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Journal of Cellular Biochemistry 112:1643–1650 (2011)

Journal of Cellular Biochemistry

# Cucurbitacin B Suppresses the Transactivation Activity of RelA/p65

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## ABSTRACT

Cucurbitacin B, a natural triterpenoid is well-known for its strong anticancer activity, and recent studies showed that the compound inhibits JAK/STAT3 pathway. In this study, we demonstrate for the first time that cucurbitacin B is also a potent inhibitor of NF-κB activation. Our results showed that cucurbitacin B inhibited TNF-α-induced expression of NF-κB reporter gene and NF-κB target genes in a dose-dependent manner, however, it did not prevent either stimuli-induced degradation of IκBα or nuclear translocation and DNA-binding activity of NF-κB. On the other hand, cucurbitacin B dose-dependently suppressed not only NF-κB activation induced by overexpression of RelA/p65 but also transactivation activity of RelA/p65 subunit of NF-κB. Consistently, treatment of HeLa cells with the compound significantly suppressed TNF-α-induced activation of Akt and phosphorylation of Ser536 in RelA/p65, which is required for transactivation activity. Consequently, cucurbitacin B inhibited TNF-α-induced expression of NF-κB-dependent anti-apoptotic proteins such as c-IAP1, c-IAP2, XIAP, TRAF1, and TRAF2 and sensitized TNF-α-induced cell death. Taken together, our results demonstrated that cucurbitacin B could be served as a valuable candidate for the intervention of NF-κB-dependent pathological condition such as cancer. J. Cell. Biochem. 112: 1643–1650, 2011. 

**KEY WORDS:** CUCURBITACIN B; NF-κB; RELA/p65; AKT; TRANSACTIVATION; ANTI-APOPTOSIS

Nuclear factor-κB (NF-κB) is a family of dimeric transcription factor that regulates diverse physiological processes including immune responses, cell growth and suvival, and pathological processes associated with inflammation and cancer [Karin and Greten, 2005; Vallabhapurapu and Karin, 2009]. In mammalian cells, the NF-κB family is composed of five members that form homo- and hetero-dimeric complexes including RelA/p65, RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) [Ghosh and Karin, 2002]. RelA/p65 contains a *C*-terminal transactivation domain in addition to the *N*-terminal Rel-homology domain, thus serving as a critical transactivation subunit of NF-κB while p50 serves as a regulatory subunit modulating the DNA-binding affinity of RelA/p65 [Schmitz and Baeuerle, 1991; Ballard et al., 1992]. Under resting conditions, the p65-p50 NF-κB heterodimer is normally sequestered in the cytoplasmic compartment by physical association with inhibitory proteins, including IκBα and related proteins [Karin and Ben-Neriah, 2000]. The latent cytoplasmic NF-κB p65-p50 complex can be posttranslationally activated by a variety of cellular stimuli, which trigger site-specific phosphorylation of IκBα by a multi-

Abbreviations: NF-κB, nuclear factor kappa B; TNF-α, tumor necrosis factor-α; EMSA, electrophoretic mobility shift assay; IL-6, interleukin-6; IL-8, interleukin-8; MCP1, monocyte chemotactic protein 1; c-IAP1, cellular inhibitor of apoptosis 1; c-IAP2, cellular inhibitor of apoptosis 2; XIAP, X-linked inhibitor of apoptosis protein; TRAF1, TNF receptor-associated factor 1; TRAF2, TNF receptor-associated factor 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Korea Research Institute of Bioscience and Biotechnology Research Initiative Program; Grant number: PF0320701-00; Grant sponsor: Plant Diversity Research Center of 21st Frontier Research Program (Korean Ministry of Science and Technology)..

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Received 29 October 2010; Accepted 11 February 2011 • DOI 10.1002/jcb.23078 • © 2011 Wiley-Liss, Inc.
Published online 22 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

subunit IκB kinase. The phosphorylated IκBα becomes rapidly ubiquitinated and degraded by the proteasome complex [Ben-Neriah, 2002]. Following IκBα degradation, the p65-p50 NF-κB heterodimer is translocated to the nucleus and then subjected to further regulation of transactivation activity of RelA/p65 mainly through phosphorylation which is required for full induction of NF-κB target genes [Li and Stark, 2002].

The wide variety of genes regulated by NF-κB include those encoding cytokines (e.g., IL-1, IL-2, IL-6, IL-12, TNF-α), chemokines (e.g., IL-8, MIP-1α, MCP1, RANTES, and eotaxin), adhesion molecules (e.g., ICAM, VCAM, and E-selectin), and inducible effector enzymes (e.g., iNOS and COX-2) [Zhang and Ghosh, 2001; Aggarwal, 2004]. In addition to regulating the expression of genes important for immune and inflammatory responses, NF-κB also controls the transcription of genes that confer resistance to death-inducing signals. Candidate target genes include those encoding the cellular inhibitor of apoptosis c-IAP1, c-IAP2, X-linked inhibitor of apoptosis protein XIAP, the TNF receptor-associated factors TRAF1 and TRAF2, the zinc finger protein A20, the immediate-early response gene IEX-1L, and the pro-survival Bcl-2 homolog Bfl-1/A1 [Karin and Lin, 2002].

