

Calyculin A induces apoptosis and stimulates phosphorylation of p65NF- κ B in human osteoblastic osteosarcoma MG63 cells

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Abstract. Previously, we reported that okadaic acid, a specific inhibitor of serine/threonine protein phosphatases, induced apoptosis in human osteoblastic cells. However, it is not clear whether calyculin A, another inhibitor of protein phosphatases, would induce apoptosis in human osteoblastic cells and if so, which mechanisms are involved and whether the phosphorylation status of NF- κ B could be affected by the treatment with calyculin A. In this report, we demonstrate that calyculin A induced apoptosis in MG63 cells, as judged by WST-8 assay, nuclear fragmentation, and DNA ladder formation. Expression of *PTEN*, *FasL*, and *FasR* mRNA was stimulated by calyculin A treatment in MG63 cells. Calyculin A also enhanced the phosphorylation level of NF- κ B, as judged from the results of Western blot analysis and an *in vitro* dephosphorylation assay. Western blot analysis with anti-phospho-p65NF- κ B antibody disclosed that the NF- κ B was phosphorylated on serine 536 in cytosol and translocated into nucleus with calyculin A-treatment. The phosphorylation status of p65NF- κ B was further confirmed by using the phosphorylation site-mutated p65NF- κ B gene transfected into HEK293 cells. Unlike TNF- α , calyculin A treatment did not degraded I κ B α within 10 min, while it degraded I κ B α at 2-h treatment. Our findings indicate that calyculin A elicit phosphorylation of NF- κ B on serine 536 in MG63 cells, resulting in the translocation of phospho-NF- κ B to the nucleus, thereby promoting transcriptional activity of NF- κ B-related genes.

Introduction

Apoptosis is an important and well-controlled form of self-regulated cell death that plays a major role during embryogenesis, carcinogenesis, cancer treatment, and immune and toxic cell killing (1,2). Apoptosis can be regulated by extrinsic factors, including hormones, growth factors, cell-surface receptors, and various forms of cellular stress. The action of apoptosis-related factors is often affected by the modulation of the phosphorylation status of key elements active in the apoptotic processes (3).

Nuclear factor-kappa B (NF- κ B) is an essential transcription factor in the control of expression of genes involved in cell growth, differentiation, inflammation, and neoplastic transformation (4-6). NF- κ B has also been shown to have a role in regulating the apoptotic program, either as essential for the induction of apoptosis or as a blocker of apoptosis (7-11). NF- κ B is present in the cytoplasm as two major precursor forms, either as the p50-p65 complex with the inhibitor of κ B α (I κ B α) or as p65-p105 complex. For activation of NF- κ B, I κ B α is phosphorylated, ubiquitinated, and then degraded by the proteasome, thereby allowing translocation of the liberated NF- κ B from the cytoplasm to the nucleus. In the nucleus, NF- κ B regulates the expression of the target genes that modulate biological responses. Several kinases and phosphatases are considered as candidate enzymes for involvement in the phosphorylation of NF- κ B (12-14). However, whether and, if so, how phosphorylation of NF- κ B modulates its function remains unclear.

Calyculin A (CA) and okadaic acid (OA) are potent inhibitors of protein phosphatases type 1 (PP1) and type 2A (PP2A), which dephosphorylate serine and threonine residues in eukaryotic cells (15,16). The use of these agents has led to the understanding that the phosphorylation and dephosphorylation status is related to cellular regulation including apoptosis (17-20). We recently reported that the double-stranded RNA-dependent protein kinase (PKR) was activated and NF- κ B translocation occurred during the OA-induced apoptosis (20,21). However, the mechanisms whereby CA acts on apoptosis were not studied in detail. In the present study, we examined whether CA could induce apoptosis in

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human osteoblastic MG63 cells. We also examined the effect of CA on the phosphorylation status of NF- κ B in MG63 cells. Our results indicate that the CA treatment induced apoptosis in MG63 cells and enhanced the phosphorylation status of serine residue positioned at 536 in p65NF- κ B in these cells.

Materials and methods

Materials. CA and OA were purchased from Wako Chemicals (Osaka, Japan), and stock solutions (100 μ M), prepared in dimethyl sulfoxide and protected from light, were diluted to the appropriate concentrations with medium. TNF- α was purchased from Sigma Chemicals (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS, USA); and α -modified Eagle's minimal essential medium (α -MEM) and Dulbecco's modified Eagle's minimum essential medium (D-MEM) from Gibco BRL (Gaithersburg, MD, USA). Plastic dishes were from Iwaki (Chiba, Japan). Anti-p65NF- κ B and anti-Eps15 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-p65NF- κ B (Ser536) and anti-phospho-p65NF- κ B (Ser276) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-p65NF- κ B (Ser529) and I κ B α antibodies were obtained from BD Biosciences (Palo Alto, CA, USA). Anti-Lamin B1 antibody was from Zymed Laboratories (South San Francisco, CA, USA). Other materials used were of the highest grade commercially available.

