Inhibition of Nuclear Translocation of Nuclear Factor-κB Contributes to 3,3′-Diindolylmethane-Induced Apoptosis in Breast Cancer Cells

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Abstract

Dietary indole-3-carbinol (I3C), a natural compound present in vegetables of the genus *Brassica*, showed clinical benefits and caused apoptosis in breast cancer cells. Our laboratory and others have shown that I3C induces apoptosis in breast cancer cells mediated by inactivation of Akt and nuclear factor-κB (NF-κB) pathway. 3,3′-Diindolylmethane (DIM), a major *in vivo* acid-catalyzed condensation product of I3C, also showed some benefit in breast cancer. However, the precise molecular mechanism(s) by which DIM induces apoptosis in breast cancer cells has not been fully elucidated. Hence, we investigated whether DIM-induced apoptosis of breast cancer cells could also be mediated by inactivation of Akt and NF-κB. We found that DIM induces apoptotic processes in MCF10A derived malignant (MCF10CA1a) cell lines but not in nontumorigenic parental MCF10A cells. DIM specifically inhibits Akt kinase activity and abrogates the epidermal growth factor–induced activation of Akt in breast cancer cells, similar to those observed for I3C. We also found that DIM reduces phosphorylation of IκBα, an inhibitor of NF-κB. Our confocal microscopy study clearly showed that DIM blocks the translocation of p65, a subunit of NF-κB to the nucleus. DNA binding analysis and transfection studies with IκB kinase cDNA revealed that overexpression of IκB kinase mediates IκBα phosphorylation, which activates NF-κB, and this activation was completely abrogated by DIM treatment. Taken together, these results showed for the first time that the inactivation of Akt and NF-κB activity also plays important roles in DIM-induced apoptosis in breast cancer cells, which seems to be more relevant to *in vivo* situations. (Cancer Res 2005; 65(1): 364-71)

Introduction

Breast cancer is the second leading cause of cancer-related deaths in women and is steadily increasing in both developed and developing countries (1). Epidemiologic studies have shown that high dietary intake of fruits and vegetables protect against carcinogenesis (2, 3). The dietary indoles present in the *Brassica* plants, including turnips, kale, broccoli, cabbage, Brussels sprouts, and cauliflower, have shown to be protective against several cancers (4, 5). Indole-3-carbinol (I3C) is an autolysis product of glucosinolate found in *Brassica* food plants and exhibits antitumor activities *in vitro* and *in vivo* by inducing apoptotic cell death especially associated with mammary neoplasia (6–10). I3C has also shown to suppress the growth of both estrogen-dependent and estrogen-independent human breast cancer cell lines (11, 12). The use of dietary botanicals for inhibiting cancer cell growth and induction of apoptosis is increasingly being appreciated; however, the question remains regarding the usefulness of these plant-derived agents, such as I3C/3,3′-Diindolylmethane (DIM), in preclinical models and to determine whether physiologically attainable concentration could be shown from cell culture studies.

I3C is chemically unstable in an acidic environment and is rapidly converted in the stomach to a variety of condensation products. Among those, DIM is a major acid condensation product of I3C that is readily detectable in the liver and feces of rodents fed with I3C (13). The parent I3C could not be detected in tissues of I3C-treated rodents, suggesting that DIM may mediate the physiologic effects of dietary I3C (14). It has been shown that I3C and its dimeric product DIM possess anticarcinogenic effects in experimental animals and inhibit the growth of human cancer cells (12, 15). It has also been reported that DIM exerts its chemopreventive effects in estrogen-responsive tissues, and DIM-induced G_1 arrest occurs by up-regulation of $p21^{WAF1/CIP1}$ in breast cancer cells, suggesting its inhibitory effects on hormone-related cancer (15, 16). These findings led to significant interest in the past few years to explore the potential utility of DIM as a chemopreventive agent (15, 17, 18). However, the molecular mechanism(s) of antiproliferative and anticancer effects of DIM have not been fully elucidated. The mechanism(s) by which DIM induces apoptosis in human breast cancer cells could potentially lead to the development of novel approaches for the prevention and/or treatment of breast cancer.

Phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathway is an important signal transduction pathway in cells and plays a critical role in controlling cell survival and apoptosis. It has been shown that Akt, a serine/threonine kinase, regulates nuclear factor-κB (NF-κB) activation directly through activation of IκB kinase (IKK) or phosphorylation of RelA (19, 20). NF-κB is a key regulator of genes involved in cell activation and proliferation (21). The activation of NF-κB involves the phosphorylation of IκB, an inhibitory binding partner of NF-κB complex, for ubiquitination and degradation through proteosome degradation pathway. This allows the translocation of NF-κB into the nucleus, where it activates transcription of genes (22). A key regulatory step in this pathway of NF-κB activation is the activation of a high molecular weight IKK complex in which catalysis is thought to be done by kinases, including IKKα and IKKβ, which directly phosphorylates IκB proteins. Exactly how these IKKs are activated is the subject of intense investigation.

Studies from our laboratory and others have shown that I3C is a potent inducer of apoptosis and inhibits NF-κB and Akt activation in breast and prostate cancer cells, suggesting that I3C could serve as a preventive and/or therapeutic agent against breast and prostate cancer (6, 7, 23, 24). However, little is known regarding the Akt and NF-κB gene alteration in breast cancer cells after DIM treatment. Therefore, we hypothesized that DIM may inhibit NF-κB activation by inhibiting IKK and Akt activity in breast cancer cells

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leading to apoptotic cell death. Here, we report for the first time that DIM inhibits IKK-mediated IκBα phosphorylation, resulting in the inactivation of both Akt and NF-κB during apoptotic cell death in breast cancer cells.

Materials and Methods

Cell Line and Culture. In recent years, the breast cancer program of the Comprehensive Cancer Center at Karmanos Cancer Institute, Wayne State University, Detroit, MI, has developed a new and unique model of early human breast cancer progression, the "MCF10AneoT" model. MCF10AneoT is a line derived from MCF10A (a breast epithelial cell line that is nontumorigenic and considered to be closely related to the normal breast epithelial cells) by transfecting activated H-*ras* oncogene and transforming the phenotype (25). *Ras*-transformed MCF10A cells are still considered to be a preneoplastic human breast epithelial cell line, which are able to grow in nude/beige mice where they undergo a sequence of progressive histologic changes, culminating in a certain percentage of carcinomas. Thus, MCF10AneoT is a transplantable, xenograft model of human proliferative breast disease with proven neoplastic potential, giving us a unique opportunity to study cellular and molecular changes required for the development and progression of breast cancer.

