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# Genistein reduces NF-κB in T lymphoma cells *via* a caspase-mediated cleavage of IκBα

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**Abstract**

The transcription factor NF-κB is elevated in murine T-cell lymphoma lines compared with normal thymic lymphocytes, and may play a role in the neoplastic transformation of these cells. When T lymphoma cells were treated with the soy isoflavone genistein, a marked reduction in nuclear NF-κB levels was detectable predominantly for the p50/p50 homodimer and p50/p65 heterodimer. To examine the mechanism by which NF-κB is reduced by genistein, we analyzed the NF-κB inhibitor, IκBα, and detected a 34 kDa cleavage product ΔIκBα, which was induced by genistein in a dose-dependent manner. Our observation that a pan-caspase inhibitor could inhibit the induction of ΔIκBα by genistein suggested that caspase activity was responsible for this cleavage product. In support of this idea, we detected an increase in caspase-3 activity in response to increasing time of genistein exposure. When the induction of ΔIκBα was prevented, we detected no reduction of NF-κB levels by genistein. These results support a direct role for ΔIκBα in the reduction of NF-κB by genistein. To determine the effect of genistein on some NF-κB target gene products, we examined the antiapoptotic proteins Bcl-2, Bcl-$X_L$, A1, and cIAP-1. Only changes in A1 and cIAP-1 levels were affected with significant reductions in response to genistein. Generation of the repressive activity of ΔIκBα on NF-κB is a novel mechanism for the reduction of this transcription factor by genistein and the possible effect this may have on the ability of genistein to induce apoptosis in tumor cells.


*Keywords:* T-cell lymphoma; Genistein; NF-κB; IκBα; Caspase; Retrovirus

## 1. Introduction

The transcription factor, NF-κB, is elevated in a variety of tumors and transformed cells [1–3]. NF-κB is a dimer comprised of either homo- or heterosubunits consisting of p50, p52, p65, c-Rel and RelB [4]. The most frequent dimer formed is composed of p50 and p65 [5]. The C-terminal portion of p50 and p65 consists of a rel homology domain (RHD) which contains a nuclear localization signal responsible for targeting NF-κB to the nucleus when receiving an activating signal [6]. NF-κB dimers are rendered transcriptionally inactive within the cytoplasm of the cell by the IκB inhibitors [7,8]. IκB proteins contain 5 to 7 ankyrin repeats consisting of 33 amino acids [9]. These repeats function as protein–protein interaction domains that are required for binding of IκB to NF-κB dimers. These IκB proteins serve to prevent nuclear localization of NF-κB by blocking the nuclear localization signal within the RHD [6]. They also prevent NF-κB from binding to its DNA target sequence [10].

IκBα is the best studied of the five IκB proteins. IκBα resides in the cytosol where it associates with NF-κB. Upon receiving an activation signal transmitted through a series of cell signaling pathways, IκBα is phosphorylated at two serine residues, ser32 and ser36 [10]. These phosphorylation events result in the release and nuclear translocation of NF-κB. IκBα is subsequently ubiquitinated at lys21 and lys22 and then rapidly degraded by the 26S proteosome [11]. The activation of NF-κB results in the increased transcription of genes that harbor a NF-κB DNA-binding site.

In investigating the generation of thymic lymphomas in mice as a result of retroviral infection by the mink cell focus-inducing murine leukemia virus (MCF MLV), we observed that these T-cell tumors have significantly higher nuclear levels of NF-κB compared to normal thymocytes as measured by electrophoretic mobility shift assays.[1] A

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role for NF-κB in promoting tumorigenesis may be due to its activation of target genes involved in the regulation of antiapoptotic activities [12]. Such genes include those that encode the Bcl-2 family of proteins and the inhibitors of apoptosis (IAPs), whose antiapoptotic activities protect the mitochondria from depolarization and prevent the activity of certain caspases [13–16]. The inhibition of NF-κB could eliminate the protective effects of these antiapoptotic gene products that may promote tumorigenesis.

Genistein is an isoflavone obtained primarily from soy products [17]. The recorded activities of genistein at pharmacological concentrations are numerous and include protein tyrosine kinase inhibition, topoisomerase II inhibition, antioxidant activities, G2/M phase cell cycle inhibition, induced differentiation, and deregulation of mitochondrial membrane pore permeability [18–26]. Furthermore, it has been demonstrated that genistein can alter the regulation of nuclear transcription factors, such as members of the forkhead-related transcription factor family, cAMP-responsive element binding protein (CREB), signal transducers and activators of transcription (STATs), and nuclear factor-κB (NF-κB), through its ability to inhibit tyrosine kinases [27–30]. Genistein has been shown to induce apoptosis and reduce cell growth in several cancer cell types [22,24,26,29,31,32]. This effect has led to its use to treat different types of cancers in animal models [33–35].

In this study, we examined the effect of genistein at pharmacological concentrations on cell lines derived from T-cell lymphomas generated by infection with MCF247 MLV. We show that genistein is able to reduce NF-κB nuclear levels through a novel regulatory mechanism involving the activation of caspase-3 and generation of a cleaved form of IκBα (ΔIκBα) [36,37]. We further demonstrate that the reduction of nuclear NF-κB correlates with the reduction of the antiapoptotic proteins A1 and cIAP-1.