Cell culture. Human osteosarcoma MG63 cells and human embryonic kidney HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The MG63 cells and HEK 293 cells were cultured in α -MEM and D-MEM, respectively supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The medium was replenished every 3 days. Confluent cells were treated with various concentrations of reagents for variable periods of time. Cell modification was monitored with the use of an Olympus IMT-2 phase-contrast microscope.

WST-8 assay. The cytotoxicity of MG63 cells was analyzed by the WST-8 quantitative colorimetric assay for cell survival (Dojindo Laboratory, Kumamoto, Japan). The assay detects living cells and the generated signal depends on the degree of activation of the cells. MG63 cells grown in 96-well culture plates were treated with the agents for various time periods and washed once with medium. Ten μ l of the reaction solution was immediately added to 100 μ l of a culture medium per well and the cells were then incubated for an additional 90 min at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The absorbance of each sample was measured at 450 nm with an ImmunoMini NJ-2300 microplate reader (Japan Intermed, Tokyo, Japan). Data were expressed as mean \pm SEM. The statistical significance was determined using the Student's t-test calculated by Microsoft Excel (Microsoft, Redmond, WA, USA). The level of significance was set at $P < 0.01$.

Nuclear fragmentation assay with Hoechst staining. The cells were plated on sterile 18-mm round glass coverslips placed in 60-mm plastic dishes and cultured. After appropriate incubation with CA, the coverslips were removed from the dishes and placed directly into 10% formalin in PBS for 10 min at ambient temperature. The fixed cells were washed three times with PBS and permeabilized with methanol for 10 min at -20°C. After washing three times with PBS the coverslips were incubated with Hoechst 33342 (10 μ g/ml) for 10 min at ambient temperature, rinsed with PBS, and the coverslips were mounted while wet in aqueous mounting medium (Biomedex, Foster City, CA, USA). Cells were examined under an Olympus BX50 microscope equipped for epifluorescent illumination (BX-FLA). Photomicrographs were recorded on digital equipment (Olympus, DP70-WPCXP).

DNA isolation and agarose gel electrophoresis. Cells were washed twice in PBS followed by lysis in cold 10 mM Tris-HCl buffer (pH 7.5), 10 mM EDTA, and 0.5% Triton X-100. After cell lysis, debris was removed by centrifugation at 15,000 g for 20 min. DNase-free RNase was added to the lysates at a final concentration of 20 μ g/ml, and incubated for 1 h at 37°C with gentle shaking, followed by the addition of Proteinase K at a final concentration of 20 μ g/ml for another 1 h at 37°C. DNA was precipitated with 50% 2-propanol and 0.5 M NaCl overnight at -20°C. After centrifugation and drying, the DNA was dissolved in TE-buffer (10 mM Tris, pH 8.0, containing 1 mM EDTA). Agarose gel electrophoresis of DNA was performed through a 2.0% agarose gel. DNA markers (100 bp) (New England BioLabs, Beverly, MA, USA) were run in the same gels. The gels were stained for 10 min with 10 μ g/ml ethidium bromide. To visualize apoptotic alterations to DNA integrity, we observed the DNA bands on a UV trans-illuminator (Vilber Lourmat, Marnela Vallee, France). Photographs were taken with a Polaroid DS-300 camera.

RNA preparation and RT-PCR. After the appropriate treatment, total cellular RNA was isolated from MG63 cells using Isogen (Nippon Gene, Tokyo, Japan). The purified RNA was further incubated with DNase I to digest the contaminating DNA followed by phenol extraction and ethanol precipitation. cDNA was synthesized by using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, Uppsala, Sweden). PCR was performed on the cDNA with AmpliTaq Gold® DNA Polymerase, LD (Applied Biosystems, Foster City, CA, USA) and the following primers: PTEN (forward) 5'-ACCGCCAAATTTAATTGCAG-3'; PTEN (reverse) 5'-GGGTCTGAATTGGGAFFAAT-3'. FasL (forward) 5'-TCTCAGACGTTTTCGGCTT-3'; FasL (reverse) 5'-AAGACAGTCCCCCTTGAGGT-3'. FasR (forward) 5'-CAAGGGATTGGAATTGAGGA-3'; FasR (reverse) 5'-GACAAAGCCACCCCAAGTTA-3'. GAPDH (forward) 5'-ACCACAGTCCATGCCATCAC-3'; GAPDH (reverse) 5'-TCCACCACCTGTTGCTGTA-3'.

After denaturation and activation of the enzyme at 95°C for 10 min, PCR amplification was performed under the following condition: 94°C for 50 sec, 55°C for 50 sec, and 72°C for 50 sec, for total cycles indicated. The reaction was terminated after a 5-min elongation step at 72°C. PCR products were analyzed on 2% agarose gels, stained with ethidium

bromide, and visualized with a UV illuminator following ethidium bromide staining. Photographs were taken with a Polaroid DS-300 camera.