NF-κB is frequently activated in patients with chronic inflammatory conditions such as cancer and pulmonary, cardiovascular, autoimmune, skin, and neurodegenerative diseases [Sethi et al., 2008]. The ability of NF-κB to control multiple genes involved in human diseases makes NF-κB signaling pathway a novel target for therapy [Karin et al., 2004; Nagashima et al., 2006]. We recently identified cucurbitacin B as a NF-κB inhibitor from *Trichosanthes kirilowii* [Dat et al., 2010], which has significant anti-inflammatory activity and is used traditionally to treat liver disease, like hepatitis [Peters et al., 1997; Agil et al., 1999]. Recently, it has been reported that cucurbitacin B inhibits the growth of numerous human cancer cell lines and tumor xenografts [Liu et al., 2008; Wakimoto et al., 2008; Zhang et al., 2009]. Particular attention has been given to cucurbitacin B by its inhibition of JAK/STAT pathway [Thoennissen et al., 2009]. We here demonstrate that cucurbitacin B potently inhibits NF-κB activation via targeting transactivation activity of RelA/p65 subunit of NF-κB without affecting TNF-α-induced degradation of IκBα as well as nuclear translocation and DNA-binding activity of NF-κB. Cucurbitacin B significantly suppressed TNF-α-induced activation of Akt and phosphorylation of Ser536 in RelA/p65 subunit of NF-κB. Consequently, cucurbitacin B inhibits TNF-α-induced expression of anti-apoptotic NF-κB target genes such as c-IAP1, c-IAP2, XIAP, TRAF1, and TRAF2, thereby potentiating TNF-α-induced cell death.

## MATERIALS AND METHODS

### CELL CULTURE AND REAGENTS

HeLa cells were maintained in Dulbecco's Modified Essential Medium (Invitrogen, Carlsbad, CA) supplemented with penicillin (100 units/ml)–streptomycin (100 μg/ml; Invitrogen) and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). Cells were grown in an incubator at 37°C and 5% $CO_2$. TNF-α and recombinant human LIGHT were obtained from R&D Systems (Minneapolis, MN). LY294002 was obtained from Calbiochem-Novabiochem (San Diego, CA). Annexin V-FITC Apoptosis kit was obtained from Sigma (St. Louis, MO). Cucurbitacin B was isolated from *T. kirilowii* and its structure is shown in Figure 1A. The purity of cucurbitacin B was more than 98% in HPLC analysis [Dat et al., 2010].

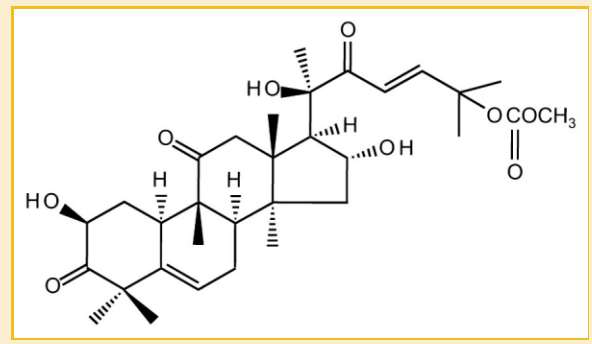


Fig. 1. Chemical structure of cucurbitacin B isolated from the *Trichosanthes kirilowii*.

### PLASMIDS, TRANSFECTIONS, AND LUCIFERASE ASSAY

A pNF-κB-Luc and an expression plasmid for RelA/p65 have been previously described [Lee et al., 2002]. A vector encoding a fusion protein between the DNA-binding domain of Gal4 and transactivation domains of RelA/p65, Gal4-RelA$^{268-551}$, was constructed by inserting cDNA for Gal4-RelA$^{268-551}$ into pFA-CMV (Stratagene, La Jolla, CA). A 5XGal4-luciferase reporter gene was obtained from Stratagene. Flag-Akt was kindly provided by Dr. Jeong-Hyung Lee (Kangwon National University, Korea). Transfections were performed using Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). Luciferase activity was measured using luciferase assay system according to the instructions of the manufacturer (Promega, Madison, WI). In brief, HeLa cells ($1 \times 10^5$ cells/well) were seeded in a 96-well plate for 24 h. The cells were then transfected with plasmids for each well and then incubated a transfection period of 24 h. After that, the cell culture medium was removed and replaced with fresh medium containing various concentrations of cucurbitacin B for 30 min, followed by treatment with 20 ng/ml of TNF-α for 18 h. Luciferase activity was determined in Microlumat Plus luminometer (EG&G Berthold, Germany) by injecting 100 μl of assay buffer containing luciferin and measuring light emission for 10 s. The results were normalized to the activity of *Renilla* expressed by cotransfected *Rluc* gene under the control of a constitutive promoter.