In addition, subsequent transplant generation has ultimately given rise to an established cell line, named MCF10ACA1a, which is fully tumorigenic in mice (25). Hence, this model provides a unique *in vitro* and *in vivo* model to test the chemopreventive role of dietary I3C in breast cancer development and progression in future studies. For the present study, we have used an isogenic pair of human breast epithelial cells, one of which is tumorigenic (MCF10CA1a, hereafter known as CA1a) and the other is nontumorigenic (MCF01A) breast epithelial cells. All cells were cultured in 95% air, 5% CO_2 at 37°C. MCF10A cells were cultured in DMEM/F-12 (1:1, Life Technologies, Grand Island, NY) supplemented with 5% horse serum (Life Technologies), 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 1 μg/mL insulin, 0.1 μg/mL cholera toxin, 0.5 μg/mL hydrocortisone (Sigma, St. Louis, MO), 0.5 μg/mL fungizone, and 0.02 μg/mL epidermal growth factor (EGF; Life Technologies). CA1a cells were cultured in DMEM/F-12 (1:1) supplemented with 5% horse serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.

DIM was kindly provided by Michael Zeligs (Bio Response, Boulder, CO) and was dissolved in DMSO to make a 10 mmol/L stock solution and was added directly to the culture medium at different concentrations. Results of several studies have indicated that DIM exhibits promising cancer protective activities, especially against mammary neoplasia (14, 18, 19, 26). Therefore, based on these previous studies, we have chosen different concentrations of DIM for this study, which is relevant and achievable *in vivo*. Wherever indicated, the PI3-K inhibitors LY294002 and wortmannin (Sigma), which inhibits Akt kinase activity, were dissolved in DMSO and added to the culture medium at a final concentration of 25 and 1 μmol/L, respectively. Wherever indicated, EGF (Invitrogen, Carlsbad, CA) was also added to the medium at a final concentration of 100 ng/mL. Control cultures received the same concentration of DMSO, similar to those used for the experimental cultures.

Cell Growth Inhibition Studies by 3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide Assay. The MCF10AneoT and CA1a cells were seeded at a density of 1×10^3 per well in 96-well culture dishes. After 24 hours, the cells were treated with 15, 30, 60, and 100 μmol/L DIM or DMSO as vehicle control. Cells treated with DIM or DMSO for 1 to 3 days were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/mL, Sigma) at 37°C for 4 hours and then with DMSO at room temperature for 1 hour. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan, Durham, NC) at 495 nm.

DNA Ladder Analysis for Detecting Apoptosis. Cellular cytoplasmic DNA from MCF10AneoT and CA1a cells treated with 30, 60, or 100 μmol/L DIM or DMSO (vehicle control) for 24, 48, or 72 hours was extracted using 10 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, and 0.2% Triton X-100. These concentrations have been extensively used by other investigators for many *in vitro* studies, and these concentrations could be achievable *in vivo* (13, 14, 18, 19, 26, 27). The lysate was centrifuged for 15 minutes at 13,000 × *g* to separate the fragmented DNA (soluble) from intact chromatin (nuclear pellet). The supernatant from the lysate was treated with RNase followed by SDS-proteinase K digestion, phenol-chloroform extraction, and isopropanol precipitation. DNA was separated through a 1.5% agarose gel. After electrophoresis, gels were stained with ethidium bromide and the DNA was visualized under UV light and photographed.

Histone/DNA ELISA for Detecting Apoptosis. The cell apoptosis ELISA detection kit (Roche, Palo Alto, CA) was used to detect apoptosis in breast cancer cells treated with DIM according to manufacturer's protocol. Briefly, the cytoplasmic histone/DNA fragments from MCF10AneoT and CA1a cells treated with 60 μmol/L DIM or DMSO (vehicle control) for 24, 48, or 72 hours were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader at 405 nm.

ELISA Assay for Detection of PI3-K Activity. The ELISA detection kit (Echelon Bioscience, Salt Lake City, UT) was used to detect PI3-K activity in breast cancer cells treated with DIM according to manufacturer's protocol. Briefly, the assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3,4,5)P_3 produced. After PI3-K reactions are complete, reaction products are first mixed and incubated with a PI(3,4,5)P_3 detector protein and then added to the PI(3,4,5)P_3-coated microplate for competitive binding. A peroxidase-linked detection reagent and colorimetric detection is used to detect PI(3,4,5)P_3 detector protein binding to the plate. The colorimetric signal is inversely proportional to the amount of PI(3,4,5)P_3 produced by PI3-K activity.

Western Blot Analysis. The MCF10AneoT and CA1a cells were plated on culture dishes and allowed to attach for 24 hours followed by the addition of 60 or 100 μmol/L DIM and incubated for 24, 48, and 72 hours. Control cells were incubated in the medium with DMSO for similar times. After incubation, the cells were lysed in 62.5 mmol/L Tris-HCl and 2% SDS. Protein concentration was then measured using BCA protein assay (Pierce, Rockford, IL). Cell extracts were subjected to 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Membranes were incubated with the following monoclonal antibodies: NF-κB p65 (1:2,000, Chemicon, Temecula, CA), anti-phospho-Akt Ser^{473} (1:1,000, Cell Signaling, Beverly, MA), IKKβ and IκBα (1:100 and 1:500, respectively, Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (1:5,000, Sigma). The membranes were washed with TTBS and incubated with secondary antibodies conjugated with peroxidase. The signal was then detected using chemiluminescence detection system (Pierce).

Immunoprecipitation and Akt Kinase Assay. The Akt kinase activity of CA1a cells treated with DIM, EGF, and DIM followed by EGF and LY294002 followed by EGF, wortmannin, or DMSO was measured using Akt kinase assay kit (Cell Signaling) according to manufacturer's protocol with modification and the method described by our laboratory previously (8).