SDS-PAGE and Western blotting. Cultured cells were washed twice with phosphate-buffered saline (PBS), scraped into lysate buffer containing 1 mM DTT, 1 mM PMSF, 1 μ g/ml leupeptin, 2 μ g/ml aprotinin, 5 mM EGTA, and protein phosphatase inhibitor cocktails (Sigma) in PBS. Nuclear and cytosol fractions were obtained from the cells by using a CellLytic NuCLEAR extraction kit (Sigma). The protein concentration was evaluated by using Protein Assay Reagent (Bio-Rad, Hercules, CA, USA), and each sample was diluted to a protein concentration of 1 mg/ml with lysate buffer followed by the addition of Laemmli's 5X SDS-sample buffer. Each sample of proteins and prestained molecular weight markers (Gibco BRL) were separated by SDS-PAGE and then transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were incubated for 2 h at 20-22°C in a blocking solution for phosphorylated proteins (Blocking One-P, Nacalai Tesque, Kyoto, Japan). Then they were washed briefly in PBS-Tween and incubated overnight at 4°C in a blocking solution containing specific antibodies. Next, the membranes were washed 4 times for 30 min with PBS-Tween and subsequently incubated for 1 h at ambient temperature in 5% skim milk in PBS-Tween containing horseradish peroxidase-conjugated second antibodies. The membranes were washed again and the interacting proteins were identified using an ECL detection kit (Amersham Pharmacia). To re-use the membrane, the antibody was stripped off by treatment with 2% SDS and 0.35% 2-mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 50°C. The antibody stripped-membranes were then blocked again and re-incubated with another appropriate antibody.

In vitro dephosphorylation. Whole cell lysates were prepared from MG63 cells untreated or treated with 2 nM CA. Cell lysates (10 μ g) were incubated for 60 min at 30°C with 1,000 U of λ protein phosphatase (New England BioLabs), a residue-non-specific protein phosphatase, in the reaction buffer. After incubation, 5X SDS sample buffer was added to the reaction mixture, and the proteins were denatured for 5 min in boiling water. The samples were subjected to SDS-PAGE and analyzed by Western blotting.

DNA construction and transfection. Plasmid pEGFP-p65NF- κ B (human) was kindly provided by Dr J.A. Schmid. A phosphorylation site mutation of p65NF- κ B S536A (carrying a mutation of serine to alanine at position 536) was introduced by QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The nucleotide sequences were confirmed by DNA sequencing. Transfection was performed with Lipofectamine 2000 system (Invitrogen, Carlsbad, CA, USA). HEK293 cells were plated in 60-mm plastic dishes and maintained in D-MEM containing 10% FBS until they had reached 70-80% confluence. Before transfection, the cells were washed once with serum-free medium. DNA (10 μ g) or Lipofectamine 2000 reagent was mixed with 1.5 ml OPTI-MEM for 5 min. The mixtures were added together, incubated for 30 min at room temperature, and the complex

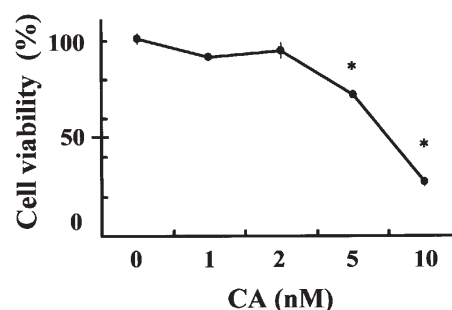


Figure 1. Effects of CA on cell viability in MG63 cells. MG63 cells grown in 96-well plates were treated for 24 h with various concentrations of CA and the cell viability was determined by the WST-8 assay. The activity was compared to the control well of the same cell line and results are expressed as a percentage of the control (mean \pm SEM) (N=3). The absorbance at 450 nm of the control cultures were 0.604 ± 0.017 . Significant differences from the control cultures are indicated by an asterisk; *P<0.01 (Student's t-test).

was overlaid onto the cells. After incubation at 37°C for 4 h, the medium was replaced with fresh D-MEM containing 10% FBS and incubation was continued for another 24 h and treated with CA.

Results

Induction of apoptosis in MG63 cells by CA. Treatment with 0.1 nM CA for 24 h had a minimal effect on MG63 cell survival as observed under a phase-contrast microscope. Cell rounding and shrinking were obvious in the cultures treated for 24 h with 5 nM CA. To further quantify the CA-induced cell death in MG63 cells, cytotoxicity was measured by the WST-8 cell viability assay. Fig. 1 shows that CA induced cytotoxicity in MG63 cells in a dose-dependent manner up to 10 nM. The level of the cell viability treated with 10 nM CA was 27% that of the control cultures. To determine if CA-induced cytotoxicity in MG63 cells was due to apoptosis, we evaluated the presence of nuclear fragmentation and condensation in MG63 cells treated for 48 h with various concentrations of CA. The control cultures of MG63 cells did not show any apoptotic features. However, nucleic acid staining with Hoechst 33342 revealed typical apoptotic nuclei, which exhibited highly fluorescent condensed chromatin in the cells treated with 5 nM CA (Fig. 2A). The number of the cells with nuclear fragmentation increased in response to CA in dose- and time-dependent manner (data not shown). In MG63 cells treated for 48 h with CA, a DNA fragmentation pattern forming a ladder of multiples of 185-200 bp was observed (Fig. 2B). The DNA laddering pattern of the CA-treated cells reached a maximal level at 10 nM. The dose- and time-dependence of CA-induced DNA ladder formation were comparable to that of the cytotoxicity obtained from WST-8 assay.