## 2. Materials and methods

### 2.1. Cell culture

Dr. Nancy DiFronzo (Children's National Medical Center, Washington, DC) graciously provided T-cell lymphoma lines (92316T, 92284T, 92290T) that were isolated from thymic lymphomas induced by MCF247 MLV. 92316T and 92284T are clonal cell lines. Cell line 92290T is polyclonal. Cells were maintained in RPMI 1640 supplemented with L-glutamine, 10% FBS, 2 mM sodium pyruvate, 50 μM 2-β-mercaptoethanol, 15 mM Hepes, 20 units/mL penicillin–streptomycin at 37° and 5% $CO_2$ in 75 $cm^2$ tissue culture flasks. Cells were split and cultured at $5 \times 10^5$ cells per mL 24 hr prior to the addition of genistein (Toronto Research Chemicals). Cells were washed once with RPMI 1640 and resuspended at $1 \times 10^6$ cells per mL at the time of genistein exposure. A genistein stock solution was prepared in DMSO at a concentration of 125 mM. The same volume of DMSO was added to untreated control cells, which produced a final concentration of DMSO less than 0.05%. For caspase inhibition studies, cells were first exposed to 50 μM pan-caspase inhibitor Boc-D-FMK (Calbiochem) for 1 hr at 37° prior to the addition of genistein to the culture. Boc-D-FMK was prepared as a stock solution in DMSO at a concentration of 50 mM.

Single cell suspensions of lymphocytes were prepared from thymuses of AKR/J mice (Jackson Laboratories) by pressing thymic tissue through a wire screen into cold RPMI 1640 containing 2% FBS. Thymic lymphocyte viability at isolation was greater than 90% as determined by trypan blue exclusion.

### 2.2. Electrophoretic mobility shift assay (EMSA) of NF-κB

Nuclear protein extracts were prepared as previously described [38]. Isolation of nuclei from cells was performed by treatment with 0.5% Nonidet P-40 in lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes; pH 8.0), 50 mM NaCl, 7 mM β-mercaptoethanol, 0.5 M sucrose, 1 mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine), containing 0.5 mM phenylmethylsulfonyl fluoride, aprotinin (3 μg/mL), leupeptin (1 μg/mL), and pepstatin A (1 μg/mL). Nuclei were pelleted by centrifugation and protein was isolated in extraction buffer (same as lysis buffer except that 10% glycerol is substituted for sucrose). An equal volume of extraction buffer that contained 0.55 N NaCl was then added and nuclear debris was removed by centrifugation. Nuclear protein was precipitated from the supernatant by the addition of ammonium sulfate to a final concentration of 70% saturation. Protein amounts were measured using the bicinchoninic acid protein assay (Pierce). An NF-κB consensus oligonucleotide [5′-AGT TGA GGG GAC TTT CCC AGG C-3′] was purchased from Promega and end-labeled with [γ-$^{32}$P]ATP and T4 polynucleotide kinase (Promega). The EMSA binding reaction consisted of gel shift buffer (2 mM Hepes; pH 8.0, 10 mM NaCl, 0.2 mM EDTA, 0.05 mM EGTA, 1.4 mM 2-β-mercaptoethanol, and 2% glycerol), 2 μg polydI/dC (Boehringer), and 10 μg nuclear protein in a final volume of 25 μL. Samples were incubated at room temperature for 30 min and electrophoresed through a 6% polyacrylamide gel with 15 mM Tris-base, 114 mM glycine (pH 8.5), 0.6 mM EDTA running buffer at room temperature for 2.5 hr. Gels were vacuum dried and exposed to X-ray film (Kodak) or a phosphorimager screen (Molecular Dynamics Amersham Biosciences). Recombinant NF-κB (p50) protein was purchased from Promega.

Gel mobility supershift assays were conducted by incubation of nuclear protein with 2 μg of antibody specific for p50, p52, p65, c-Rel, or RelB (Santa Cruz Biotechnology) for 20 min at room temperature prior to the addition of [γ-$^{32}$P]ATP-labeled NF-κB oligonucleotide to the reaction

mentioned above. The reaction was allowed to continue for an additional 20 min before gel electrophoresis.

### 2.3. Western blot analysis

Total cell or nuclear protein extracts were prepared as described [39]. 20 μg was added to reducing buffer (62.5 mM Tris–HCl pH 6.8, 25% glycerol, 2% sodium dodecylsulfate (SDS), 0.01% bromophenol blue, 5% β-mercaptoethanol) and boiled for 4 min. Samples were electrophoresed in a running buffer of 25 mM Tris, 192 mM glycine, 1% SDS (w/v), pH 8.3, through an 8 or 10% SDS–polyacrylamide gel at 120 V for 1.5 hr and transferred to polyvinylidene difluoride (PVDF) membrane (BioRad) at 350 mA for 2 hr at 4°. Membranes were probed with rabbit antibodies to NF-κB (#3032, Cell Signaling Technology), IκBα (#sc-371, Santa Cruz Biotechnology), Bcl-2 (#PC68, Oncogene), Bcl-$X_L$ (#2762, Cell Signaling Technology), A1 (#sc-8351, Santa Cruz Biotechnology) and cIAP-1 (Santa Cruz Biotechnology) overnight at 4°. After washing, membranes were incubated with horseradish peroxidase-conjugated goat antirabbit serum (Pierce) for 1 hr at room temperature. Protein bands were detected by enhanced chemiluminescence (ECL) and visualized on Biomax MR film (Kodak).