NF-κB DNA Binding Activity Measurement. MCF10AneoT and CA1a cells were plated at a density of 1×10^6 cells in 100-mm dishes and cultured for 24 hours. Subsequently, the cultures were treated with 30 and 60 μmol/L DIM or DMSO for 24 hours. Following treatment, cells were resuspended in 10 mmol/L Tris-HCl (pH 7.5)/5 mmol/L $MgCl_2$/0.05% (v/v) Triton X-100 and lysed with Dounce homogenizer. The homogenate was centrifuged at 3,000 × *g* for 15 minutes at 4°C. The nuclear pellet was resuspended in an equal volume of 10 mmol/L Tris-HCl (pH 7.4)/5 mmol/L $MgCl_2$ followed by the addition of one nuclei pellet volume of 1 mol/L NaCl/10 mmol/L Tris-HCl (pH 7.4)/4 mmol/L $MgCl_2$. The lysing nuclei were left on ice for 30 minutes before centrifugation at 10,000 × *g* for 15 minutes at 4°C. The supernatant (nuclear extract) was removed and protein concentration was measured by using BCA protein assay. Nuclear protein (10 μg) was subjected to electrophoretic mobility shift assay as described earlier (8).

Densitometric and Statistical Analysis. Autoradiograms of the Western blots for Akt phospho-Akt Ser^{473} and phospho-GSK-3α/β, β-actin protein expression, and NF-κB electrophoretic mobility shift assay were scanned with Gel Doc 1000 image scanner (Bio-Rad, Hercules, CA). The bidimensional absorbance were quantified and analyzed using Molecular Analyst software (Bio-Rad). The ratios of Akt, phospho-Akt Ser^{473}, or phospho-GSK-3 against β-actin were calculated. A comparative value of $P < 0.05$ was considered statistically significant.

Fluorescence Staining for Confocal Imaging. Cells (5×10^4) were plated on coverslips in each well of a six-well plate. The cells were treated with 30, 60, or 100 μmol/L DIM for 6, 12, 24, 48, and 72 hours with or without 20 ng/mL tumor necrosis factor-α (TNF-α) for 10 minutes. For staurosporine treatment, the medium was supplemented with 1 to 3 μmol/L staurosporine and cells were incubated for 24 hours and used as positive control for induction of apoptosis. Cells were fixed in ice-cold 100% methanol for 10 minutes and left at 4°C until the day of staining. Cells were incubated in PBS-0.1% saponin solution containing 1 μg/mL NF-κB p65 antibody (Chemicon) for 2 hours and the cells were stained by the methods described by our laboratory previously (6, 7). Excitation wavelength/detection filter settings were as follows: 585/665 nm long pass and Alexa Flour 488, 495/519 nm for NF-κB p65 visualization. Laser time and irradiation time were minimized to avoid photobleaching and possible photodynamic effects (28). Cells were visualized in dual channel imaging where NF-κB p65 staining was used to compensate for effects of one channel on another.

Reporter Gene Constructs and Transfection. A study has suggested that overexpression of IKKβ consistently leads to greater activation of the NF-κB reporter gene than IKKα at equivalent expression levels (29). CA1a cells were transiently cotransfected with IKK constitutive expression construct (IKKβ wild-type/mutant) provided by Tularik, Inc. (South San Francisco, CA) at 50% confluence using the ExGen 500 (Fermentas, Hanover, MD). The transfected cells were treated with DIM for 37 hours. The samples were then subjected to NF-κB DNA binding activity measurement using method as described earlier.

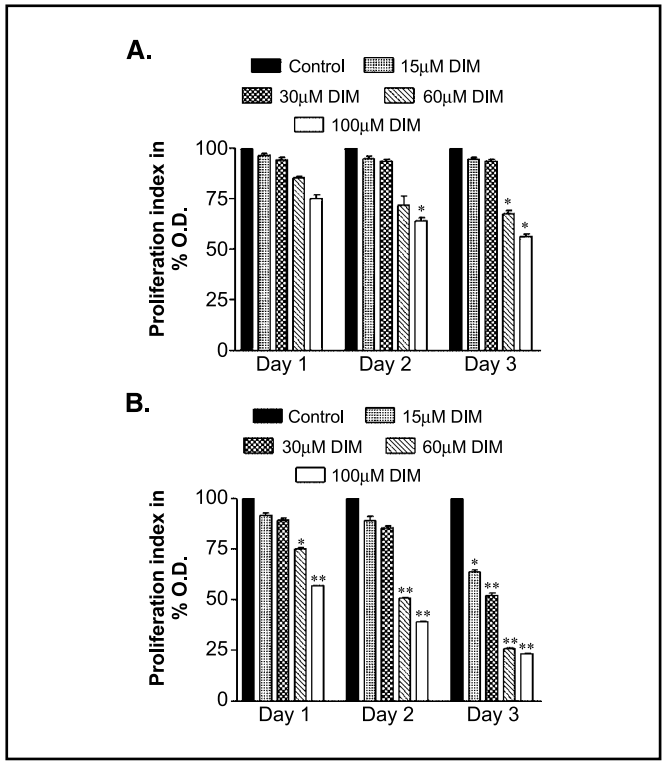

Figure 1. Inhibitory effect of DIM on the growth of MCF10AneoT (*A*) and CA1a (*B*) cells tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *Control,* cells treated with DMSO; *days 1*, *2*, and *3,* cells treated with 15, 30, 60, or 100 μmol/L DIM for 24, 48, or 72 hours, respectively. *, $P < 0.05$; **, $P < 0.01$.