Expression of PTEN, FasL, and FasR mRNA in MG63 cells treated with CA. We examined mRNA expression of PTEN, FasL, and FasR in MG63 cells treated with 2 nM CA. Approximately 460-, 410-, and 440-bp bands corresponding to PTEN, FasL, and FasR respectively, were detected in MG63 cells treated for 1 h with CA after amplification of cDNA for 32

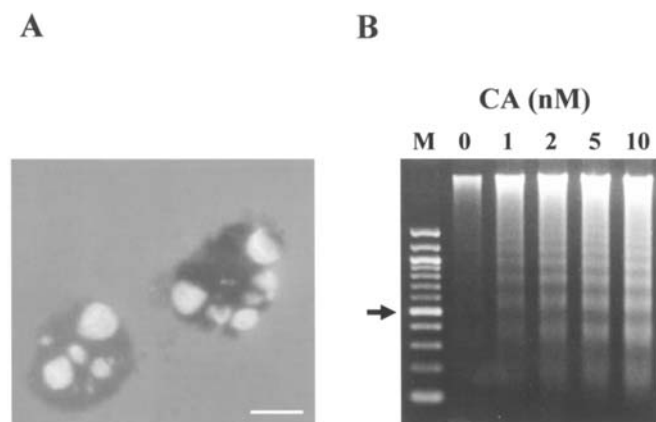


Figure 2. Nuclear fragmentation of MG63 cells treated with CA. (A) Nuclear morphology of MG63 cells treated with CA. MG63 cells were treated for 48 h with 5 nM CA. Cells were stained with Hoechst 33342 and observed under a fluorescent microscope. Bar represents 10 μ m. (B) DNA ladder formation in CA-treated MG63 cells. MG63 cells were exposed for 48 h to various concentrations of CA indicated. DNA was extracted and analyzed on an agarose gel. Lane M, standard DNA markers; arrow indicates 500 bp.

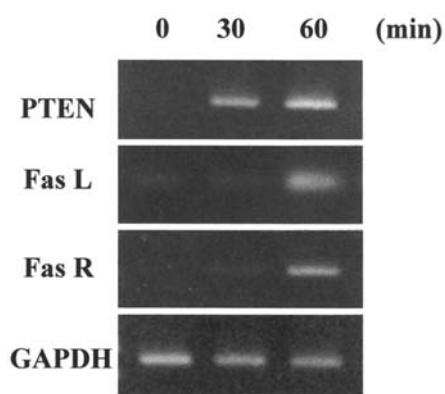


Figure 3. Expression of *PTEN*, *FasL*, and *FasR* mRNA in MG63 cells treated with CA. After having reached confluence, MG63 cells were treated with 2 nM CA for various time-points indicated and semiquantitative analysis of *PTEN*, *FasL*, and *FasR* mRNA was done with the mRNA amplified by 32 cycles in MG63 cells. The RT-PCR product of *GAPDH* prepared from the same samples and amplified for 29 cycles is also shown.

cycles. The RT-PCR exponential phase was determined from 30 to 34 cycles to allow semiquantitative comparisons among cDNAs developed from identical reactions. Fig. 3 shows that the expression of *PTEN*, *FasL*, and *FasR* mRNAs in MG63 cells treated with CA increased in a time-dependent manner. The RT-PCR product of *GAPDH* prepared from the same cells and amplified for 29 cycles is also shown in Fig. 3. The *GAPDH* mRNA was constitutively expressed, and its level was not affected by CA-treatment (Fig. 3).

Regulation of I κ B α in MG63 cells treated with CA. Whole cell lysates were prepared from MG63 cells treated for various time periods with 2 nM CA. The cells were also treated with 10 ng/ml TNF- α for the control. The expression level of I κ B α was examined by Western blot analysis by using anti-I κ B α antibody. Fig. 4 shows that the anti-I κ B α antibody interacted with a protein of 38 kDa in the unstimulated cells. CA treatment decreased the staining intensity of I κ B α up to 2 h, at which

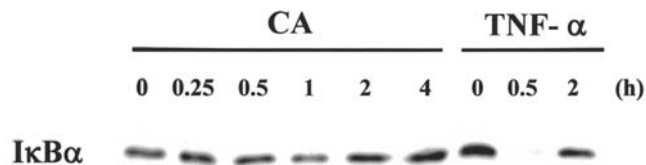


Figure 4. Regulation of I κ B α protein in MG63 cells treated with CA. MG63 cells were treated with 2 nM CA or 10 ng/ml TNF- α for the variable time periods indicated and cell lysates were prepared from each type of culture. Samples (10 μ g) were separated on a 10% of SDS-PAGE gel, transferred to a PVDF membrane, and analyzed for I κ B α expression by Western blotting.