### 2.4. Immunoprecipitation

Total cell protein extracts from untreated and 60 μM genistein treated cells were prepared by incubation of cells with lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40) containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, aprotinin (3 μg/mL), leupeptin (1 μg/mL), and pepstatin A (1 μg/mL)) for 30 min on ice. Cell lysates were centrifuged at 10,000 *g* for 15 min at 4°. Supernatants were collected and protein measured using the Pierce Protein Assay. 200 μg of total protein was precleared with 50 μL of prepared Protein A agarose beads (Pierce) and 1 μL goat antirabbit IgG (Pierce) for 3 hr at 4°. 5 μg of antibody to either p50 or Bax (Santa Cruz Biotechnology) or rabbit antigoat IgG (Sigma-Aldrich) was added to precleared lysates and incubated at 4° overnight on a rotator. 50 μL of fresh Protein A agarose beads was added, and samples were incubated at 4° for 3 hr. The agarose beads were washed once with cold 0.5 M NaCl and pelleted by centrifugation at 10,000 *g* for 30 s. The beads were subsequently washed an additional four times with cold lysis buffer. Antibody-complexed proteins were eluted from the beads by boiling for 10 min in 1× Laemmli buffer. The beads were pelleted and the supernatant was subjected to Western blotting with antibody to IκBα.

### 2.5. Caspase-3 activity assay

$2 \times 10^7$ cells were collected, washed twice with phosphate buffered saline (PBS), and lysed in 200 μL 50 mM Tris–HCl, pH 7.5, containing 0.03% Nonidet P-40 and 1 mM dithiothreitol (DTT). Cell suspensions were centrifuged at 1200 *g* for 5 min to pellet nuclei. Cytosolic fractions were recovered and stored at −70°. 50 μL of the cellular extract was used to assay for caspase-3 activity using the EnzChek caspase-3 assay kit (Molecular Probes) in a 96-well plate format. After a 30 min incubation, fluoromethylcoumarin fluorescence, resulting from the cleavage of the Z-DEVD-AMC peptide substrate by caspase-3 activity, was detected with a Spectra MAX Gemini fluorometer (Molecular Devices) with an excitation wave length of 350 nm and emission wave length detection at 450 nm. An AMC standard curve was prepared in the range of 0.137–17.5 μg of fluorophore. Protein amounts were measured as described previously.

## 3. Results

### 3.1. Analysis of NF-κB in T lymphoma cells

We have observed that nuclear NF-κB levels are highly elevated in murine T-cell lymphomas compared with normal thymic lymphocytes (see Footnote 1). We determined that NF-κB levels in three cell lines derived from three different lymphomas of this type (92316T, 92284T, 92290T) also had increased nuclear NF-κB levels as evaluated by EMSA (Fig. 1A). Nuclear protein extracts from all three thymic lymphoma cell lines possessed greater NF-κB levels compared with normal thymocytes. By this assay, three protein–DNA complexes were detectable (lanes 2–4). The 92316T cell line (Fig. 1A, lane 2) was selected for more detailed NF-κB analysis due to its having the greatest detectable NF-κB levels.

To determine which of the three protein–DNA complexes represented specific NF-κB binding, we performed EMSA competition assays (Fig. 1B). These assays indicated that while all three complexes were reduced by an excess of unlabeled NF-κB specific oligonucleotide, only two complexes, identified as I and II, were completely eliminated (Fig 1B, lanes 3–5). From these results we concluded that the third complex represented nonspecific protein binding. A similar nonspecific complex of unknown origin that was detectable by NF-κB EMSA analysis has been reported for primary T lymphocytes [40]. In contrast, addition of a 200-fold excess of unlabeled oligonucleotide containing the consensus binding site for the Sp1 DNA-binding factor (Promega) (lane 6) had no effect on the formation of NF-κB complexes.

To identify the NF-κB protein subunits representing specific binding in complexes I and II, an EMSA supershift was performed using antibodies to p50, p52, p65, c-Rel, and RelB. Fig. 1C shows that the antip50 antibody produced a supershifted band and a reduction in intensity of both complexes I and II. This result indicated that both complexes were comprised of p50. We also observed that

