, indicating that genistein may be modulating the localization of the NF-κB heterodimer.

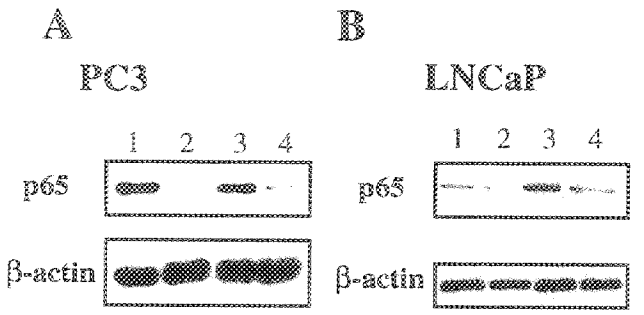

Figure 3. Genistein inhibits NF-κB p65 protein expression in nuclear extracts after TNF-α stimulation. PC3 (A) and LNCaP (B) cells were pretreated with 0 (Lanes 1 and 3) or 50 μM genistein (Lanes 2 and 4) for 48 h. Samples in Lanes 3 and 4 were then treated with 20 ng/ml TNF-α. Nuclear extracts were prepared, and 20 μg of protein were loaded onto a 12% polyacrylamide gel and subjected to electrophoresis. Gel was transferred to a nitrocellulose membrane and probed with an anti-p65 antibody, then incubated with a goat anti-rabbit secondary antibody. Signal was detected by enhanced chemiluminescence detection method.

Genistein Blocks Nuclear Translocation of NF-κB

Under nonstimulating conditions, NF-κB exists in the cytoplasm as a trimer made up of primarily the p50 and p65 subunits and the IκBα inhibitory protein (13,14). After stimulation, IκBα is phosphorylated, ubiquitinated, and degraded, allowing the NF-κB dimer to translocate to the nucleus, bind to the DNA, and transactivate genes (22). Using antibodies to NF-κB p50 and p65 subunits, we were able to visualize, by confocal microscopy, both NF-κB subunits translocate to the nucleus after H_2O_2 or TNF-α stimulation. In contrast, when cells were pretreated for 48 hours with 50 μM genistein and then stimulated with either NF-κB-inducing agent, genistein blocked the translocation of p50 and p65 subunits to the nucleus, thereby preventing the NF-κB complex from

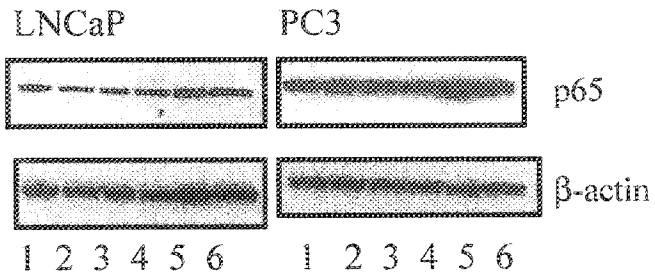

Figure 4. Genistein does not alter total protein expression levels of p65 subunit. PC3 and LNCaP cells were pretreated with 0 (Lanes 1, 3, and 5) or 50 μM genistein (Lanes 2, 4, and 6) for 48 h. Samples in Lanes 3 and 4 were subsequently treated with 20 ng/ml TNF-α, and samples in Lanes 5 and 6 were treated with 250 μM H_2O_2. Whole cell extracts were prepared, and 20 μg of protein were subjected to electrophoresis, transferred to a nitrocellulose membrane, probed with an anti-p65 antibody, and then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody. Signal was detected by enhanced chemiluminescence detection method.