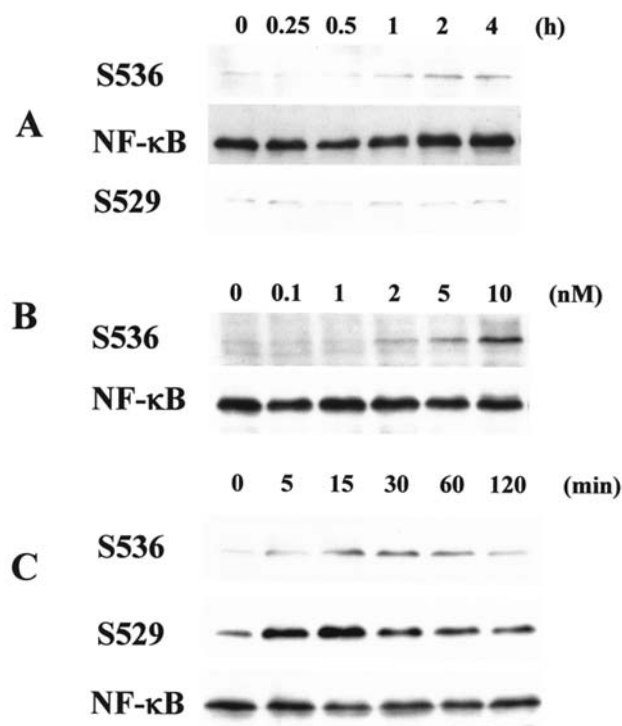


Figure 5. Western blot analysis of phospho-NF- κ B in MG63 cells. (A) Cell lysates were prepared from MG63 cells treated with 2 nM for various times indicated. The blotted membrane was incubated with the anti-phospho-Ser536 p65NF- κ B (Ser536), anti-p65NF- κ B (NF- κ B), and anti-phospho-Ser529 p65NF- κ B (S529) antibodies. (B) The cells were treated for 3 h with various concentrations of CA as indicated. The samples were analyzed by Western blotting using anti-phospho-Ser536 p65NF- κ B antibody (S536). The antibody was stripped off the membrane and replaced with anti-p65NF- κ B antibody (NF- κ B). (C) MG63 cells were treated with 10 ng/ml TNF- α for the variable time periods indicated, cell lysates were prepared from each type of culture and analyzed by Western blotting by using anti-phospho-Ser536 p65NF- κ B (Ser536), anti-phospho-Ser529 p65NF- κ B (S529) or anti-p65NF- κ B (NF- κ B) antibodies.

time the staining level was minimum, then the staining level increased time dependently up to 4 h. Fig. 4 also shows that I κ B α was completely degraded by the TNF- α treatment within 30 min. Then the expression level of I κ B α increased by 2 h, at which point the staining was almost at the same level as that of the unstimulated control cells.

Phosphorylation of NF- κ B on serine 536 residue by CA treatment. Fig. 5A shows the anti-phospho-Ser536 p65NF- κ B

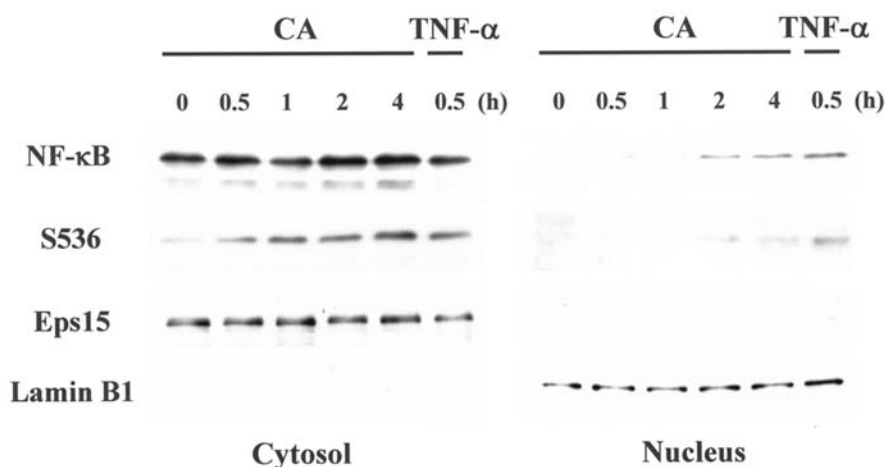


Figure 6. Nuclear translocation of NF- κ B in MG63 cells treated with CA. MG63 cells were treated with 2 nM CA or 10 ng/ml TNF- α for various time-points indicated. Cell fractionation was done to prepare the cytosolic (left panel) and nuclear (right panel) fractions. Proteins prepared from each fraction were subjected to Western blot analysis using anti-p65NF- κ B antibody anti-phospho-Ser536 p65NF- κ B antibodies as indicated. Each fraction was also interacted with anti-Eps15 and anti-Lamin B1 antibodies to ensure the purity of cytosolic and nuclear fractions, respectively.