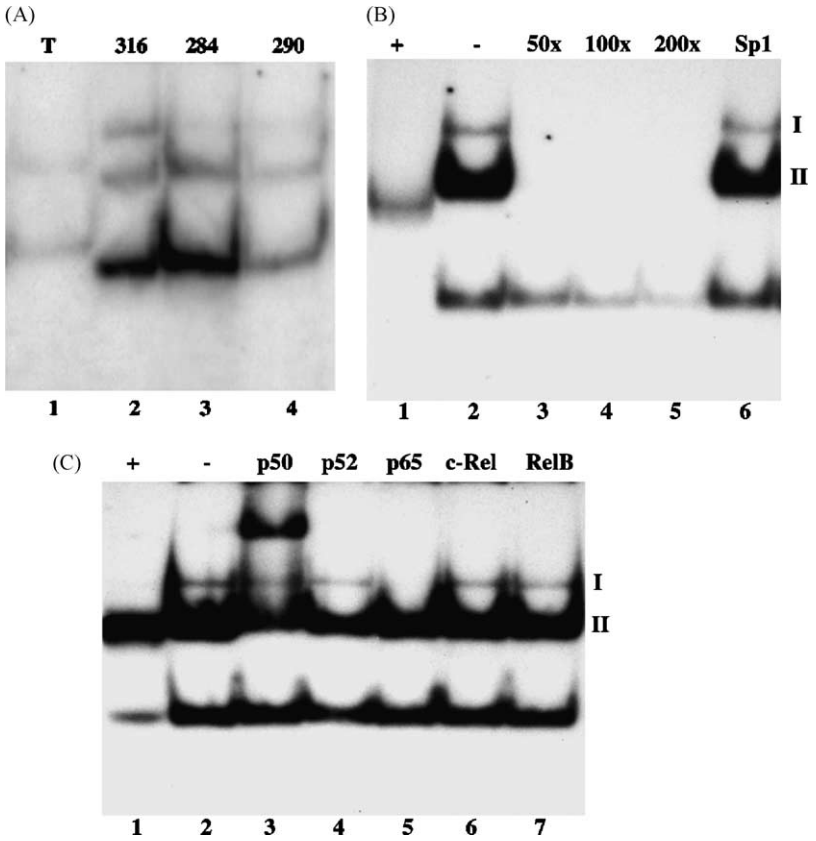


Fig. 1. Increase of NF-κB in T lymphoma cell lines. (A) EMSA comparing NF-κB levels in normal thymocytes and lymphoma cell lines. Nuclear protein extracts from normal mouse thymocytes (lane 1), nuclear protein extracts from 92316T (lane 2), 92284T (lane 3), and 92290T (lane 4) cells. (B) Competition EMSA. 10 μg of nuclear protein extract from 92316T cells were pretreated 20 min with an excess of unlabeled NF-κB oligonucleotide or nonspecific Sp1 oligonucleotide. (+), p50 recombinant protein control (lane 1); (−), nuclear extract without cold oligonucleotide (lane 2); 50×, 50-fold excess (lane 3); 100×, 100-fold excess (lane 4); 200×, 200-fold excess (lane 5) of unlabeled NF-κB oligonucleotide; Sp1, 200-fold excess Sp1 oligonucleotide (lane 6). (C) EMSA supershift. Nuclear protein was isolated from 921316T cells and subjected to 2 μg of each monoclonal antibody for 20 min prior to NF-κB binding reaction. (+), p50 recombinant protein control (lane 1); (−), nuclear extract without antibody (lane 2); p50 antibody treated extract (lane 3); p52 antibody treated extract (lane 4); p65 antibody treated extract (lane 5); c-Rel antibody treated extract (lane 6); RelB antibody treated extract (lane 7).

the antibody to p65 eliminated complex I, but did not alter the band intensity of complex II (lane 5), suggesting that this antibody interfered with the binding of this protein to the DNA probe. Neither supershifting nor effect on band intensities was detectable as a result of addition of antibodies to p52, c-Rel, or RelB. Thus, our data demonstrated that complex I is comprised of a p50/p65 heterodimer and that complex II is comprised of a p50 homodimer. These results are similar to what other investigators have observed for NF-κB complexes which have been detected for thymic lymphocytes and other T-cells [41].

### 3.2. Reduction of nuclear NF-κB levels in T lymphoma cells

To assess the effect of genistein on the levels of NF-κB in T lymphoma cells, nuclear protein extracts were prepared from 92316T cells exposed to 15, 30, and 60 μM genistein for 24 hr (Fig. 2A). A decrease in both NF-κB specific complexes I and II was seen for extracts from cells treated with 15 μM and greater genistein concentrations as compared with untreated cells. The decrease of these complexes occurred in a dose-dependent manner. As an independent assay, the same samples were subjected to Western blot analysis for p65 and p50 (Fig. 2B). A similar genistein dose-dependent reduction in nuclear NF-κB levels was observed.

Because these studies in which we detected a reduction in NF-κB by different concentrations of genistein were done only at 24 hr of exposure, we performed a time-course analysis of NF-κB levels to determine whether its reduction occurred at an earlier time after exposure to genistein. 92316T cells were treated with 60 μM genistein and nuclear protein extracts were obtained at 0, 2, 4, 6, 8, 12, and 24 hr of exposure. Western blot analysis of the nuclear protein extracts showed that the reduction of nuclear NF-κB levels as a result of exposure to genistein occurred between 12 and 24 hr of exposure (Fig. 2C). Reductions occurred for both the p65 protein and p50 protein as seen previously. Densitometric analysis showed an approximately 4-fold decrease in these NF-κB subunits in cells treated with 60 μM genistein at the 24 hr time-point as compared to untreated cells. These data demonstrate that there is a reduction in nuclear NF-κB levels in response to

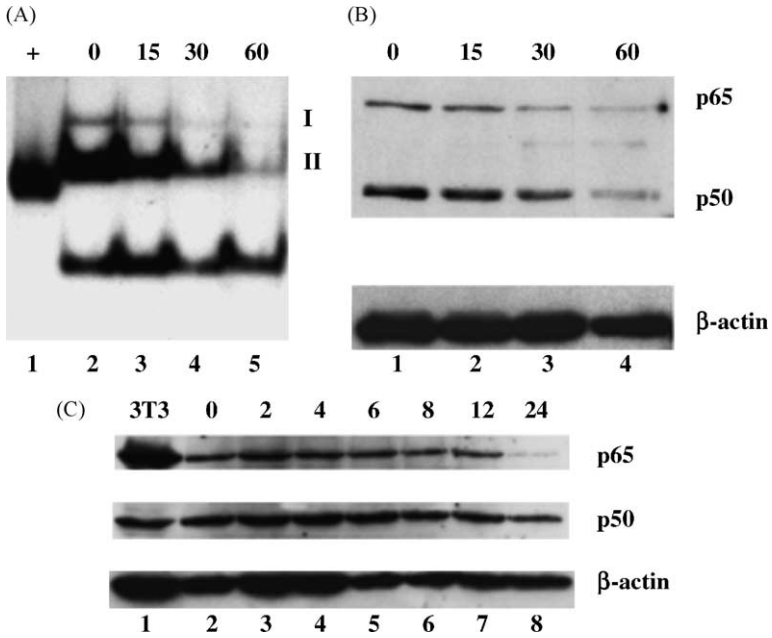


Fig. 2. Reduction of nuclear NF-κB in T lymphoma cells. (A) EMSA of nuclear extract from 92316T cells. 10 μg nuclear extract from 92316T cells treated with concentrations of genistein at 0, 15, 30, and 60 μM for 24 hr (lanes 2–5); (+), p50 recombinant protein control (lane 1); I and II refer to NF-κB specific protein–DNA complexes. (B) Western blot of nuclear extract from 92316T cells. 20 μg nuclear extract used in the EMSA assays (A) were evaluated (lanes 1–4). The upper panel shows bands for NF-κB p65 and p50 subunits. The lower panel shows β-actin that was used to verify equal loading of protein. (C) Kinetic Western blot of 92316T cell nuclear extracts. Cells were treated with 60 μM genistein and 20 μg nuclear protein was examined at 0, 2, 4, 6, 8, 12, and 24 hr of exposure (lanes 2–8). NIH3T3 total cell extract was used as a positive control (lane 1).