### WESTERN BLOT ANALYSIS

HeLa cells ($1 \times 10^7$ cells) were cultured in 10 cm-dishes and allowed to adhere for 24 h. The culture medium was removed and replaced with fresh medium containing cucurbitacin B at 100 nM for 12 h and then treatment with 20 ng/ml of TNF-α for indicated times. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride,

1 μg/ml leupeptin, 1 mM sodium vanadate, 150 mM NaCl). The cell lysates were incubated on ice for 1 h followed by a 30 min centrifugation at 15,000 rpm. The protein concentration of cell lysates were determined by the Bradford method standardized with BSA. Sample aliquots were separated on SDS-polyacrylamide gels and followed by transferring to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked with 5% non-fat skim milk in TTBS buffer (0.1% Tween-20 in Tris-buffer saline) to block non-specific binding, and then incubated with the corresponding antibody. Antibodies for IκBα, Akt, phospho-(Ser473)-specific Akt, p65, phospho-(Ser536)-specific p65, p52, c-IAP1, c-IAP2, and XIAP were purchased from Cell Signaling Technology (Beverly, MA). Antibody for TRAF1, p38, phospho-specific p38, and TopoI were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for TRAF2 was obtained from R&D system (Minneapolis, MN) and antibody for tubulin was obtained from Sigma. After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Biosciences, Buckinghamshire, UK).

### PREPARATION OF NUCLEAR EXTRACTS AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Electrophoretic mobility shift assay was performed as described previously [Lee et al., 2002]. HeLa cells ($3 \times 10^6$ cells) were cultured in 6-cm dishes and allowed to adhere for 24 h and then treatment with 100 nM cucurbitacin B for 12 h following stimulated with TNF-α (20 ng/ml) for the indicated times. The cell were then harvested and washed twice with ice-cold PBS, and then nuclear extracts were prepared using NE-PER reagent (PIERCE, Rockford, IL), according to the instructions of manufacture. Electrophoretic mobility shift assay was performed using gel shift assay system (Promega), according to the instructions of manufacturer. A double-stranded oligonucleotide for NF-κB (Promega) was end-labeled with [γ-$^{32}$P] ATP and purified with a G-25 spin column (Boehringer Mannheim, Mannheim, Germany). Approximately 20,000 cpm of probe were used per assay. Nuclear extracts (10 μg) were incubated for 20 min at room temperature with a gel shift-binding buffer [5% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 50 μg/ml poly(dI-dC)], and $^{32}$P-labeled oligonucleotide. The DNA–protein complex formed was separated on 4% native polyacrylamide gels. The gel was transferred to Whatman 3 mm paper, dried, and exposed to X-ray film at −70°C with an intensifying screen.

### REAL-TIME PCR

Real-time PCR was performed as described previously [Jin et al., 2010]. In brief, HeLa cells were preincubated with the indicated concentrations of cucurbitacin B at 37°C for 12 h. In following, HeLa cells were stimulated with TNF-α (20 ng/ml) for 1 h, harvested and washed twice with ice-cold PBS, and then total RNA was isolated from cells using RNeasy Mini kits according to the manufacturer's instructions (Qiagen, Valencia, CA). Complementary DNA was synthesized from 1 μg of total RNA in a 20 μl reverse transcription reaction mixture according to the manufacturer's protocol (TaKaRa Bio, Kyoto, Japan). The following primer pairs were used for real-time PCR amplification: human interleukin-6 (IL-6), 5′-GAACTCCTTCTCCACAAGCGCCTT-3′ and 5′-CAAAA-GACCAGTGATGATTTTCACC AGG-3′; human interleukin-8 (IL-8), 5′-TCTGCAGCTCTGTGTGAAGG-3′ and 5′-ACTTCTCCACAACCCTCTG-3′; human monocyte chemotactic protein 1 (MCP1), 5′-CCCCAGT-CACCTGCTGTTAT-3′ and 5′-AGATCTCCTTGGCCACAATG-3′; human EBI-1-ligand chemokine (CCL19), 5′-GCCTGCTG GTTCTCTGGAC-3′;5′-GGATGGGTT TCTGGGTCAC-3′; GAPDH, 5′-ACCAGGTGGTCTCCTCT-GAC-3′ and 5′-TGCTGTAGC CAAATTCGTTG-3′. GAPDH was used as the housekeeping gene control. Real-time PCR was performed with a SYBR green (BIO-RAD, Hercules, CA) with iQSYBR Green Supermix (BIO-RAD). The reactions were performed in 25 μl (total volume) mixtures containing primers at a concentration of 400 nM. The reaction conditions consisted of 10 min at 95°C and then 40 cycles of 15 s at 95°C, 15 s at 58°C, and 30 s at 72°C. Melting curve analysis was used to determine PCR specificity. All reactions were carried out triplicate for each sample. The standard curve method was used to determine the amount of each transcript. Relative induction was determined by normalizing the relative expression to the untreated control samples.

### DETERMINATION OF CELL VIABILITY

The cell viability was performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma–Aldrich). MTT was dissolved in PBS at 5 mg/ml as a stock solution and sterilized using 0.2 mm filter. HeLa cells were exposed to TNF-α and/or the indicated concentrations of cucurbitacin B in a 96-well plate for 48 h followed by the addition of MTT solution (0.5 mg/ml) to the cells, and then the solution was incubated at 37°C until blue deposits were visible. The colored metabolite was dissolved in dimethyl sulfoxide (DMSO). Optical densities were determined on a microplate reader (Molecular Devices, Sunnyvale, CA).