antibody interacted with a band having an estimated molecular weight of 65 kDa. It appeared after 1-2 h of CA-treatment, and the level of staining increased up to 4 h (Fig. 5A, upper panel). The bound antibody was stripped off the membrane and the membrane was re-incubated with anti-p65NF- κ B antibody. This antibody recognized a 65-kDa band and the staining intensity was not affected by CA-treatment (Fig. 5A, middle panel). The anti-phospho-Ser529 p65NF- κ B antibody also recognized a 65 kDa band (Fig. 5A, bottom panel). However, the level of the staining intensity was not affected by CA-treatment. The anti-phospho-Ser276 p65NF- κ B antibody did not react with any proteins (data not shown). Fig. 5B shows the reaction between the anti-phospho-Ser536 p65NF- κ B antibody and the proteins extracted from the MG63 cells treated for 3 h with various concentrations of CA. Staining intensity was weak in the extracts prepared from the untreated control cells. The anti-phospho-Ser536 p65NF- κ B antibody interacted with a 65 kDa band and the intensity of this band increased in a dose-dependent manner up to 10 nM CA. When the bound antibody was stripped off the membrane and re-incubated with anti-p65NF- κ B antibody, the staining intensity of this band was not changed. The cells were also treated for various time-points with 10 ng/ml of TNF- α . Fig. 5C shows that anti phospho-Ser536 and anti-phospho-Ser529 p65NF- κ B antibodies interacted with a protein of 65 kDa at 5-min treatment and the staining intensity increased up to 60 and 15 min, respectively.

Translocation of NF- κ B in MG63 cells treated with CA. Cytosolic and nuclear fractions were prepared from MG63 cells treated for various time periods with 2 nM CA and 10 ng/ml TNF- α . Anti-p65NF- κ B antibody recognized a 65 kDa band in the cytosolic fractions of unstimulated or CA-treated cells (Fig. 6). The staining reaction of anti-p65NF- κ B antibody was not detected in the nuclear fraction of the unstimulated cells, however, p65NF- κ B appeared in the nuclear fraction with CA-treatment and the staining intensity increased (Fig. 6). The anti-phospho-Ser536 p65NF- κ B antibody did not react with any proteins in the cytosolic and nuclear fractions of

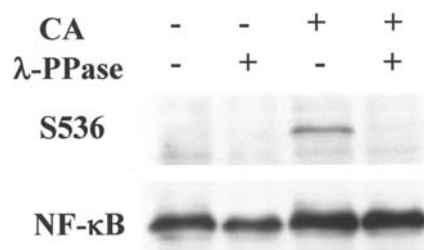


Figure 7. Dephosphorylation of phospho-NF- κ B with λ protein phosphatase. MG63 cells were untreated or treated for 4 h with or without 2 nM CA as indicated. Cell lysates prepared from each type of culture were incubated for 60 min at 30°C with or without 1,000 units of λ protein phosphatase (λ -PPase) as indicated. The samples were then analyzed by Western blotting by using the anti-phospho-Ser536 p65NF- κ B (upper panel) and anti-p65NF- κ B (bottom panel) antibodies.

the control cells (Fig. 6). However, the staining intensity of phospho-Ser536 p65NF- κ B increased both in the cytosolic and nuclear fractions of the cells treated with CA. In the cells treated with TNF- α , anti-phospho-Ser536 p65NF- κ B antibody reacted with the proteins in the cytosolic and nuclear fractions. The anti-Eps15 antibody interacted with a 140-kDa band in the cytosolic fractions of CA- or TNF- α -treated cells while this antibody did not interact with any proteins in the nuclear fractions (Fig. 6). The anti-Lamin B1 antibody did not interact with any proteins in the cytosolic fractions whereas this antibody interacted with a protein of 68 kDa in the nuclear fractions, indicating that cell fractionation in the present study validate the high purity of each fraction.