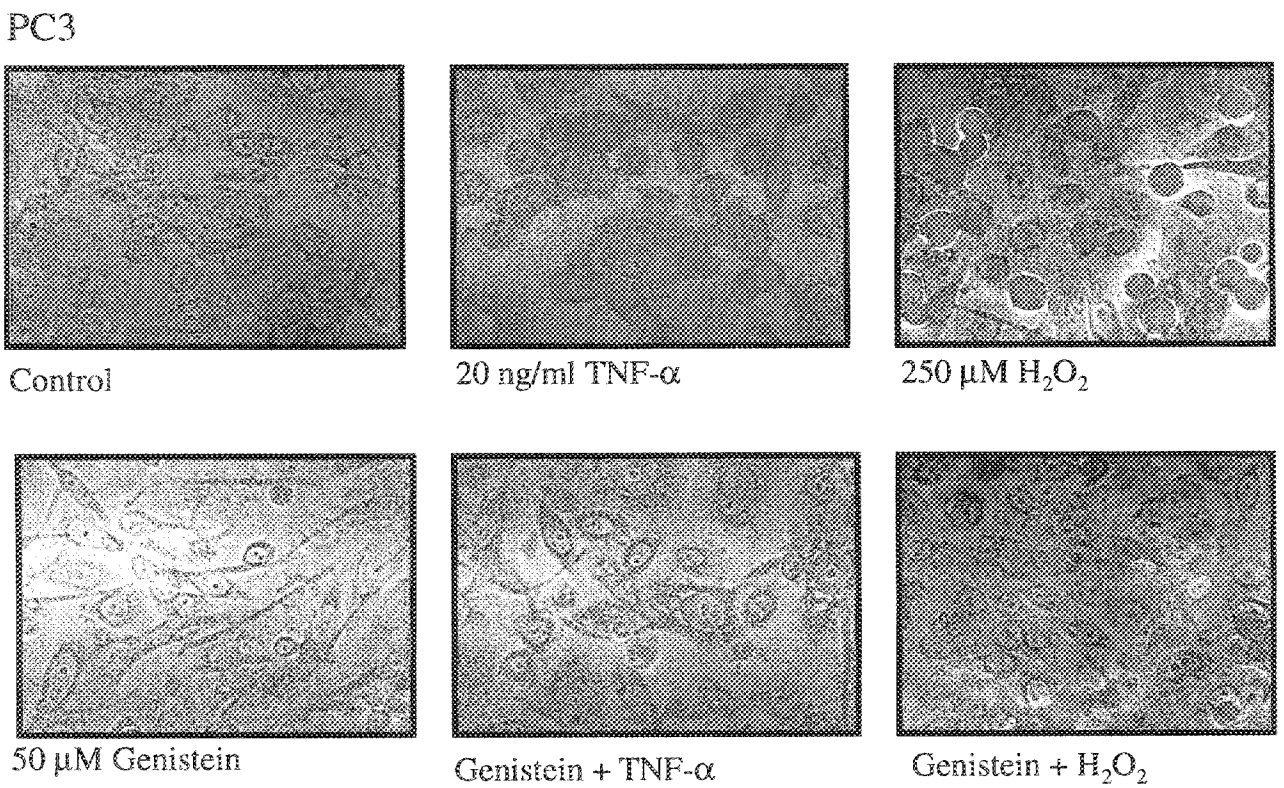

Figure 5. Genistein blocks nuclear translocation of NF-κB. PC3 prostate cancer cells were grown on coverslips and pretreated with 0 or 50 μM genistein for 48 h. Cells were then stimulated with 20 ng/ml of TNF-α for 15 min or 250 μM H_2O_2 for 75 min. Cells were fixed in methanol, then incubated first with anti-p65 polycolonal antibody and then with Texas red-conjugated secondary antibody. Cells were visualized under a confocal microscope.

binding to the DNA. Figure 5 represents the data for the p65 subunit in PC3 cells; similar results were obtained in the LNCaP cell line.

Genistein Inhibits Phosphorylation of IκBα

We next investigated whether genistein blocks phosphorylation of the inhibitory protein IκB. Stimulation of cells with TNF-α results in rapid serine phosphorylation of the IκBα NH_2 terminus (21), an event coupled to the rapid degradation and proteolysis of phospho-IκBα through the 26S proteosome. The proteosome inhibitor MG-132 has been shown to block IκBα proteolysis in TNF-α-stimulated cells (22,23). Therefore, to detect phosphorylated and unphosphorylated forms of IκBα in our system, we incubated PC3 and LNCaP cells with MG-132 before stimulation with TNF-α or H_2O_2. The cells were then stimulated with TNF-α or H_2O_2. Using an antibody that recognizes the phosphorylated and unphosphorylated forms of IκBα, we have demonstrated that genistein treatment inhibits the phosphorylated form of IκBα (Figure 6). There is a significant increase in phosphorylated and unphosphorylated IκBα after TNF-α and H_2O_2 treatment. However, genistein pretreatment reduced the amount of phosphorylated IκBα, suggesting that unphosphorylated IκBα remains bound to the NF-κB complex, sequestering the NF-κB in the cytoplasm, and ultimately prevents translocation to the nucleus.

