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Oleandrin suppresses activation of nuclear transcription factor-κB and activator protein-1 and potentiates apoptosis induced by ceramide

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Abstract

Ceramide (*N*-acetyl-D-sphingosine), a second messenger for cell signaling induces transcription factors, like nuclear factor-kappa B (NF-κB), and activator protein-1 (AP-1) and is involved in inflammation and apoptosis. Agents that can suppress these transcription factors may be able to block tumorigenesis and inflammation. Oleandrin (*trans*-3,4′,5-trihydroxystilbene), a polyphenolic cardiac glycoside derived from the leaves of *Nerium oleander*, has been used in the treatment of cardiac abnormalities in Russia and China for years. We investigated the effect of oleandrin on NF-κB and AP-1 activation and apoptosis induced by ceramide. Oleandrin blocked ceramide-induced NF-κB activation. Oleandrin-mediated suppression of NF-κB was not restricted to human epithelial cells; it was also observed in human lymphoid, insect, and murine macrophage cells. The suppression of NF-κB coincided with suppression of AP-1. Ceramide-induced reactive intermediates generation, lipid peroxidation, cytotoxicity, caspase activation, and DNA fragmentation were potentiated by oleandrin. Oleandrin did not show its activity in primary cells. Oleandrin's anticarcinogenic, anti-inflammatory, and growth-modulatory effects may thus be partially ascribed to the inhibition of activation of NF-κB and AP-1 and potentiation of apoptosis.

Keywords: Oleandrin; NF-κB; Cell signaling; Inflammation; Apoptosis; Ceramide

1. Introduction

Resistance of tumors to chemotherapy is a common clinical problem in human cancer that may develop mostly by unknown mechanisms. Apoptosis seems to be the main mechanism to kill tumor cells induced by chemotherapy, radiation, and cytokines, such as TNF, which require activation of the caspase cascade, leading to proteolytic cleavage of a variety of important proteins and ultimately to fragmentation of cellular DNA.

NF-κB, a nuclear transcription factor first identified by Sen and Baltimore [1] as it regulates the expression of various genes that play critical roles in inflammation, viral replication, tumorigenesis, and apoptosis [1–4], therefore, this factor is a current target of pharmaceutical interest [5]. NF-κB is also an ideal target for anticancer drug development as it has been shown to block apoptosis and promote proliferation [6,7]. NF-κB activation induces resistance to chemotherapeutic agents [8,9] and constitutive expression of NF-κB induces proliferation in tumor cells [10,11]. NF-κB is a heterodimer of two subunits p50 (NF-κB1) and p65 (RelA), that is normally present in the cytoplasm in an inactive state in complex with an inhibitory subunit IκBα. Upon phosphorylation and subsequent degradation of IκBα, a nuclear localization signal on the p50–p65 heterodimer is exposed, leading to nuclear translocation and transcription of dependent genes [12]. AP-1, another transcription factor composed of *fos* and *jun* is activated by those agents that activate NF-κB and also show similar function like NF-κB [13]. AP-1 has been shown to be involved in tumor promotion and progression of various types of cancers. Therefore, inhibitors of AP-1 have been shown to have potential in blocking carcinogenesis [14]. Most of these inducers activate NF-κB, AP-1,

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Abbreviations: AP-1, activator protein-1; CE, cytoplasmic extract; Ceramide, *N*-acetyl-D-sphingosine; DOC, deoxycholate; FBS, fetal bovine serum; Mn-SOD, manganese superoxide dismutase; MTT, 3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; NE, nuclear extract; NIK, NF-κB-inducing kinase; oleandrin, *trans*-3,4′,5-trihydroxystilbene; PARP, poly(ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cells; PIS, pre-immune serum; ROI, reactive oxygen intermediate; SEAP, secretory alkaline phosphatase; TBA, thiobarbituric acid.

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c-*jun* N-terminal kinase (JNK), and apoptosis through the production of a signaling intermediate ceramide [15]. We are interested in identifying specific inhibitors of NF-κB and AP-1 from natural sources and dissect their mechanism of action.