In vitro dephosphorylation and phosphorylation of mutant gene products. To confirm that the band recognized by the anti-phospho-Ser536 p65NF- κ B antibody was the phosphorylated form of p65NF- κ B, we prepared lysates from CA-treated cells and incubated them with λ protein phosphatase. Fig. 7 shows that treatment of the cell lysates with λ protein phosphatase resulted in the disappearance of the band interacted with anti-phospho-Ser536 p65NF- κ B antibody whereas this

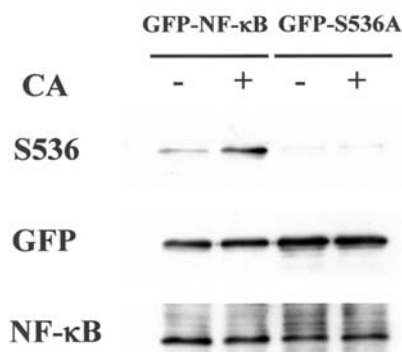


Figure 8. Phosphorylation of NF- κ B in the cells transfected with the mutant p65NF- κ B. HEK293 cells were transfected with GFP-NF- κ B and GFP-S536A plasmid and treated with or without 2 nM CA as indicated. Cell lysates were prepared from each type of culture and analyzed by Western blotting using the anti-phospho-Ser536 p65NF- κ B, anti-GFP, and anti-p65NF- κ B antibodies as indicated.

band was retained in the lysates from the untreated control cells. To confirm further that CA could stimulate phosphorylation of Ser536 of p65NF- κ B, we constructed mutant p65NF- κ B gene in which the serine at 536 was replaced with alanine (GFP-S536A). We also used GFP-p65NF- κ B (GFP-NF- κ B) as control. The plasmids were transfected into HEK293 cells, cultured with or without CA, and cell lysates were prepared. Fig. 8 shows that the anti-phospho-Ser536 p65NF- κ B did not interact with the cell lysates prepared from the cells transfected with GFP-S536A mutant gene and treated with or without CA. However, the antibody interacted with the cell lysates from the GFP-NF- κ B transfected cells and the staining intensity increased with CA-treatment (Fig. 8). The anti-GFP and anti-p65NF- κ B antibodies interacted with the proteins prepared from these cells and the staining intensity did not change with CA-treatment (Fig. 8). These results together with those described above indicate that p65NF- κ B was phosphorylated at serine 536 residue of p65NF- κ B by CA treatment.

Discussion

In the present study, we treated human osteoblastic MG63 cells with various concentrations of CA for various time-points to examine whether this inhibitor of protein phosphatases could induce apoptosis in these cells. Under a phase-contrast microscopy, obvious morphological changes were observed in MG63 cells treated with CA. Loss of cell viability in CA-treated cells was demonstrated by the WST-8 cell viability assay. By using the Hoechst 33342 staining, marked nuclear condensation and fragmentation into spherical bodies were demonstrated. Moreover, DNA ladder formation was detected in MG63 cells treated with CA. These results indicate that CA induced apoptosis in MG63 cells. Effect of CA on induction of apoptosis is not limited to MG63 cells because CA induced apoptosis in human osteoblastic Saos-2 cells and human squamous carcinoma SCC-25 cells (22-24). Because the expression of *PTEN*, *FasL*, and *FasR* mRNA in MG63 cells increased with CA-treatment, apoptosis would be induced by the apoptosis inducing factors. The induction of cell death genes and ongoing protein synthesis might be

needed in CA-induced apoptosis in MG63 cells. It might be possible that PP1 and/or PP2A are responsible for CA-induced apoptosis. Inhibition of protein phosphatase activity by CA and related substances may be a general way of triggering apoptosis via the cell death inducing genes in some kinds of cells. However, it was reported that new protein synthesis was not required for CA-induced apoptosis (25). Most cells might have machinery by which they commit apoptosis under an inappropriate phosphorylation status. Several reports have pointed to PTEN, FasR, and FasL as important regulators of NF- κ B-mediated apoptosis (9,26). Upregulation of PTEN, FasR and FasL by NF- κ B was found to be critical to apoptosis (18,27). Although the expression of apoptosis inducing substances were not examined in detail PTEN or FasR and FasL system should cause the induction of apoptosis in MG63 cells.

We explored the effects of CA on the phosphorylation status of NF- κ B in MG63 cells. We demonstrated that p65NF- κ B was phosphorylated on serine residue at position 536 by the CA treatment. The phosphorylation of serine 536 at NF- κ B was further confirmed by the λ protein phosphatase treatment and by using the cells transfected with the mutated gene of p65NF- κ B S536A. Besides CA, NF- κ B is activated by a wide variety of other agents, including LPS and TNF. Phosphorylation of p65NF- κ B at serine 536 residue by TNF- α becomes evident 5-10 min in MG63 cells demonstrated in the present study. We also demonstrated that TNF- α stimulated phosphorylation of p65NF- κ B at serine 529 residue within 10 min in MG63 cells. These findings are in good agreement with the past reports concerning their biological effects in other systems (28-30). The CA-mediated phosphorylation of p65NF- κ B occurs slower than that induced by specific receptors associated with TNF- α or LPS. Unlike TNF- α or LPS, CA penetrates the cell membrane and inhibits the intracellular protein phosphatases, thus promoting phosphorylation of serine 536 on p65NF- κ B. The induced signal transduction pathways should be differing among the agents used. The nature of molecules in the pathways of p65NF- κ B phosphorylation on which CA might have an affect is not clear.