### DETECTION OF CELL APOPTOSIS

Detection of cell apoptosis was performed by Annexin V-FITC staining [Moon et al., 2010]. In brief, $0.25 \times 10^6$ HeLa cells seeded into each well of the six-well plate were treated with TNF-α and/or the 100 nM cucurbitacin B for 48 h. The treated cells were harvested by 0.25% trypsin, washed twice with binding buffer containing 10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, and then resuspended at a concentration of $1 \times 10^6$/ml in binding buffer. Cell suspension was mixed with 5 μl Annexin V/FITC incubated at room temperature for 15 min. The apoptotic cells were analyzed using FACScan (BD Bioscience).

### STATISTICAL ANALYSIS

All assays were carried out in triplicate with three independent experiments and results were expressed as the mean ± SDs. The 50% inhibitory concentration (IC50) of cucurbitacin B on expression of NF-κB reporter gene was obtained by linear regression analysis of concentration–response curve plotting between percentage of inhibition and sample concentration of three independent assays. Comparison data was analyzed by Student's *t*-test, and a *P*-value $< 0.01$ was considered statistically significant.

## RESULTS

### CUCURBITACIN B INHIBITS TNF-α-INDUCED NF-κB ACTIVATION

In an effort to identify natural NF-κB inhibitors, we identified cucurbitacin B (Fig. 1) from a traditional medicinal plant, *T. kirilowii* [Dat et al., 2010]. To investigate the effect of cucurbitacin B on the induced NF-κB activation by TNF-α, we performed NF-κB reporter assay. As shown in Figure 2A, cucurbitacin B dose-dependently inhibited the TNF-α-induced expression of NF-κB reporter gene construct with $IC_{50}$ value of 14.7 nM. To further confirm the NF-κB inhibitory activity of cucurbitacin B, we analyzed the effect of cucurbitacin B on expression of NF-κB target genes such as IL-6, IL-8, and MCP1 using quantitative real-time PCR. After preincubation with indicated concentrations of cucurbitacin B for 12 h, and subsequent stimulation with TNF-α (20 ng/ml) for 1 h, RNAs were isolated from cells, reverse-transcribed, and analyzed by real-time PCR. As shown in Figure 2B, the TNF-α-induced mRNA expression of IL-6, IL-8, or MCP1 was potently suppressed by cucurbitacin B in a dose-dependent manner.

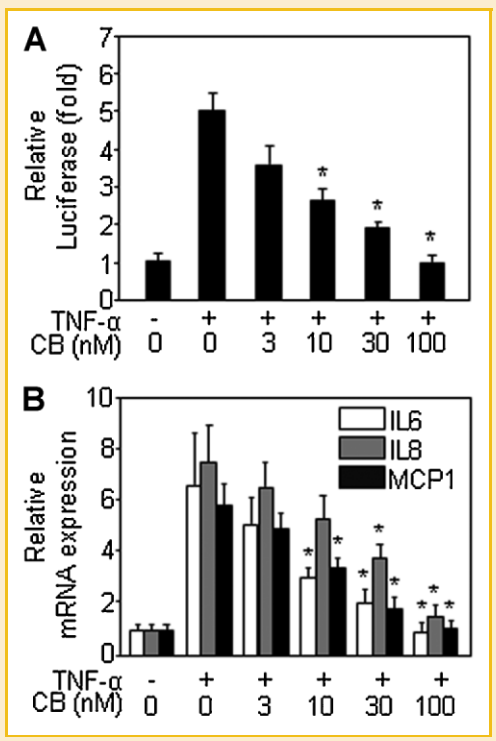


Fig. 2. Cucurbitacin B (CB) inhibits TNF-α-induced expression of NF-κB-dependent reporter gene and NF-κB target genes. A: HeLa cells, transiently transfected with a NF-κB-dependent reporter gene for 24 h, were pretreated with indicated concentrations of cucurbitacin B for 0.5 h followed by stimulation for 18 h with TNF-α (20 ng/ml). Luciferase activity was determined as described in Materials and Methods section; mean values from three independent experiments performed in triplicated are shown; bar indicate the SDs. Statistical significance ($^*P < 0.01$) compared with the control. B: HeLa cells pretreated with indicated concentrations of cucurbitacin B for 12 h were stimulated with TNF-α (20 ng/ml) for 1 h. RNAs were isolated from cells, reverse-transcribed, and analyzed by real-time PCR for IL-6, IL-8, and MCP1. Mean values from three independent experiments performed in triplicated are shown; bar indicate the SDs. Statistical significance ($^*P < 0.01$) compared with the control.

### CUCURBITACIN B DOES NOT INTERFERE WITH THE TNF-α-INDUCED IκBα DEGRADATION OR NUCLEAR TRANSLOCATION AND DNA-BINDING ACTIVITY OF NF-κB

Since degradation of IκBα protein is an essential step for NF-κB activation, we examined the effect of cucurbitacin B on the TNF-α-induced degradation of IκBα protein. HeLa cells were pretreated with 100 nM cucurbitacin B for 12 h, and subsequently stimulated with TNF-α (20 ng/ml) for indicated times. Cytoplasmic extracts were analyzed for the presence of IκBα with Western blot analysis. As shown in Figure 3A, IκBα was almost completely degraded in 30 min after stimulation with TNF-α and resynthesized in 1 h. Preincubation with cucurbitacin B, however, could not significantly prevent TNF-α-induced degradation and resynthesis of IκBα. Next, we measured the effect of cucurbitacin B on TNF-α-induced nuclear translocation and DNA-binding activity of NF-κB. HeLa cells stimulated with TNF-α (20 ng/ml) showed strong nuclear translocation and DNA-binding activity of NF-κB. However, pretreatment with cucurbitacin B did not inhibit nuclear translocation (Fig. 3B) and DNA-binding activity of NF-κB induced