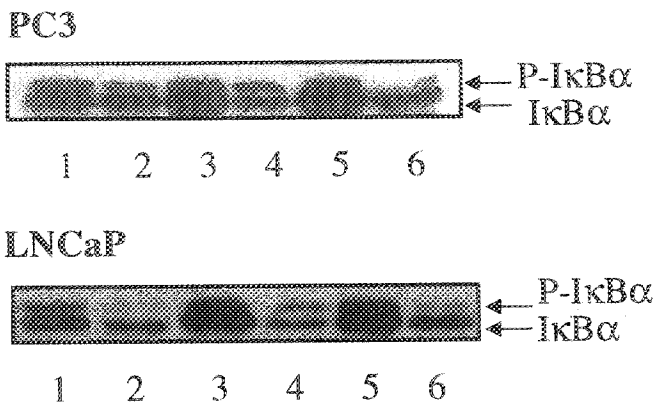

Figure 6. Genistein inhibits phosphorylation of IκBα in PC3 and LNCaP cells. Prostate cancer cells were pretreated with 0 (Lanes 1, 3, and 5) or 50 μM genistein (Lanes 2, 4, and 6) for 48 h. Samples were then treated with 20 ng/ml TNF-α (Lanes 3 and 4) or 250 μM H_2O_2 (Lanes 5 and 6). Cytoplasmic extracts were prepared, and 20 μg of protein were loaded onto a 16% polyacrylamide gel and subjected to electrophoresis. Gel was transferred to a nitrocellulose membrane and probed with a primary antibody to NH_2 terminus of IκBα, then incubated with a goat anti-rabbit secondary antibody. Signals were developed employing enhanced chemiluminescence reaction. Top band, phosphorylated form of IκBα (P- IκBα); bottom band, unphosphorylated form of IκBα.

Discussion

The aim of this report was to investigate the mechanism by which genistein induces an apoptotic response in prostate cancer cells, particularly the inactivation of NF-κB. We have shown that genistein inhibits NF-κB DNA binding by blocking the phosphorylation of the inhibitory protein IκBα, thereby preventing the nuclear translocation of the NF-κB complex. In addition, genistein significantly inhibited NF-κB activation

by the DNA-damaging agents TNF-α and H_2O_2. These data suggest two important effects of genistein. First, genistein downregulates NF-κB function, promoting apoptotic signaling. Second, genistein may protect cells from DNA-damaging agents, providing evidence for its chemopreventive ability.

There is strong evidence that NF-κB plays an antiapoptotic role (17,18,24). The activation of NF-κB by TNF-α blocks the activation of caspase-8, a key enzyme of the caspase pathway that further activates caspase-3, linking death domain protein signaling with caspase activation. Additionally, inhibitor of apoptosis proteins cIAP-1 and cIAP-2 have been identified as gene targets for NF-κB transcriptional activity (25). Accordingly, inactivation of NF-κB promotes apoptosis and enhances the apoptotic effect in response to TNF-α, ultraviolet radiation, and anticancer agents (17). These results are in full agreement with our results as reported here. It has been suggested that failure of anticancer agents is due to their resistance to apoptosis and that NF-κB-deficient cells are more susceptible to cell death (15). Therefore, identifying agents that inactivate NF-κB may be useful in the prevention and treatment of cancer. Anti-inflammatory drugs, salicylates, and glucocorticoids, which are known inhibitors of NF-κB, are being used as part of therapy for hematologic malignancies (26,27).

We have previously shown that the soy isoflavone genistein induces apoptosis in lung and prostate cancer cells and activates caspase-3 (4,7). In this report, we provide a mechanism by which genistein induces a proapoptotic response in prostate cancer cells by inhibiting NF-κB. Treatment of PC3 and LNCaP cells with genistein blocked the phosphorylation of the inhibitory protein IκBα. Unphosphorylated IκBα remains bound to the p50-p65 complex and prevents the heterodimer from translocating from the cytoplasm to the nucleus, binding to the DNA consensus sequence, and transactivating genes. IκBα is phosphorylated at its regulatory NH_2 terminus on serine residues 32 and 36 by IKK (22). Because genistein is a tyrosine kinase inhibitor, it is unlikely that genistein is directly blocking phosphorylation of IκBα by IKK. It is more likely that genistein is modulating upstream cell-signaling molecules; however, the exact mechanism by which genistein sustains underphosphorylation forms of IκBα remains to be determined.