Anti-phospho-Ser536 p65NF- κ B antibody interacted with the 65-kDa band, which was also recognized by anti-p65NF- κ B antibody. This finding indicates that phosphorylation of this site does not influence the migration of p65NF- κ B when subjected to SDS-PAGE. However, anti-phospho-Ser536 p65NF- κ B and anti-p65NF- κ B antibodies recognized the slower migrating band in addition to the 65-kDa band in MG63 cells treated with OA. Because the slower migrating band disappeared on the treatment with λ protein phosphatase it should be a super-phosphorylated form of p65NF- κ B (unpublished data). Another explanation is that the slower migrating band might be the acetylation form of p65NF- κ B (31).

The majority of p65NF- κ B phosphorylation occurs at serine residues, however, only a few phosphorylation sites have been mapped precisely. Phosphorylation of Ser276, situated in the Rel-homology-domain (RHD) in the N-terminus of NF- κ B, was reported to be involved in sequence-specific DNA binding, dimerization, and nuclear translocation (32). However, in our study, phosphorylation of Ser276 did not

occur in MG63 cells by CA or OA treatment, indicating that phosphorylation of Ser276 is not essential for the CA- or OA-activated NF- κ B transcription. Ser536 on p65NF- κ B was phosphorylated by IKK complex (28,33,34). Over-expression studies with the activated Akt revealed that IKK is necessary for enhanced p65NF- κ B transactivation, whereas mutation of Ser536 abolishes this effect (34). The involvement of both IKK α and IKK β in phosphorylation of Ser536 on p65NF- κ B was confirmed by using IKK $\alpha^{-/-}$ or IKK $\beta^{-/-}$ mouse embryonic fibroblasts (35). In the present study, we demonstrated that CA enhanced the phosphorylation level of Ser536 but not Ser276 and Ser529 on p65NF- κ B in MG63 cells. These findings indicate that phosphorylation of Ser536 on p65NF- κ B functions in NF- κ B activation in the CA-treated cells. In earlier experiments, the phosphorylation of Ser529 on p65NF- κ B was shown not to contribute to NF- κ B activity in response to TNF- α or LPS, suggesting only a minor role for Ser529 phosphorylation on p65NF- κ B in NF- κ B function (28,35). As CA enhanced the phosphorylation level of p65NF- κ B, mapping the phosphorylation sites targeted by CA is quite important to understand how this phosphatase inhibitor functions in the cells. We demonstrated that the translocation of p65NF- κ B from the cytosol to the nucleus was stimulated by CA treatment. Phosphorylation of Ser536 on p65NF- κ B can be detected both in the cytoplasm and nucleus. However, it is unclear if this was due to the shuttling of phosphorylated p65NF- κ B or to the presence of Ser536 protein kinase in both the cytoplasm and nucleus (28,36). More detailed experiments are needed to clarify this point.

In response to certain stimuli, I κ B α is phosphorylated, ubiquitinated, and targeted for proteasomal degradation allowing the complex to translocate to the nucleus and bind to κ B-response element. One of the target genes of NF- κ B is its inhibitor I κ B α , resynthesis of which gives rise to an autoregulatory loop that terminates the activation process of NF- κ B (37). In MG63 cells TNF- α stimulated the rapid degradation of I κ B α and translocation of NF- κ B to the nucleus. Although I κ B α was degraded by CA-treatment, it was not completely degraded and the process was slower than that of TNF- α . Previously we demonstrated that the degradation of I κ B α did not occur in the OA-treated MG63 cells (38). It has also been reported that phosphorylation of Ser536 on p65NF- κ B is not involved in the process leading to I κ B α degradation (37). There is evidence that the phosphatidylinositol-3 kinase pathway is involved in I κ B-independent regulation of NF- κ B (39). These findings together with our results indicate that, phosphorylation of NF- κ B does not depend on I κ B α degradation in certain type of signal transduction.

Changes in NF- κ B transcriptional activity have been attributed to the phosphorylation of p65NF- κ B by a large variety of kinases in response to different stimuli (40-42). The N-terminal domain of p65NF- κ B was shown to be phosphorylated by PKC- ζ (40,41). In fact, PKC- ζ can directly and efficiently phosphorylate p65NF- κ B. More importantly, TNF- α -induced phosphorylation of p65NF- κ B was seriously inhibited in PKC- $\zeta^{-/-}$ embryo fibroblasts (41). It should be remembered that the phosphorylation status of p65NF- κ B is determined not only by kinase activities but also by the interplay of both kinases and phosphatases. Indeed, PP2A

is physically associated with p65NF- κ B in unstimulated melanocytes and is able to dephosphorylate p65NF- κ B after IL-1 stimulation (43).

Gene expression varies in response to different signals (44,45). Different kinases and phosphatase inhibitors may be acting at various stages of the transcription process, including nuclear transport, DNA-binding capacity, and transcriptional initiation or elongation. The phosphorylation of p65NF- κ B is a process highly regulated by both cell- and stimulus-dependent activating kinases and phosphatases.

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