genistein exposure in this T lymphoma cell line and that this reduction occurs between 12 and 24 hr of exposure.

### 3.3. Cleavage of IκBα

It has been shown that IκBα regulates the activation of NF-κB by sequestering it in the cytoplasm [7]. Nuclear translocation of NF-κB occurs as a result of ubiquitination and subsequent proteasomal degradation of IκBα. To examine whether IκBα may have a role in the reduction of nuclear NF-κB levels by genistein, we performed Western blot analysis of whole cell protein extracts from 92316T cells exposed to different concentrations of genistein for 24 hr, utilizing an antibody that specifically recognizes the C-terminus of IκBα. Beginning at 15 μM genistein, we detected two protein species of 37 and 34 kDa (Fig. 3A). The 37 kDa band, which corresponded to the predicted size of IκBα, further decreased while the 34 kDa band increased in intensity with increasing genistein concentrations. These results suggest that genistein induced the cleavage of IκBα to a 34 kDa product (ΔIκBα).

Because the IκBα cleavage product, ΔIκBα, which others have shown lacks the ubiquitination sites, can produce a super-repressor effect on NF-κB by stably sequestering it within the cytosol [37], we performed a kinetic study to determine whether the appearance of ΔIκBα occurred before NF-κB reduction. Total cell protein from 92316T cells treated with 60 μM genistein was extracted at 0, 2, 4, 6, 8, 12, and 24 hr of exposure and subjected to Western blot analysis using an antibody to IκBα (Fig. 3B). By 6 hr of genistein exposure, we clearly detected the 34 kDa band, which increased in intensity with time (lanes 5–8). During this time, we detected a concomitant decrease of the 37 kDa form of IκBα. Our data demonstrated that the appearance of ΔIκBα occurred well before reductions in nuclear NF-κB levels were detectable at 24 hr (Fig. 2).

To test if cleaved IκBα associates with NF-κB, whole cell lysates were prepared from 92316 T cells exposed to 60 μM genistein for 24 hr. Lysates were subjected to immunoprecipitation with an antibody specific for p50 because it was the predominant NF-κB protein detectable in these cells (Fig. 1). For immunoprecipitations, antibody–protein complexes were eluted from Protein A agarose beads and analyzed by Western blotting using an antibody to IκBα (Fig. 3C). As observed previously, total cell lysates from untreated cells that were not subjected to immunoprecipitation contained only the uncleaved 37 kDa IκBα (lane 1). Nonimmunoprecipitated lysates from cells treated with 60 μM genistein for 24 hr had both the 37 kDa IκBα and 34 kDa ΔIκBα (lane 2). Immunoprecipitation with p50 antibody of cell lysates from untreated cells produced predominantly the 37 kDa IκBα band (lane 3), whereas lysates from genistein-treated cells produced predominantly the 34 kDa ΔIκBα band (lane 4). Controls included immunoprecipitation with either antibody to the unrelated Bax protein (lane 5) or preimmune serum (lane 6). These results demonstrate that ΔIκBα is predominantly associated with NF-κB in cells treated with