There is some evidence indicating the involvement of tyrosine kinases in the activation of NF-κB. Staurosporine, a tyrosine kinase inhibitor, can inhibit NF-κB complex formation. On the other hand, interleukin (IL)-1-induced NF-κB has been associated with a transient increase in protein tyrosine kinase activity. This activation can be blocked by herbamycin A (28). Tyrosine kinase inhibitors, herbamycin A and tyrphostin, completely inhibited phorbol myristate acetate- and TNF-α-induced NF-κB activation in Jurkat T cells (29). Tyrosine kinases have been suggested to play a role in NF-κB activation, but the specific tyrosine kinase directly responsible for NF-κB activation is unknown. Studies have implied that mitogen-activated protein kinase signaling may be involved in NF-κB activation. Overexpression of MEKK1 is able to directly phosphorylate and activate IKK, which is responsible for IκBα phosphorylation (30). Dominant negative kinase dead mutant mitogen-activated protein kinase kinase 1 was able to inhibit TNF-α-inducing activation of NF-κB. Recently, NF-κB-inducing kinase was characterized and found to induce NF-κB activation, whereas kinase-inactive NF-κB-inducing kinase mutants blocked TNF-α- and IL-1-induced NF-κB activation (31,32). These observations, along with a recent study in which genistein has been shown to block the activation of mitogen-activated protein kinase by TNF-α (33), strongly suggest the role of several signaling molecules in mediating the activation of NF-κB. This report (33) clearly documents the inactivation of NF-κB activity by genistein in nonepithelial cells, and our results report similar observations in epithelial cells, which provide strong evidence for the role of NF-κB in mediating the effect of genistein in prostate cancer cells.

There are some conflicting studies in which tyrosine kinase inhibitors failed to inactivate NF-κB. Genistein, tyrphostin 47, herbamycin A, and erbstatin failed to block translocation of NF-κB and degradation of IκBα after TNF-α stimulation in MCA-101 fibrosarcoma cells (34), and tyrosine kinase inhibitors failed to block IL-1β-induced activation of NF-κB in renal mesangial cells (35). These studies indicate that there are differences in the mechanism of NF-κB activation that may be cell type dependent. Our studies, however, clearly show that genistein inhibits NF-κB activation in PC3 and LNCaP prostate cancer cells.

Further studies have shown that the overexpression of NF-κB is correlated with the transformation process and neoplastic phenotype *in vitro*. A significant increase in expression of the p65 subunit was observed in thyroid carcinoma cell lines compared with normal thyroid cultures. Antisense inhibition of p65 reduced the ability of anaplastic thyroid carcinoma cells to form colonies on soft agar, which represents a main feature of the neoplastic phenotype (36). Li and colleagues (37) demonstrated that transcription factors AP-1 and NF-κB contribute to the process of tumor promoter-induced transformation by 12-*O*-tetradecanoylphorbol 13-acetate or TNF-α in AP-1/ NF-κB reporter cell lines, with NF-κB activation occurring first. Inhibition of either transcription factor blocked transformation (37). The exact role of NF-κB in the transformation process remains to be determined. However, these studies, in conjunction with our results, in which genistein inhibits NF-κB activation, provide evidence to support the role of genistein as a chemopreventive agent.

In conclusion, our results indicate that genistein inhibits NF-κB prosurvival mechanisms while promoting apoptotic signaling mechanisms in androgen-sensitive (LNCaP) and androgen-insensitive (PC3) prostate cancer cell lines. One of the major challenges in the clinical management of prostate cancer is that essentially all human prostate cancer cells are initially sensitive to androgen ablation; however, over time they become androgen independent, and thus further treat-

ment options become limited. Our results clearly indicate that genistein can inhibit NF-κB activation regardless of androgen sensitivity, therefore making cells more susceptible to cell death. However, the physiological concentrations of genistein reported *in vivo* are different from those used by many investigators *in vitro*. The physiological concentration of genistein varies (276 nM–6 μM) depending on the patient and the isoflavone source (1,38). Our results demonstrate that 25–50 μM genistein is an effective inhibitor of NF-κB after only three days of exposure, whereas humans consuming soy-rich diets are chronically exposed to lower concentrations of isoflavones. Because of the inability to do long-term culture experiments, we have shortened the exposure time to three days. Thus the *in vitro* data may be relevant to the *in vivo* data; however, further *in vivo* studies are required to establish the role of genistein *in vivo*. Overall, our *in vitro* data, along with the epidemiological studies in which men who consume high levels of isoflavones have reduced prostate cancer incidence and mortality rates, provide strong evidence for the potential utility of genistein in the prevention and treatment of prostate cancer, even in hormone-independent patients.

Acknowledgments and Notes

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