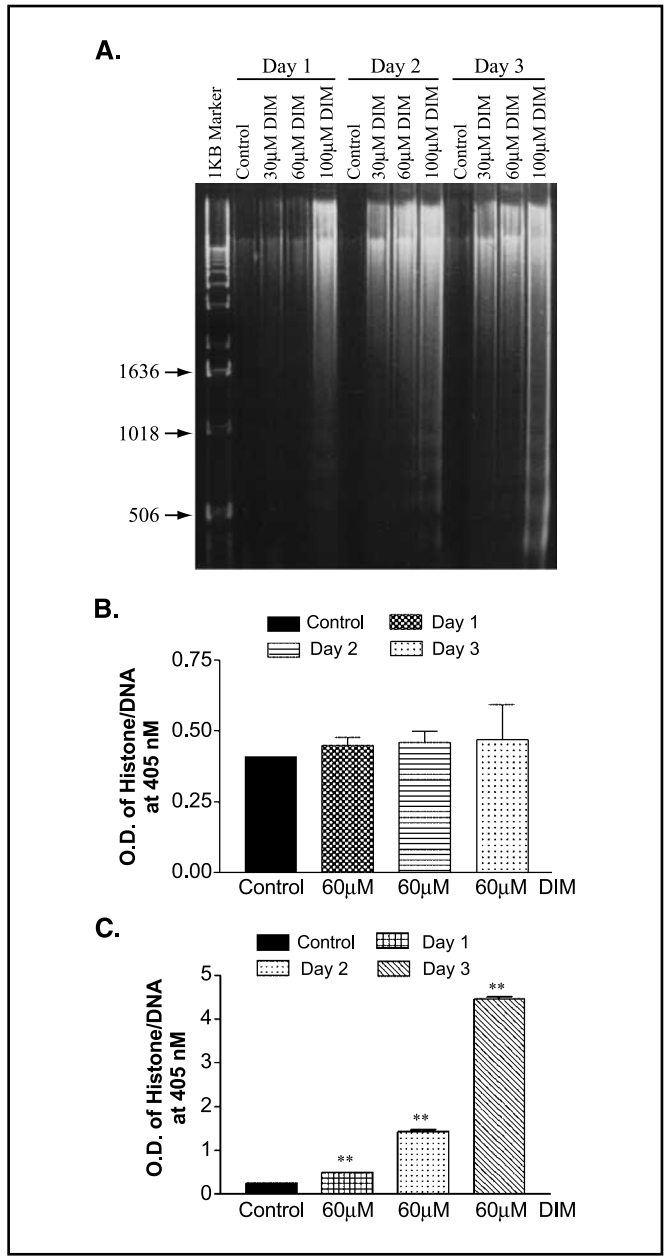

Figure 2. DIM-induced apoptosis in breast cancer cells. *Control,* CA1a cells treated with DMSO; *days 1*, *2*, and *3,* cells treated with 15, 30, 60, or 100 μmol/L DIM for 24, 48, or 72 hours, respectively, as illustrated by the DNA ladder (*A*). Histone/DNA fragment analysis by ELISA in nontumorigenic MCF10ANeoT (*B*) and tumorigenic CA1a (*C*) breast epithelial cells treated with 60 μmol/L DIM for 24 to 72 hours. **, $P < 0.01$.

Results

Effects of DIM on Cell Growth. The effect of DIM on cell growth of MCF10AneoT (nontumorigenic breast epithelial cell) and CA1a (breast cancer cells) is depicted in Fig. 1. The treatment of MCF10AneoT (Fig. 1*A*) and CA1a (Fig. 1*B*) cells incubated with 15, 30, 60, and 100 μmol/L DIM for 24 to 72 hours resulted in

inhibition of cell proliferation, which was dose dependent. However, there seems to be more pronounced growth inhibition by low concentration of DIM in CA1a cells compared with MCF10AneoT cells (Fig. 1), and these results are consistent with our previous observation with I3C. This inhibition of cell proliferation, however, could be due to cell cycle arrest, resulting in the inhibition of cell growth. Alternatively, the inhibition of cell growth could be attributed to the induction of apoptotic cell death induced by DIM in CA1a breast cancer cells. We therefore investigated whether DIM could induce apoptosis in these cells.

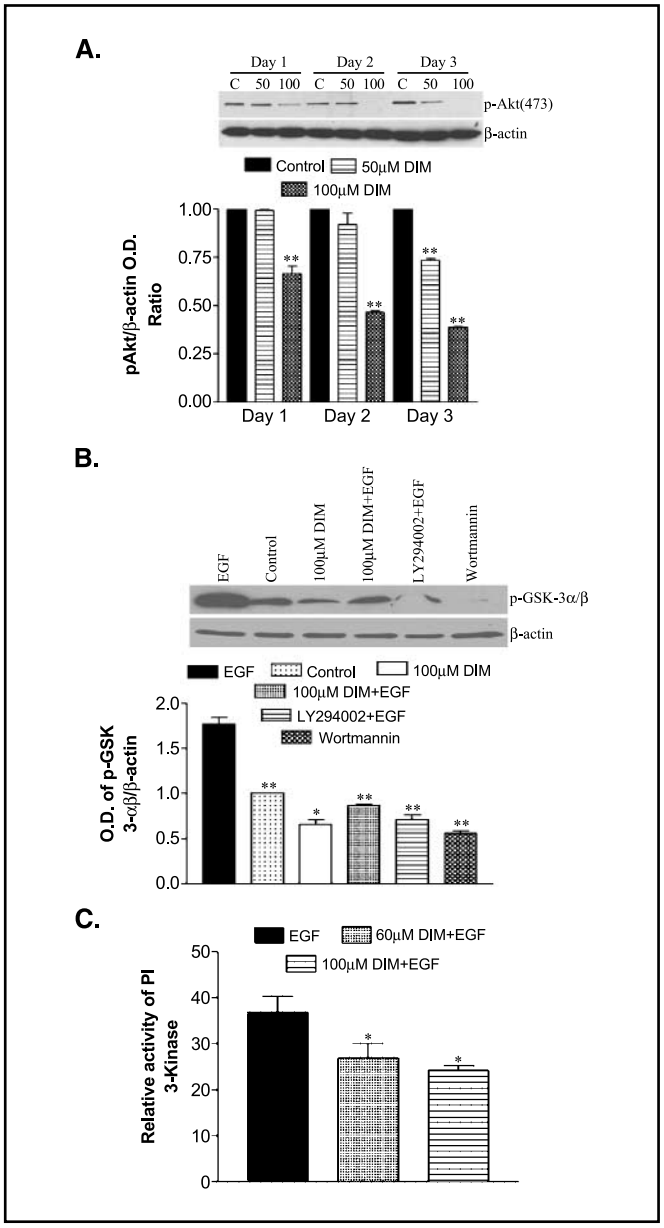

Figure 3. Inhibition in the phosphorylated Akt (*pAkt*) in DIM-treated tumorigenic CA1a breast epithelial cells. *A, Control,* CA1a cells treated with DMSO; *days 1*, *2*, and *3,* cells treated with 50 or 100 μmol/L DIM for 24, 48, or 72 hours, respectively, as illustrated by the Western blot and densitometric analysis. *B,* immunoprecipitation, kinase assay, Western blot, and densitometric analysis of Akt kinase activity in CA1a cells treated with DIM. Immunoprecipitation was accomplished with Akt antibody; subsequently, Akt kinase assay was done using GSK-3 protein as kinase substrate, and the phosphorylation of GSK-3 was detected by Western blot analysis with phospho-GSK-3α/β antibody. *B, lanes 1* to *6,* EGF 100 ng/mL, control, 100 μmol/L DIM treatment for 48 hours, EGF treatment of DIM-pretreated cells, and PI3-K inhibitors LY294002 and wortmannin, respectively. EGF and LY294002 served as positive and negative controls for Akt phosphorylation. *C,* DIM inhibits PI3-K activity in breast cancer cells. ELISA kit was used for detection of EGF-induced PI3-K activity in DIM-treated CA1a breast cancer cells. Cells were treated with EGF and 60 or 100 μmol/L DIM with 100 ng/mL EGF for 48 hours. PI3-K activity was measured as described in Materials and Methods. *, $P < 0.05$; **, $P < 0.01$.