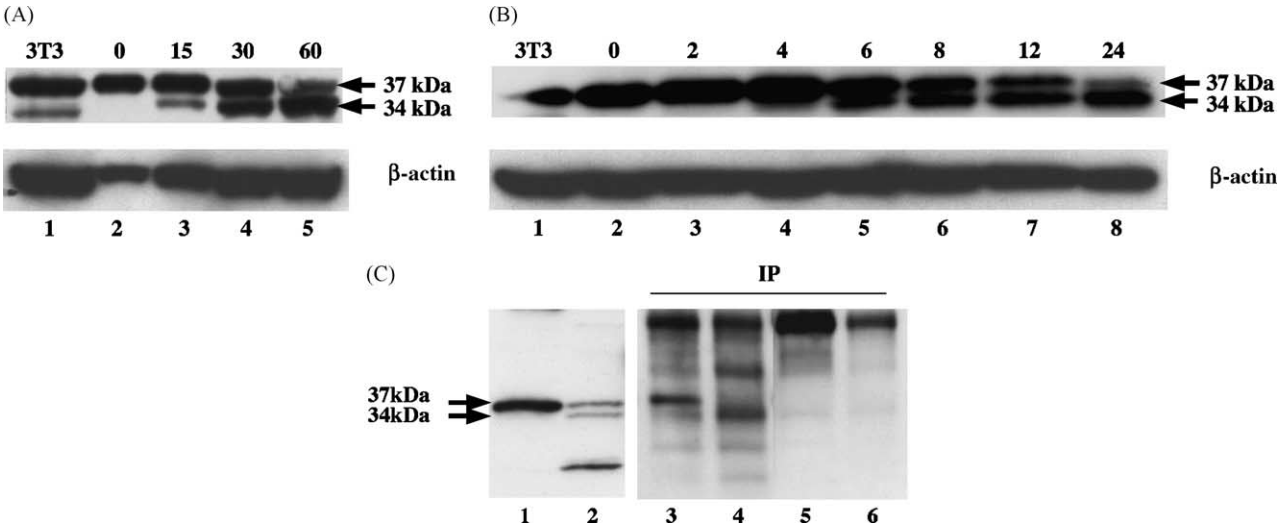


Fig. 3. Cleavage of IκBα resulting from genistein treatment. (A) Western blot of IκBα. 92316T cells were treated with genistein concentrations of 0, 15, 30, and 60 μM for 24 hr (lanes 2–5). 20 μg total cell protein extracts was denatured in a SDS Tris–glycine buffer containing β-mercaptoethanol. Extracts were electrophoresed through an 8% SDS–polyacrylamide gel. Separated proteins were then transferred to a PVDF membrane. The upper panel displays the 37 and 34 kDa IκBα bands indicated by arrows. The lower panel includes β-actin staining as a loading control. NIH3T3 total cell extract was utilized as a positive control (lane 1). (B) Kinetic Western blot of IκBα. 92316T cells were treated with 60 μM genistein over 24 hr. Total cell extracts were obtained at 0, 2, 4, 6, 8, 12, and 24 hr of exposure. 20 μg of protein extract was electrophoresed through a 10% SDS–polyacrylamide gel. The upper panel shows the IκBα 37 and 34 kDa bands. The bottom panel shows the β-actin detection. (C) Western blot of IκBα in immunoprecipitated extracts. 92316T cells were treated with 60 μM genistein for 24 hr. Total cell extracts were immunoprecipitated with antibody to p50. Untreated and 60 μM genistein treated cellular extracts without prior immunoprecipitation were subjected to Western blot analysis for IκBα (lanes 1 and 2). Untreated and genistein-treated cellular extracts immunoprecipitated with p50 antibody (lanes 3 and 4, respectively). Genistein-treated cellular extracts immunoprecipitated with either antibody to Bax or rabbit antigoat IgG (lanes 5 and 6, respectively). The 37 and 34 kDa IκBα bands are indicated by arrows.

genistein and further support the involvement of ΔIκBα in the reduction of NF-κB in these cells.

It has been reported previously that a caspase-3-like activity plays a role in the cleavage of IκBα [36]. To determine whether the cleavage of IκBα in genistein treated cells is also due to caspase activity, we compared cytosolic protein extracts from cells treated with 60 μM genistein for 24 hr to those pretreated with the pan-caspase inhibitor Boc-D-FMK for 1 hr prior to genistein exposure (Fig. 4A). In cells untreated with genistein, only the 37 kDa

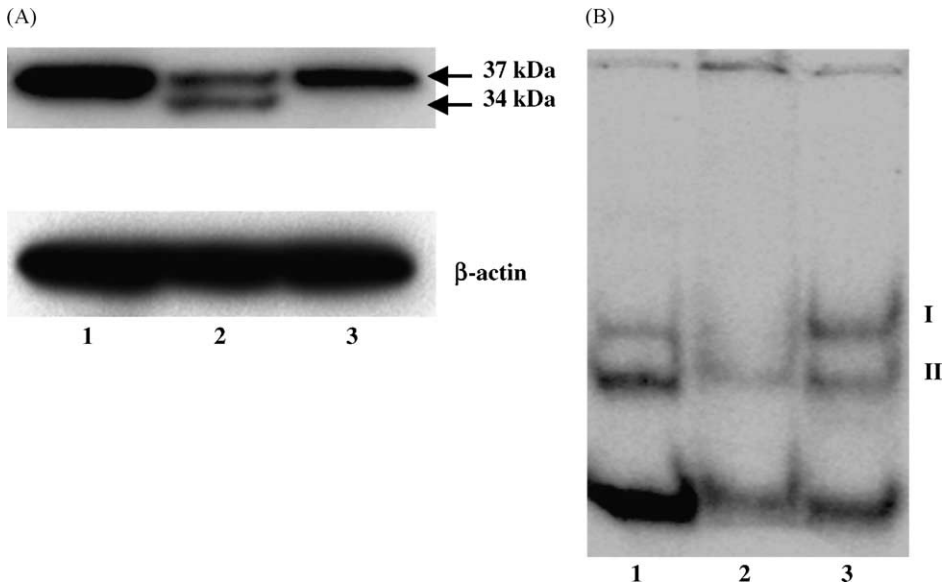


Fig. 4. Prevention of IκBα cleavage by caspase inhibition. (A) Western blot of 92316T cytosolic protein extracts. Protein extracts were obtained from untreated cells (lane 1), cells exposed to 60 μM genistein for 24 hr (lane 2), and cells pretreated with 50 μM Boc-D-FMK prior to 24 hr treatment with 60 μM genistein (lane 3). The upper panel shows the 34 and 37 kDa IκBα bands. The lower panel shows the β-actin used to verify equal protein loading. (B) EMSA of nuclear protein extracts from 92316T cells. Untreated cells (lane 1), cells exposed to 60 μM genistein for 24 hr (lane 2), and cells pretreated with Boc-D-FMK prior to genistein exposure (lane 3) were compared. I and II refer to specific NF-κB binding complexes.

IκBα band was detectable (lane 1), whereas the 34 kDa cleavage product was observed in protein extract from cells treated with genistein (lane 2) as was seen previously. Pretreatment of cells with the pan-caspase inhibitor prevented the appearance of the 34 kDa band in cells that were subsequently exposed to 60 μM genistein (lane 3). This result indicated that the generation of the ΔIκBα was dependent on caspase cleavage.