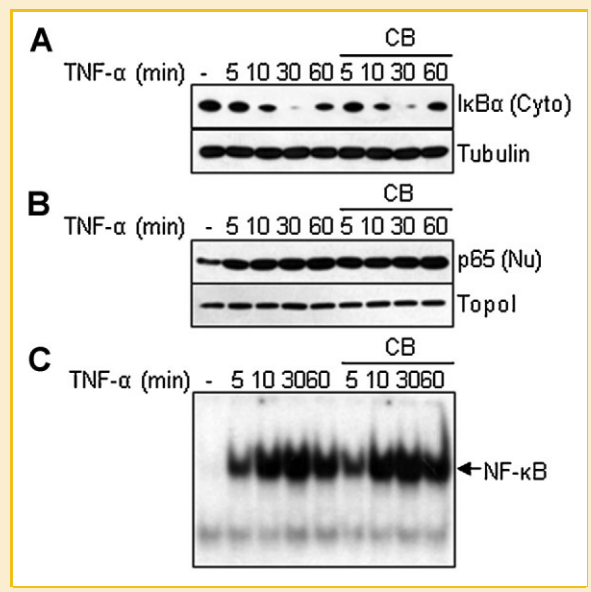


Fig. 3. Cucurbitacin B (CB) does not interfere with TNF-α-induced IκBα degradation or nuclear translocation and DNA-binding activity of NF-κB. A: HeLa cells were pretreated for 12 h with 100 nM cucurbitacin B and stimulated with TNF-α (20 ng/ml). Cells were harvested at the indicated time points and cytosol extracts were prepared. IκBα protein was detected by western blot analysis. B: HeLa cells were pretreated for 12 h with 100 nM cucurbitacin B and stimulated with TNF-α (20 ng/ml). Cells were harvested at the indicated time points and nuclear extracts were prepared. p65 protein was detected by Western blot analysis. C: HeLa cells were pretreated for 12 h with 100 nM cucurbitacin B and stimulated with TNF-α (20 ng/ml). Cells were harvested at the indicated time points and subsequently nuclear extracts were prepared and tested for DNA-binding of activated NF-κB by EMSA as described in Materials and Methods section.

by TNF-α (Fig. 3C). To know whether cucurbitacin B could inhibit activation of non-canonical pathway, we investigated the effect of cucurbitacin B on the LIGHT-induced p52 nuclear translocation and the expression of a non-canonical pathway target CCL19 mRNA [Jin et al., 2010]. Cucurbitacin B (100 nM), however, could not significantly inhibit LIGHT-induced p52 nuclear translocation and CCL19 mRNA expression (Supplementary Fig. 1A,B).

### CUCURBITACIN B INHIBITS TRANSACTIVATION ACTIVITY OF RELA/p65 SUBUNIT

To further investigate how cucurbitacin B prevents NF-κB activation, we examined the effect of cucurbitacin B on the induced NF-κB activity by overexpression of RelA/p65 subunit, which is a critical transactivation subunit of NF-κB [Schmitz and Baeuerle, 1991; Ballard et al., 1992]. HeLa cells were transiently transfected either with a NF-κB-dependent reporter gene alone or in combination with an expression vector encoding RelA/p65. As shown in Figure 4A, NF-κB-dependent transcription induced by overexpression of RelA/p65 was significantly affected by cucurbitacin B in a dose-dependent manner. This result suggested that cucurbitacin B could influence the transactivation activity of RelA/p65 subunit. To test this possibility, a plasmid encoding a fusion protein of the transactivation domains of RelA, RelA$^{268-551}$, with the DNA-binding domain of the yeast transcription factor Gal4, Gal4-RelA$^{268-551}$, were transfected into HeLa cells along with a luciferase reporter containing upstream Gal4-binding sites. Cucurbitacin B blocked transactivation activity of the transactivation domains of RelA/p65 in a dose-dependent manner (Fig. 4B). Taken together, these data suggested that cucurbitacin B regulates the activation of NF-κB by suppressing transactivation activity of RelA/p65 subunit.