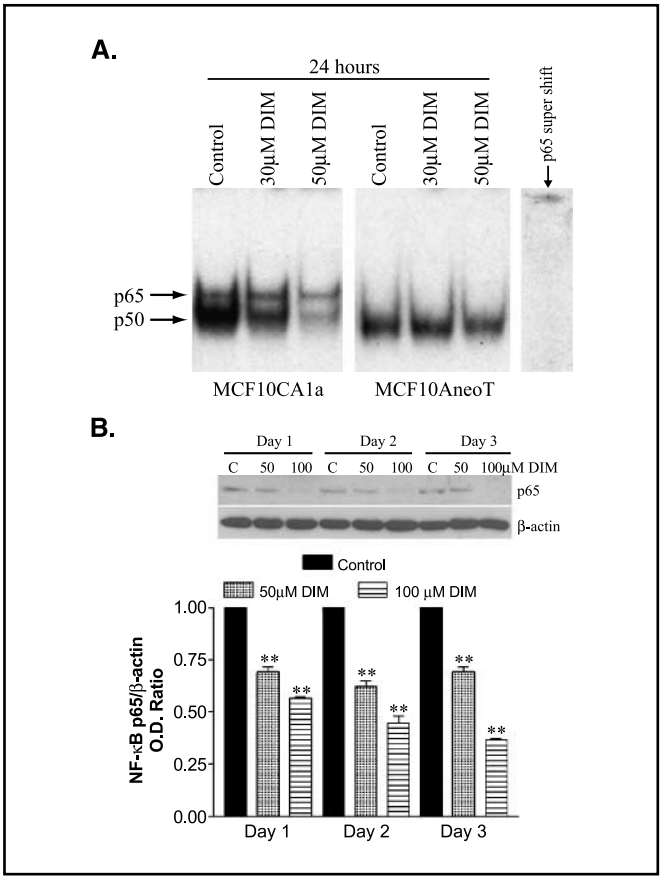

Figure 4. *A,* DIM abrogates NF-κB DNA binding activity in MCF10AneoT and CA1a breast epithelial cells. Cells were treated with 30 or 50 μmol/L DIM for 24 hours. Nuclear extracts were prepared from control and DIM-treated breast epithelial cells and subjected to analysis for NF-κB DNA binding activity as measured by electrophoretic mobility shift assay. Specificity of NF-κB DNA binding activity was confirmed by supershift assay. Slowest and faster migration of NF-κB DNA protein complex represent the p65/p50 and p50/p50 complexes, respectively. *B,* DIM inhibits NF-κB p65 protein expression in total cell lysate. *Control,* cells treated with DMSO; *days 1*, *2*, and *3,* cells treated with 50 or 100 μmol/L DIM for 24, 48, or 72 hours, respectively. Whole cell lysates were prepared and proteins were subjected to Western blot and densitometric analysis. **, $P < 0.01$.

Induction of Apoptosis by DIM in Breast Epithelial Cells. By two independent measurements of apoptotic assays, we observed induction of apoptosis in breast cancer cells treated with 60 or 100 μmol/L DIM as illustrated by the DNA ladder (Fig. 2*A*) and by the ELISA analysis of cytoplasmic histone/DNA fragments (Fig. 2*B* and *C*). The induction of apoptosis was time dependent and was directly correlated with the inhibition of cell growth. These two independent methods for the measurement of apoptosis provided similar results, suggesting that DIM unequivocally induced apoptosis in breast cancer cells. However, apoptosis was not observed in nontumorigenic MCF10AneoT breast epithelial cells as measured by ELISA. To further understand the molecular mechanisms of DIM-induced apoptosis in breast cancer cells, gene

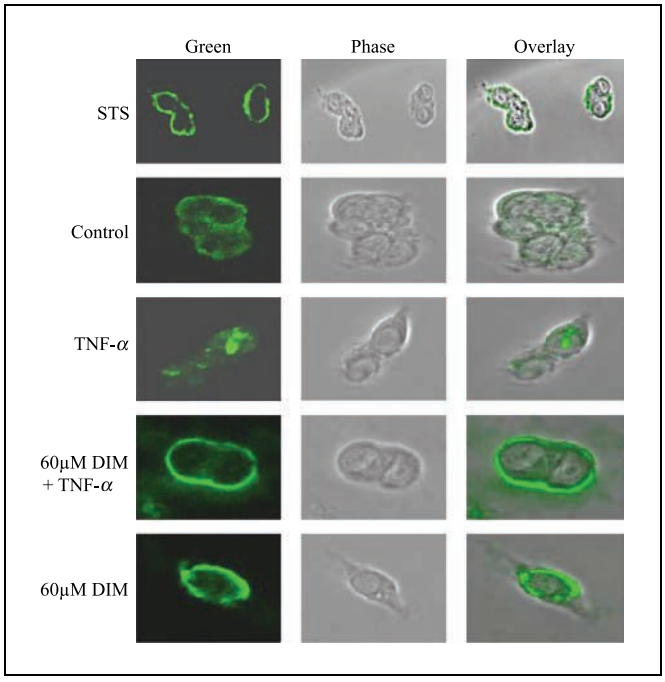

Figure 5. Nuclear translocation of NF-κB (p65) in CA1a cells. After treating the cells with DIM ± TNF-α, they were examined by confocal microscopy. p65 had a diffuse pattern in untreated (*Control*) and punctate pattern in staurosporine (*STS*)–treated cells. Treatment of CA1a cells with 60 μmol/L DIM for 24 hours and then stimulated with NF-κB-inducing agent (TNF-α) blocked the translocation of p65 subunit to the nucleus.

expression levels of the cell survival pathway related genes were investigated.