To examine the effect of inhibiting IκBα cleavage on NF-κB levels, we subjected nuclear protein extracts from cells pretreated with the pan-caspase inhibitor and subsequently exposed to genistein to EMSA to detect NF-κB binding (Fig. 4B). In untreated cells, both complexes specific for NF-κB binding as previously determined were observed (lane 1). As described above, cells exposed to 60 μM genistein for 24 hr showed a decrease in both NF-κB complexes (lane 2). When cells were pretreated with the pan-caspase inhibitor before genistein exposure, both NF-κB complexes were detectable at comparable levels (lanes 1 and 3). This observation supports the involvement of ΔIκBα in the reduction of NF-κB by genistein.

### 3.4. Caspase-3 activation in T lymphoma cells

Since inhibition of caspase activity resulted in the prevention of the appearance of ΔIκBα, we next evaluated caspase-3 activity in genistein exposed cells to determine whether its activation might be responsible for the cleavage of IκBα. We selected this particular caspase for examination because it has been shown that caspase-3 cleaved IκBα in an *in vitro* system using cellular extracts [36]. 92316T cells were exposed to 60 μM genistein, and caspase-3 activity was measured after 0, 4, 6, 8, and 24 hr of exposure (Fig. 5). Beginning at 6 hr of exposure to genistein and increasing to 24 hr, an elevation of caspase-3 activity was observed in cells treated with genistein compared to untreated control cells. Caspase-3 activity in genistein-treated cells at 6 hr was significantly greater than in untreated cells with a $P$ value <0.004 as determined by the Student's $t$-test. At 24 hr, we detected an approximately 7-fold increase in caspase-3 activity compared with untreated controls. These data support the idea that generation of ΔIκBα by genistein is mediated by caspase-3 activity as the initiation of its activity corresponded to the appearance of the IκBα cleavage product by 6 hr of genistein exposure.

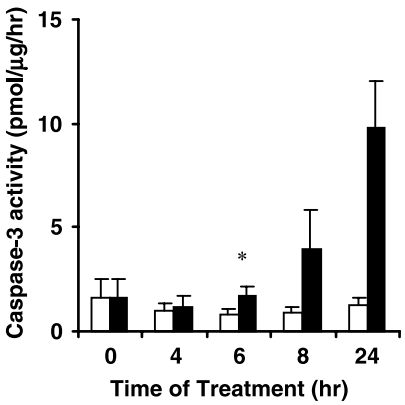


Fig. 5. Caspase-3 activation in genistein treated cells. 92316T cells were untreated (white bar), or treated with 60 μM genistein (black bar) and assayed at 0, 4, 6, 8, and 24 hr of exposure. Caspase-3 activity is reported as pmol of DEVD substrate hydrolyzed per μg of input protein per hour. The results shown are the mean values with standard deviations calculated from six independent experiments. $^{*}P < 0.004$ compared to untreated cells as determined by Student's $t$-test.

### 3.5. Reduction in the protein levels of NF-κB regulated genes

NF-κB is known to participate in the transcriptional regulation of several genes that encode antiapoptotic proteins, including Bcl-2, Bcl-$X_L$, A1, and cIAP-1 [16,42–44]. We have recently observed that genistein induces apoptosis

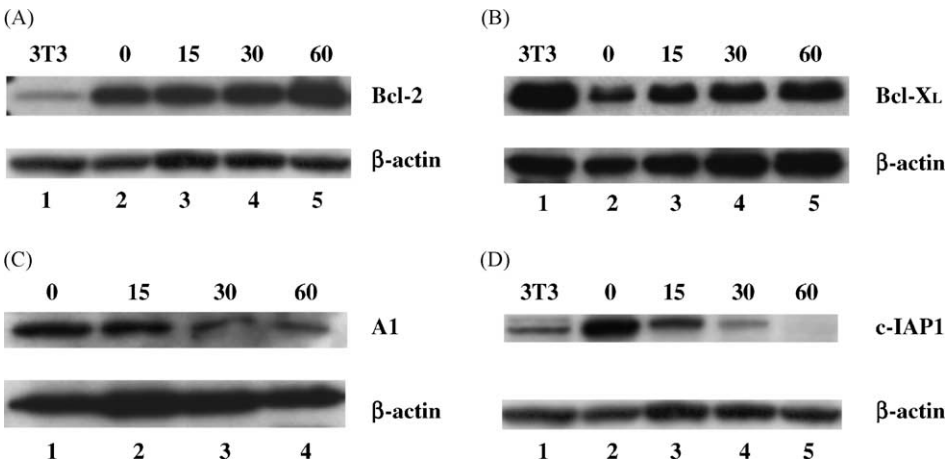


Fig. 6. Effect of genistein on antiapoptotic proteins regulated by NF-κB. 20 μg of total cell extract from 92316T cells was prepared from cells exposed to 0, 15, 30, and 60 μM genistein for 24 hr (A, B, D, lanes 2–5; and C, lanes 1–4). NIH3T3 total cell extract was utilized as a positive control (A, B, D, lane 1). The upper panels show Western blots for (A) Bcl-2; (B) Bcl-$X_L$; (C) A1; and (D), cIAP-1. The lower panel shows the β-actin staining used to verify equal loading of protein.

in the T-cell lymphoma lines used in this study (manuscript in preparation). Thus, it is possible that decreased levels of these proteins due to NF-κB reduction may contribute to the induction of apoptosis by genistein. To test this hypothesis, we examined the levels of Bcl-2, Bcl-$X_L$, A1, and cIAP-1 in protein extracts from cells treated with 15, 30, and 60 μM genistein for 24 hr by Western blot analysis. We observed no detectable change in protein levels of Bcl-2 and Bcl-$X_L$ as a result of genistein treatment (Fig. 6A and B). In contrast, a significant reduction of A1 (Fig. 6C) and cIAP-1 (Fig. 6D) was detectable as a result of exposure to genistein at concentrations above 15 μM in a dose-dependent manner. Thus, the reduction of NF-κB levels in the nucleus due to genistein corresponded to a decrease in the antiapoptosis proteins A1 and cIAP-1.