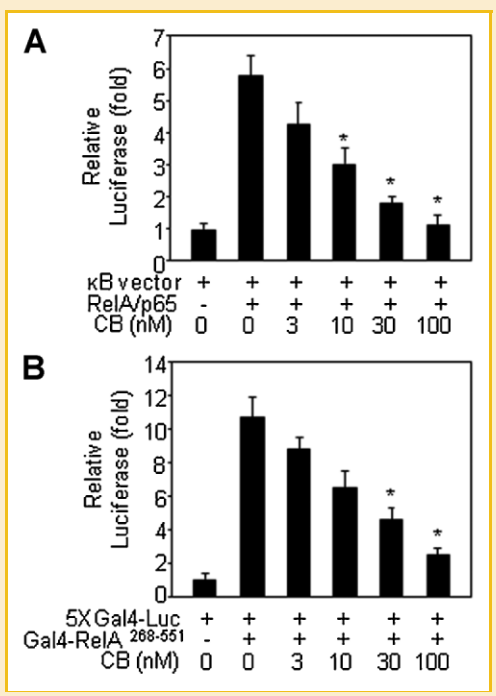


Fig. 4. Cucurbitacin B (CB) inhibits NF-κB activation induced by overexpression of RelA/p65 and transactivation activity of RelA/p65 subunit. A: HeLa cells were transiently transfected with a NF-κB-dependent reporter gene together with expression vector encoding RelA/p65. The co-transfected cells were subsequently grown for 24 h and for another 12 h in the presence of the indicated concentrations of cucurbitacin B, and the luciferase activity was determined as described in Materials and Methods section. B: HeLa cells were transiently transfected with a Gal4-luciferase reporter gene alone or in combination with a plasmid encoding a fusion protein between the DNA binding domain of Gal4 and transactivation domain of ReA, Gal4-RelA$^{268-551}$. After 24 h, the cells were incubated for another 12 h in the presence of the indicated concentrations of cucurbitacin B, and the luciferase activity was determined as described in Materials and Methods section; mean values from three independent experiments performed in triplicated are shown; bar indicate the SDs. Statistical significance ($^*P < 0.01$) compared with the control.

### CUCURBITACIN B INHIBITS AKT-INDUCED TRANSACTIVATION ACTIVITY OF RELA/p65 SUBUNIT AND TNF-α-INDUCED PHOSPHORYLATION OF AKT AND SER536 IN RELA/p65 SUBUNIT

Since it has been reported that Akt regulates transactivation activity of NF-κB through a mechanism dependent on phosphorylation of the RelA/p65 subunit of NF-κB without blocking the induced-IκBα degradation, p65 nuclear translocation, or NF-κB DNA-binding activity [Sizemore et al., 1999; Madrid et al., 2000; Mayo et al., 2002; Sizemore et al., 2002].

To determine whether cucurbitacin B inhibits RelA/p65 transactivation activity through PI3K/Akt pathway, we examined the effect of cucurbitacin B on the RelA/p65 transactivation activity induced by Akt-overexpression. As shown in Figure 5A, transient transfection with Akt led to induction of RelA/p65 transactivation activity, however, the RelA/p65 transactivation activity induced by Akt-overexpression was inhibited by cucurbutacin B dose-dependently. Next, we examined whether cucurbitacin B could act in synergy with the PI3K/Akt inhibitor LY294002 in inhibiting the transactivation activity of RelA/p65. As shown in Figure 5B, treatment with LY294002 inhibited RelA/p65 transactivation activity in a dose-dependent manner with the IC50 value of 10 μM. Comparing with single compound treatment, combined treatment with cucurbitacin B (30 nM) and LY294002 (10 μM) inhibited RelA/p65 transactivation activity more significantly with a combination index of $<1.0$, indicating that LY294002 can act in synergy with cucurbitacin B [Gu et al., 2006]. Next, we observed the effect of cucurbitacin B on the TNF-α-induced activation of Akt. HeLa cells were pretreated with 100 nM cucurbitacin B for 12 h and then stimulated with 20 ng/ml of TNF-α for indicated times. As shown in Figure 5C, stimulation of HeLa cells with TNF-α significantly induced the phosphorylation of Akt. On the other hand, pretreatment of cucurbitacin B significantly suppressed TNF-α-induced phosphorylation of Akt. Consistent with this result, cucurbitacin B also significantly suppressed the TNF-α-induced phosphorylation of Ser536 in RelA/p65, which is required for efficient activation of NF-κB.

### CUCURBITACIN B INHIBITS THE EXPRESSION OF NF-κB DEPENDENT ANTI-APOPTOTIC PROTEINS AND SENSITIZES TNF-α-INDUCED CELL DEATH

NF-κB controls the transcription of genes that confer resistance to death-inducing signals [Karin and Lin, 2002]. We therefore