Inhibition of Akt Kinase Activity. Because Akt signaling pathway is an important signal transduction pathway that plays critical roles in cell survival and apoptotic processes, we investigated the status of Akt in breast epithelial cells treated with 100 μmol/L DIM by immunoprecipitation, Western blot, and kinase assays. We did not find any alterations in the total protein expression of Akt in DIM-treated breast cancer cells (data not shown). However, a significant decrease in the phospho-Akt Ser^{473} was observed in DIM-treated CA1a breast cancer cells compared with nontumorigenic MCF10AneoT cells, suggesting inactivation of Akt kinase after DIM treatment (Fig. 3*A*). These results were further confirmed by Akt immunoprecipitation and kinase assays, which showed a decrease in the Akt kinase activity in DIM-treated CA1a breast cancer cells (Fig. 3*B*). However, no significant alteration in the phospho-Akt Ser^{473} expression was found in MCF10AneoT cells treated with DIM (data not shown), suggesting that the inactivation of Akt is specific to cancer cells and not for nontumorigenic breast epithelial cells.

Furthermore, we investigated the Akt kinase activity in the CA1a breast cancer cells pretreated with DIM followed by EGF stimulation. We found that EGF treatment alone activated Akt kinase activity as expected, whereas DIM pretreatment abrogated the EGF-induced activation of Akt (Fig. 3*B*). LY294002 and wortmannin have been shown to act as highly selective inhibitors of PI3-K and block PI3-K-dependent Akt phosphorylation and its kinase activity (30, 31). Here, LY294002 and wortmannin were used as controls to confirm the similarity between the inhibitory effects of DIM and these inhibitors on Akt kinase activity. The pretreatment with LY294002 and wortmannin followed by EGF stimulation in breast cancer cells also showed inhibition of Akt kinase activity, suggesting that DIM-induced inhibition of Akt kinase activity is mediated through the inhibition of PI3-K. However, for further in-depth investigation, we studied the role of PI3-K in mediating the effect of DIM. Our results indicate that DIM inhibits PI3-K activity in breast cancer cells (Fig. 3*C*), which is important in pathways governing cell proliferation, differentiation, apoptosis, and migration. To further elucidate the molecular mechanism(s) by which DIM elicits its effects on breast cancer cells, we focused our investigation on the inhibition of NF-κB activation, which functions as a transcription factor and is known to play important roles in the regulation of apoptotic processes (22, 32). Here, we investigated whether NF-κB signaling pathway is involved in apoptotic processes induced by DIM. To explore such mechanisms, we measured the DNA binding activity of NF-κB in breast cancer cells treated with DIM.

Inhibition of NF-κB Activation by DIM. Nuclear extracts from control and DIM-treated MCF10AneoT and CA1a breast epithelial cells were subjected to analysis for NF-κB DNA binding activity as measured by electrophoretic mobility shift assay. Autoradiography revealed that 30 or 50 μmol/L DIM significantly inhibited NF-κB DNA binding activity in CA1a cells compared with the untreated cells (Fig. 4*A*). No significant inhibition of NF-κB DNA binding activity was found in DIM-treated MCF10AneoT cells (Fig. 4*A*). The specificity of NF-κB DNA binding activity was confirmed by supershift assays. Noncompeting oligonucleotide, such as AP-1 and SP-1 DNA binding sequences, did not replace the specific binding (data not shown). These results indicate that DIM inhibits NF-κB DNA binding activity in breast cancer cells, which confirms previous results in breast cancer cells (24, 33, 34).

To determine whether the reduction of NF-κB DNA binding by DIM was due to decreased protein translation, we investigated whether DIM could affect the protein expression levels of the p65 subunit by Western blot analysis in total and cytosolic fraction. The expression of p65 was not changed in the cytosolic fraction (data not shown), but DIM pretreatment affected protein expression levels of the p65 subunit in the total cell lysate (Fig. 4*B*). This result was further confirmed by densitometric 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 activity by DIM could also be due to localization of the NF-κB heterodimer between cytoplasmic (inactive) and nuclear (active) compartments.

DIM Blocks Nuclear Translocation of NF-κB. Under nonstimulating conditions, NF-κB exists in the cytoplasm as a trimer made up primarily of the p50 and p65 subunits of NF-κB and IκBα inhibitory protein (35, 36). 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 (29, 37). Using antibodies to NF-κB p65 subunit, we were able to visualize by confocal microscopy the translocation of NF-κB to the nucleus after TNF-α stimulation, a known inducer of NF-κB activity. In contrast, when cells were pretreated for 24 hours with 60 μmol/L DIM and then stimulated with NF-κB-inducing agent, the translocation of p65 subunit to the nucleus was abrogated (Fig. 5).

DIM Inhibits Activation of IKKβ. An IκBα kinase, IKK (IKKα and IKKβ), has been identified to phosphorylate inhibitory proteins of NF-κB complex and exist in the cytoplasm in an inactive form. IKKβ seems to be critical for NF-κB activation in response to TNF-α (37). In the present study, IKKβ activation was

down-regulated after 48-hour treatment with DIM (Fig. 6*A*) in CA1a cells compared with control. Moreover, treatment of cells with TNF-α, which activates IKKβ, was significantly down-regulated by 60 μmol/L DIM (Fig. 6*B*). These results suggest that the functional IKK complex, which is important for IκB phosphorylation, could be efficiently inactivated by DIM.

DIM Inhibits Phosphorylation of IκBα. We next investigated whether DIM blocks phosphorylation of the inhibitory protein IκB. In most cell types, NF-κB is sequestered by its interaction with IκB proteins in the cytoplasm and is consequently inactive. IκB binding to the Rel homology domain of NF-κB blocks the nuclear localization signal of NF-κB. On cytokine stimulation, IκB is phosphorylated and subsequently ubiquitinated and degraded, releasing NF-κB (29, 37, 38). Therefore, to detect phosphorylated and unphosphorylated forms of IκBα in our system, we treated CA1a cells with or without DIM. Using antibodies that recognize the phosphorylated and unphosphorylated forms of IκBα, we have found that DIM treatment inhibits the phosphorylation form of IκBα (Fig. 6*B*). In DMSO-treated control, phosphorylated IκBα was observed. However, DIM 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 preventing translocation to the nucleus. To further explore the inhibitory effects of DIM on NF-κB pathways, we conducted transfection experiments with IKK constructs, which was expected to further confirm the mechanism(s) of IκB phosphorylation mediated by IKKβ, ultimately allowing nuclear translocation of NF-κB (39).