## 4. Discussion

In this study, we have observed that nuclear NF-κB levels in cell lines developed from thymic tumors generated by the oncogenic murine retrovirus MCF247 MLV are greater than in normal murine thymocytes. The predominant NF-κB complex detectable in 92316T cells and in MCF MLV generated tumors is the p50/p50 homodimer. p50 homodimers are commonly described as being transcriptionally repressive for NF-κB genes. However, p50 homodimers have also been reported to up-regulate NF-κB-regulated genes in the presence of Bcl-3 [45]. p50/p65 heterodimers are most often thought to be the complex that enhances transcriptional activation [46]. p50/p65 heterodimers were detectable in our T-cell lymphomas, albeit at much lower levels compared with p50 homodimers.

Treatment of 92316T cells with genistein concentrations of 15 μM and greater showed a substantial reduction in nuclear NF-κB. The inhibition of NF-κB by genistein in prostate cancer cell lines PC3 and LNCaP has been reported by Davis *et al.* [29]. They have suggested that this effect is due to the inhibition of tyrosine kinases by genistein. Consistent with this hypothesis is their observation that genistein produced a decrease in the phosphorylation of IκBα as well as MEKK1 within the TNFα signaling pathway [29]. Our data have demonstrated that genistein reduces NF-κB levels in T-cell lymphoma by a different mechanism. We detected the generation of a 34 kDa cleavage product of IκBα, which was dependent upon genistein concentration and occurred as early as 6 hr of exposure. This was well before the 12–24 hr period of genistein exposure that was required to detect a decrease in NF-κB levels. An IκBα cleavage product of similar size was identified by Reuther and Baldwin [37], who showed that it had a super-repressor effect on NF-κB activity. Such a cleavage of IκBα by caspase-3-like activity has been previously reported [36]. It was further shown that the generation of ΔIκBα was a result of caspase-3 activity [47]. Caspase-3 cleaves IκBα just N-terminal to ser32. This cleavage results in the loss of the ubiquitination sites and generates a nondegradable form of IκBα. This ΔIκBα acts as a super-repressor in its association with NF-κB dimers [37]. In agreement with these reports, the generation of ΔIκBα in response to genistein treatment corresponded with an increase in caspase-3 activation as measured by cleavage of the DEVD substrate. Furthermore, the pan-caspase inhibitor Boc-D-FMK was able to prevent both the generation of ΔIκBα and decrease of nuclear NF-κB. Our immunoprecipitation results demonstrated that ΔIκBα is predominantly associated with NF-κB after genistein treatment, suggesting that ΔIκBα may also function to down-regulate NF-κB by sequestering it in the cytosol. However, there are other potential mechanisms which could also account for our observations, including acceleration of the nuclear egress of NF-κB or production of an unstable NF-κB complex. Further studies will be required to elucidate the details of the mechanism by which ΔIκBα functions to down-regulate NF-κB.

The ability of genistein to induce apoptosis in a variety of cancer cell types has been well documented [22,31]. Because it has been demonstrated that induction of apoptosis by genistein involves a mitochondrial pathway in some cell types [26], we evaluated the effect of genistein on mitochondrial regulatory proteins, such as Bcl-2, Bcl-$X_L$, A1, and cIAP-1, which are regulated by NF-κB. Bcl-2 and Bcl-$X_L$, which are two antiapoptotic proteins belonging to the Bcl-2 family of mitochondrial proteins, prevent the depolarization of mitochondria by blocking pore forming or channel opening pro-apoptotic proteins from acting upon the mitochondria [43]. Neither of these proteins was observed to have altered expression levels as a result of exposure to genistein. In contrast, the level of A1, also an antiapoptotic member of the Bcl-2 family, was significantly reduced by genistein. It is of note that although all three of these Bcl-2 family proteins are transcriptionally regulated by NF-κB, only A1 is reported to be solely affected by NF-κB levels [48]. It is conceivable that both Bcl-2 and Bcl-$X_L$ levels were maintained in the face of decreased NF-κB levels by compensatory mechanisms [49,50].

The cIAP proteins function by binding to activated caspases, in particular caspase-3, -7, and -9, and inhibiting the activation of the caspase cascade [51]. We detected a decrease in cIAP-1 protein levels, which was dependent upon genistein concentration and correlated with the reduction of nuclear NF-κB. A decrease in the inhibitory activity of cIAP-1 could result in an increase in activated caspase-3 and its cleavage of target substrates, including IκBα. This idea is supported by our observation that a decrease in cIAP-1 correlated with an increase in caspase-3 activity and generation of ΔIκBα by genistein.

Our data have shown that a mechanism of inhibition of NF-κB induced by genistein involves cleavage of IκBα. This mechanism does not exclude other reported activities of genistein, such as tyrosine kinase inhibition which also produces a reduction of NF-κB. It is most likely that genistein affects a multitude of mechanisms governing

the activity of NF-κB that result in combined negative regulatory effects. In this report, we describe a novel mechanism for the down-regulation of NF-κB by genistein and propose that this has a significant role in the ability of genistein to induce apoptosis in T-cell lymphomas.

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