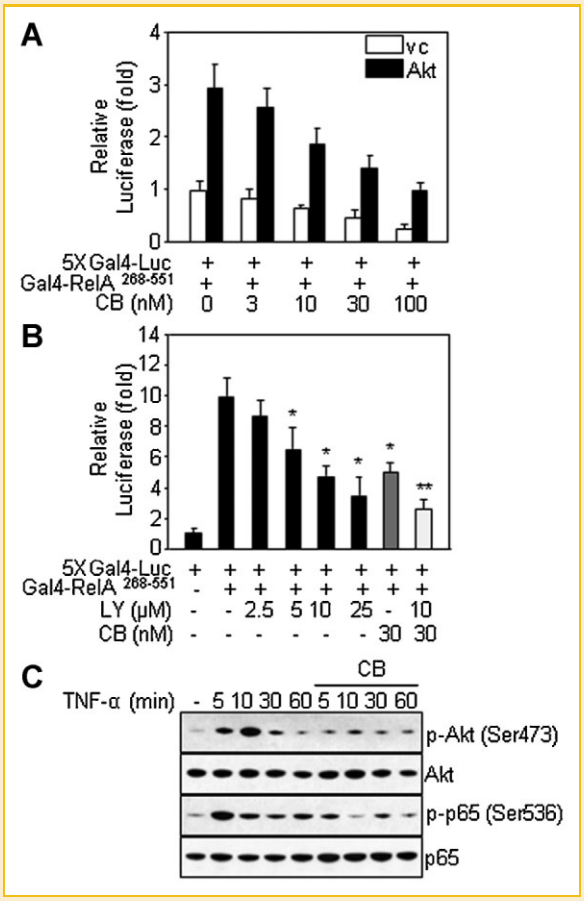


Fig. 5. Cucurbitacin B (CB) inhibits Akt-induced transactivation activity of RelA/p65 and TNF-α-induced phosphorylation of Akt and Ser536 in RelA/p65 (A) HeLa cells were transiently transfected with a Gal4-luciferase reporter gene and Gal4-RelA$^{268-551}$ and control vector or plasmid encoding Akt (1 μg of each). After 24 h, the cells were incubated for another 12 h in the presence of the indicated concentrations of cucurbitacin B, and the luciferase activity was determined as described in Materials and Methods section; Mean values from three independent experiments performed in triplicated are shown; bar indicate the SDs. B: HeLa cells were transiently transfected with a Gal4-luciferase reporter gene and Gal4-RelA$^{268-551}$. After 24 h, the cells were incubated for another 12 h in the presence of the indicated concentrations of LY294002 either in the presence or absence of indicated concentrations of cucurbitacin B, and the luciferase activity was determined as described in Materials and Methods section; mean values from three independent experiments performed in triplicated are shown; bar indicate the SDs. Statistical significance ($^{*}P < 0.01$ and $^{**}P < 0.01$) compared with the control and single treatment, respectively. C: HeLa cells were pretreated for 12 h with 100 nM cucurbitacin B and stimulated with TNF-α (20 ng/ml). Cells were harvested at the indicated time points and total extracts were prepared. Protein level of phospho-Akt, total Akt, phospho-p65, or p65 was detected by western blot analysis.

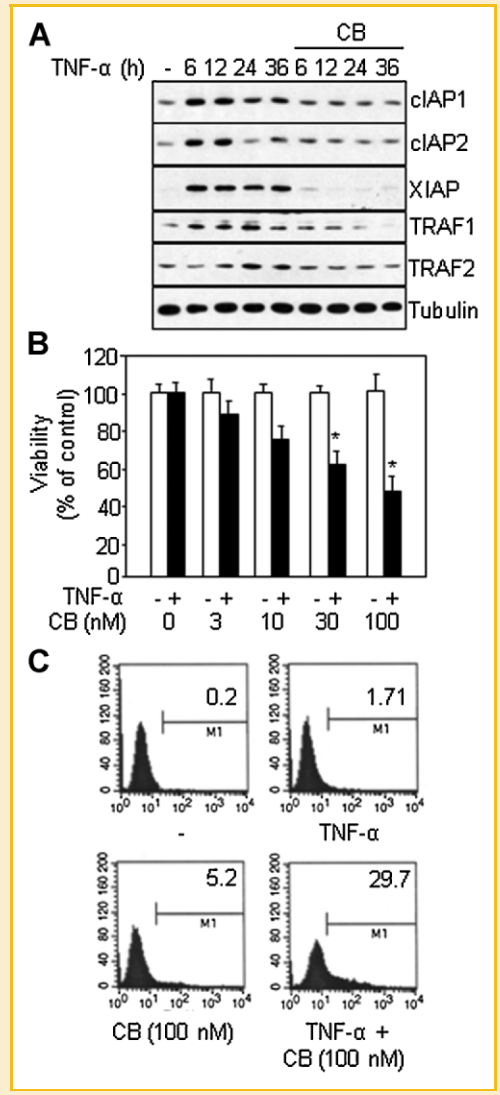


Fig. 6. Cucurbitacin B (CB) inhibits TNF-α-induced expression of NF-κB-dependent anti-apoptotic proteins and sensitizes TNF-α-induced cell death. A: HeLa cells were pretreated for 12 h with 100 nM cucurbitacin B and stimulated with TNF-α (20 ng/ml). Cells were harvested at the indicated time points and total extracts were prepared. Protein level of c-IAP1, c-IAP2, XIAP, TRAF1, or TRAF2 was detected by western blot analysis. B: HeLa cells incubated with 20 ng/ml of TNF-α for 48 h either in the presence or absence of indicated concentrations of cucurbitacin B and then cell viability was determined by MTT methods as described in Materials and Methods section; mean values from three independent experiments performed in triplicated are shown; bar indicate the SDs. Statistical significance ($^{*}P < 0.01$) compared with the control. C: HeLa cells incubated with 20 ng/ml of TNF-α for 48 h either in the presence or absence of 100 nM cucurbitacin B and then cell apoptosis was analyzed by flow cytometry after staining with Annexin V-FITC. The most representative result of three independent experiments is shown.

investigated whether cucurbitacin B could affect TNF-α-induced NF-κB-dependent anti-apoptotic proteins. After preincubation of HeLa cells with 100 nM cucurbitacin B for 12 h and stimulation with TNF-α for indicated times, as shown in Figure 6A, TNF-α-induced expression of c-IAP1, c-IAP2, XIAP, TRAF1, and TRAF2 were significantly blocked by cucurbitacin B in HeLa cells. Next, we investigated whether cucurbitacin B could sensitize TNF-α-induced cell death. HeLa cells incubated with TNF-α either in the presence or absence of the indicated concentrations of cucurbitacin B and then examined for cell viability by the MTT method. As shown in Figure 6B, TNF-α (20 ng/ml) or cucurbitacin B up to 100 nM did not induce cell death significantly. However, TNF-α-induced cell death was potentiated by cucurbitacin B in a dose-dependent manner. To

further confirm cucurbitacin B could sensitize TNF-α-induced cell death, we observed the amount of cell apoptosis with Annexin V staining in flow cytometry. As shown in Figure 6C, combined treatment resulted in a significant increased the Annexin V-positive cell population (29.7%), whereas treatment with TNF-α (1.71%) alone or cucurbitacin B (5.2%) alone has a little influence on the cell apoptosis.