IκBα Phosphorylation Is Mediated by IKKβ. NF-κB is normally retained in the cytoplasm by its natural inhibitor, IκB (40, 41); Upstream, a signaling complex consisting of two IKKs, IKKα and IKKβ, regulates IκB activity by phosphorylation of IκB (40, 41). Therefore, NF-κB could be activated through IKK pathways. In this present study, the NF-κB activity in the CA1a cells transfected with IKK constitutive expression construct (IKKβ wild-type) was increased through phosphorylation of IκB, whereas the NF-κB activity in the cells transfected with mutant IKK construct (IKKβ mutant) was decreased because of the competitive binding of kinase-dead IKK to IκB. Fig. 6*C* shows pronounced activation of NF-κB in IKK-overexpressing CA1a cells; consequently, NF-κB was down-regulated by DIM treatment in the same cells transfected with IKK, suggesting that IKKβ expression is required for NF-κB activation. As we have shown previously, Akt gene transfection leads to NF-κB activation, and our present data clearly indicate that Akt and IKK direct IκB degradation, which allows NF-κB translocation to the nucleus, and this process is abrogated in DIM-treated breast cancer cells.

Discussion

The present investigation shows that the inhibition of cell growth by DIM is a time- and dose-dependent phenomenon in nontumorigenic (MCF10AneoT) and tumorigenic (CA1a) breast epithelial cells. The cell growth of CA1a cells was significantly inhibited by 60 or 100 μmol/L DIM within 48 to 72 hours, and these results are consistent with our previous findings using I3C (6, 7). We also observed similar effect of DIM on G_1 cell cycle arrest as shown by other investigators (15). In the present study, we not only confirmed the previous observations but also showed that DIM selectively induces apoptosis in breast cancer cells as shown by DNA fragmentation analysis and ELISA compared with nontumorigenic cells. In addition, our results provide mechanistic information, for the first time, how DIM exerts its proapoptotic effects on breast cancer cells (i.e., by inhibiting PI3-K activity, phosphorylation of Akt and NF-κB DNA binding activity). Inhibition of NF-κB DNA binding activity is partly mediated by blocking the phosphorylation of the NF-κB inhibitory protein IκBα and by preventing nuclear translocation of the NF-κB complex. Moreover, DIM significantly inhibited TNF-α-induced translocation of NF-κB to the nucleus. These results suggest that DIM down-regulates NF-κB function and promotes apoptotic signaling while protecting cells from DNA-damaging agents, such as TNF-α,

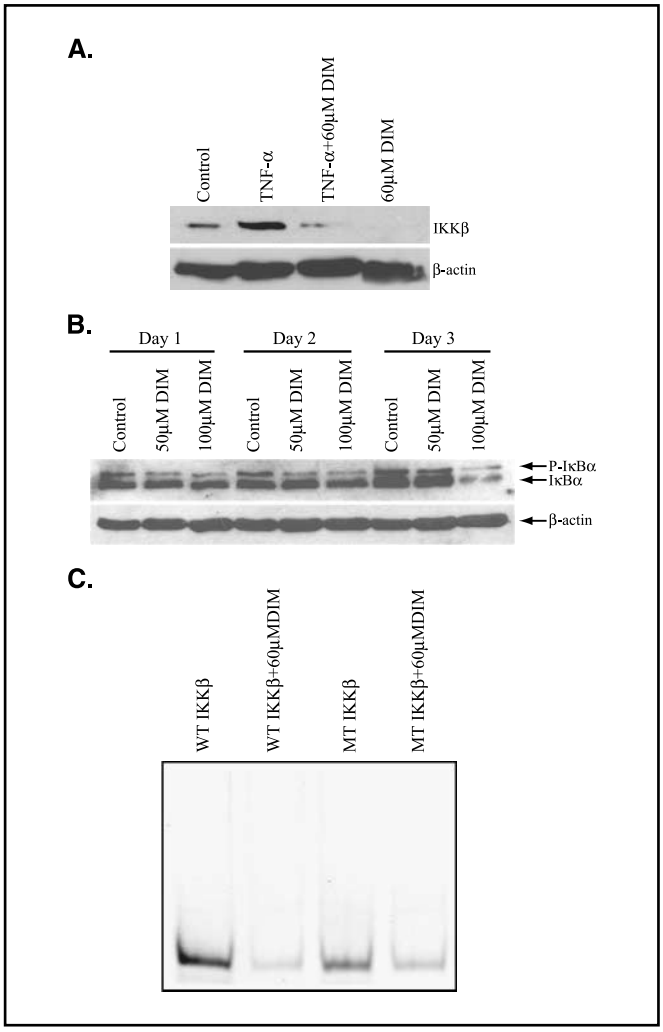

Figure 6. *A,* DIM inhibits activation of IKKβ in CA1a breast cancer cells. Cells were pretreated with 60 μmol/L DIM for 48 hours. Samples were then treated with 20 ng/mL TNF-α. Cytoplasmic extracts were prepared and assayed for IKKβ protein by Western blot analysis as described in Materials and Methods. *B,* DIM inhibits phosphorylation of IκBα in CA1a breast cancer cells. *Control,* cells treated with DMSO; *days 1*, *2*, and *3,* cells treated with 50 or 100 μmol/L DIM for 24, 48, or 72 hours, respectively. Cytoplasmic extracts were prepared and assayed for IκBα by Western blot analysis using a rabbit polyclonal antibody raised against a peptide mapping at the COOH terminus of the IκBα of human origin. *Top,* phosphorylation of IκBα (*P-IκBα*); *bottom,* unphosphorylated form of IκBα. *C,* NF-κB DNA binding activity in CA1a cells transfected with IKKβ. Cells were transfected with IKK constitutive expression construct (IKKβ wild-type and mutant) and treated with 60 μmol/L DIM for 48 hours. Nuclear extracts were prepared from transfected and DIM-treated breast epithelial cells and were subjected to analysis for NF-κB DNA binding activity as measured by electrophoretic mobility shift assay.

suggesting the potential benefit of DIM as an antioxidant as well as a powerful chemopreventive agent.