## DISCUSSION

Cucurbitacin B is found in many cucurbitaceae species and is one of the most abundant forms of cucurbitacins. It has significant anti-inflammatory activity and inhibits the growth of numerous human cancer cell lines and tumor xenografts. Despite of its various pharmacological activities, the molecular mechanism has not been sufficiently explained. Recent studies have suggested that cucurbitacin B may exert its anti-cancer and anti-inflammatory effects through suppression of STAT3 signaling pathway [Yin et al., 2008; Zhang et al., 2009]. In this present study, we demonstrate for the first time that cucurbitacin B is a potent inhibitor of NF-κB activity by targeting transactivation activity of RelA/p65 subunit of NF-κB.

Our results showed that cucurbitacin B significantly inhibit TNF-α-induced expression of NF-κB reporter gene and NF-κB target genes. Cucurbitacin B, however, did not prevent TNF-α-induced degradation of IκBα as well as nuclear translocation and DNA-binding activity of NF-κB and LIGHT-induced p52 nuclear translocation and non-canonical NF-κB target gene CCL19 mRNA expression, but inhibited NF-κB activation by RelA/p65-over-expression. These results indicate that cucurbitacin B did not regulate the non-canonical NF-κB signaling pathway. It is well-known that RelA/p65 is a critical transactivation subunit of NF-κB [Schmitz and Baeuerle, 1991; Ballard et al., 1992]. The transactivation activity of RelA/p65 subunit is regulated by posttranslational modifications such as phosphorylation and acetylation [Vermeulen et al., 2002]. It was demonstrated that the phosphorylation status of RelA/p65 determines whether it associates with CREB-binding protein/p300, which is a critical regulator of NF-κB [Zhong et al., 2002]. These observations led us to formulate a hypothesis that cucurbitacin B may modify transactivation activity of RelA/p65 subunit of NF-κB. To test this hypothesis, we investigated whether cucurbitacin B suppress the transactivation activity of RelA/p65 subunit using Gal4 system. As expected, cucurbitacin B significantly suppressed the transactivation activity of RelA/p65 subunit. In this regard, it is possible that cucurbitacin B could suppress transactivation activity of RelA/p65 by modifying the phosphorylation or acetylation status of RelA/p65 subunit. Evidences have been presented that Akt is critical for the transactivation activity of RelA/p65 induced by cytokines such as TNF and IL-1 [Sizemore et al., 1999; Madrid et al., 2000; Mayo et al., 2002; Sizemore et al., 2002]. Our results showed that the induction of transactivation activity of RelA/p65 by overexpression of Akt was reversed by cucurbitacin B and that cucurbitacin B acted in synergy with PI3K inhibitor LY294002 in inhibiting transactivation activity of RelA/p65. These results suggested that cucurbitacin B could inhibit PI3K/Akt-dependent transactivation activity of RelA/p65. However, the synergistic effect of cucurbitacin B with LY294002 may reflect that cucurbitacin B affects other signaling pathways including STAT3 activation pathway. Since previous report suggested that MAPK p38 mediated transactivation activity of RelA/p65 through phosphorylating RelA/p65 [Madrid et al., 2001], we investigated if cucurbitacin B could affect the activity of p38. Cucurbitacin B, however, did not significantly inhibit TNF-α-induced phosphorylation of p38 (Supplementary Fig. 2). Further studies remain to be elucidated how cucurbitacin B regulate the transactivation activity of RelA/p65 in detail.

Mounting studies show that NF-κB pathway plays a crucial role in a link between inflammation and cancer [Greten et al., 2004; Pikarsky et al., 2004] and demonstrated an essential role for NF-κB in various cancers and inflammatory diseases. It is now clear that several downstream effectors of NF-κB activation have been known to involve in anti-apoptosis [Karin and Lin, 2002] and activation of NF-κB by TNF-α attenuates the pro-apoptotic activity of the latter [Liu et al., 1996]. It is therefore possible to suggest that anticancer effect of cucurbitacin B might be, at least in part, connected to its NF-κB inhibitory effect. In this regard, our findings that cucurbitacin B suppresses transactivation activity of RelA/p65 and potentiates TNF-induced cell death extend our understanding on the molecular mechanisms underlying the diverse biological activities of cucurbitacin B.

## ACKNOWLEDGMENTS

This work was supported in part by a grant from the Korea Research Institute of Bioscience and Biotechnology Research Initiative Program and a research grant (PF0320701-00) from the Plant Diversity Research Center of 21st Frontier Research Program funded by the Korean Ministry of Science and Technology.

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