PI3-K/Akt is an important cell signaling pathway, which is critically needed for the regulation of cell growth, survival, and apoptosis (42). Akt is overexpressed as well as activated in numerous human malignancies (43). However, the role of Akt overexpression in the development of cancer is not fully understood. In our study, the expression of phospho-Akt Ser^{473} was down-regulated by DIM in CA1a cells. These results were further confirmed by Akt immunoprecipitation and kinase assays, which showed a decrease in the phosphorylation of GSK-3α/β by the down-regulation of phospho-Akt Ser^{473}. DIM also abrogated EGF-stimulated activation of Akt kinase as shown by inactivation of GSK-3α/β phosphorylation in CA1a cells. We also observed that DIM exerts an inhibitory effect on Akt kinase activity similar to those by LY294002 and wortmannin, suggesting that DIM serves as an inhibitor of PI3-K and Akt kinase. These results were further confirmed by ELISA assay for detection of PI3-K activity, which showed down-regulation of EGF-stimulated PI3-K activity in DIM-treated breast cancer cells. This may be one of the mechanisms by which DIM induces apoptosis in breast cancer cells. However, studies by other investigators have shown that Akt may target multiple components of the apoptotic cascade such as caspases, GSK-3, ceramide, and NF-κB (8). Thus, DIM may induce apoptosis by regulating multiple molecules in the Akt and NF-κB pathway.

Akt has been shown to activate NF-κB by phosphorylation of IKK at regulatory site Thr^{23} and subsequent phosphorylation and degradation of IκB (44). We have recently shown that Akt is constitutively activated in human prostate and breast cancer cells and may potentiate cell survival through NF-κB activation, thereby playing a key role in cancer development (8). In addition, we have provided suggestive evidence that Akt directly activates NF-κB, and this activation was completely abrogated by genistein and I3C treatments (8, 45). However, little is known about the mechanism(s) by which DIM inactivates Akt and NF-κB signaling pathway in breast cancer cells. We found that DIM inhibits NF-κB DNA binding activity in breast cancer cells, which confirms previous results in breast cancer cells treated with I3C (8, 24). The reduction of NF-κB DNA binding activity by DIM was due to decreased protein translocation in addition to its effects on p65 protein expression. Moreover, our results also showed that DIM inhibits NF-κB DNA binding by blocking the phosphorylation of the inhibitory protein IκBα, thereby preventing the nuclear translocation of the NF-κB complex. These results are consistent with our previous findings (8). 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 (46). Therefore, the inactivation of NF-κB by DIM may be useful for the prevention and/or treatment of cancer, a mechanism similar to anti-inflammatory drugs, salicylates, and glucocorticoids, which are known inhibitors of NF-κB and routinely used as part of therapy for hematologic malignancies (47).

The IKK/IκBα/NF-κB pathway is the major mechanistic molecule for NF-κB activation. Activation of IKK depends on phosphorylation at the Ser^{177} and Ser^{181} in the activation loop of IKKβ (Ser^{176} and Ser^{181} in IKKα), which are the specific sites with phosphorylation that causes a conformational change that results in kinase activation (37). In the present study, our results suggest that the functional IKK complex could be efficiently inactivated by DIM. In addition, the IKK complex, containing the catalytic subunits IKKα and IKKβ, which directly phosphorylates IκB proteins, leads to the activation of NF-κB signaling. Unphosphorylated IκBα remains bound to the p50-p65 complex and prevents nuclear translocation, binding to the DNA consensus sequence, and transactivation of genes. IκBα is phosphorylated at its regulatory NH_2 terminus on Ser^{32} and Ser^{36} by IKK (37), which was inhibited by DIM as shown by our studies.

A recent study has suggested that overexpression of IKKβ consistently leads to greater activation of the NF-κB reporter gene compared with IKKα at equivalent expression levels (29). We found increased activation of NF-κB in IKK-overexpressing CA1a cells, and this activation was down-regulated by DIM treatment, suggesting that IKKβ expression is required for NF-κB activation. Our results show for the first time that the activity of IKK is inhibited by DIM. As we have shown previously, Akt gene

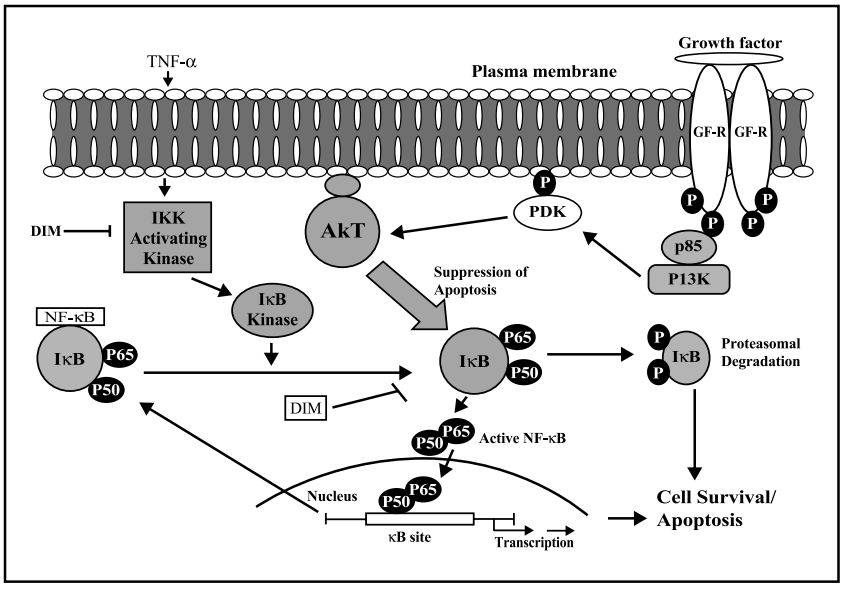

Figure 7. Schematic representation showing that Akt and IKK direct IκB degradation, which allows NF-κB translocation to the nucleus, and this process is abrogated in DIM-treated cancer cells.

transfection leads to NF-κB activation, and our present data clearly indicate that Akt and IKK direct IκB degradation, which allows NF-κB translocation to the nucleus and this process is abrogated in DIM-treated breast cancer cells (see our hypothetical schematics in Fig. 7). Recent studies also showed that I3C inhibits the phosphorylation of IκB (24), which could also be mediated through Akt signaling pathway, and these results are in direct agreement with our present results.

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