Research of the last few years has shown that different plant products exhibit chemopreventive effect [16]. As many as 70% of the therapeutic drugs in use today are derived from plants. Oleandrin is a polyphenolic cardiac glycoside derived from the leaves of *Nerium oleander*. It has been used to treat congestive heart failure and is known to be toxic to a wide variety of tumor cells [17–19]. Two of the active components of *Nerium* are the cardiac glycosides oleandrin and oleandrigenin. Previous *in vitro* studies using two human prostate cancer cell lines, DU145 and PC3, have demonstrated that cardiac glycosides may inhibit fibroblast growth factor-2 (FGF-2) export through membrane interaction with the Na^+,K^+-ATPase pump [20]. The mechanism of the cytotoxic effects of oleandrin, however, is not understood. Because NF-κB and AP-1 are known to play major roles in cell proliferation, tumor promotion, and drug resistance [9,12–14], we considered the possibility that the effects of oleandrin are mediated through suppression of NF-κB and AP-1. Therefore, in this report the effect of oleandrin on NF-κB and AP-1 activation induced by ceramide was investigated.

Given the fact that the carcinogenic, inflammatory, and growth-modulatory effects of many chemicals are mediated by NF-κB, we also hypothesized that the suppression of NF-κB activation pathway accounts for oleandrin's activities. Numerous lines of evidence suggest this possibility. For example, various agents that promote tumorigenesis are known to activate NF-κB [2], including phorbol ester, okadaic acid, and TNF, where ceramide is produced as an intermediate molecule. Most agents that activate NF-κB also activate AP-1 [13]. That AP-1 activation mediates tumorigenesis and invasiveness has also been described [21]. The activation of NF-κB and AP-1 is regulated by several protein kinases that belong to the mitogen-activated protein kinase (MAPK) family [22]. The activation of NF-κB and AP-1 and its associated kinases is in most cases dependent on the production of reactive oxygen species [23–26].

In this study, we tested the hypothesis that the anti-inflammatory and anticarcinogenic effects of oleandrin are mediated through its modulation of NF-κB and AP-1 activation, and caspase-mediated apoptosis. We demonstrated that oleandrin is a potent inhibitor of NF-κB and AP-1 activation in a variety of cell lines.

2. Materials and methods

2.1. Materials

Oleandrin, C2 ceramide, glycine, LPS, NaCl, and BSA were obtained from Sigma. Penicillin, streptomycin, RPMI 1640 medium, and FBS were obtained from Life Technologies. A 1.0 mg/mL solution of oleandrin (M_r 228.2) was prepared in DMSO and subsequent dilutions were made in medium. Antibodies (Abs) against tubulin, IκBα, p50, and p65 were obtained from Santa Cruz Biotechnology. Poly(ADP-ribose) polymerase (PARP) Ab was purchased from PharMingen.

2.2. Cell lines

HeLa (human epithelial cells), Jurkat (T cells), Daudi (B-cells), Raw 264.7 (mouse macrophages), and Vero (monkey kidney cells) cell lines were obtained from American Type Culture Collection. MCF-7 (*neo* and *Mn-SOD*) cells were collected from Professor Bharat B. Aggarwal, MD Anderson Cancer Center, Texas. HeLa, Raw 264.7, Jurkat, Daudi, MCF-7, and Vero cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). All cells were free from mycoplasma, as detected by Gen-Probe mycoplasma rapid detection kit (Fisher Scientific). Most of the experiments were carried out in HeLa cells. HeLa cells were plated in 60 mm petri dish and treated at 60% confluent state.

2.3. Neutrophil and peripheral blood mononuclear cells (PBMC) isolation

Neutrophils and PBMC were separated from fresh human peripheral venous blood of normal healthy donors by dextran T-500 sedimentation method, followed by histopaque gradient centrifugation [27]. The purity of the cells was examined by Giemsa stain and the viability of the cells was checked with the trypan blue dye exclusion test.

2.4. NF-κB and AP-1 activation assays

To determine NF-κB activation, EMSA were conducted essentially as described [28]. Briefly, 8 μg nuclear extract proteins were incubated with ^{32}P end-labeled 45-mer double-stranded NF-κB oligonucleotide of HIV-LTR, 5′-TTG TTA CAA **GGG ACT TT**C CGC TG**G GGA CTT TC**C AGG GAG GCG TGG-3′ (bold indicates NF-κB-binding site) for 30 min at 37°, and the DNA–protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. Similarly, 6 μg of nuclear extract proteins was assayed for AP-1 assayed using ^{32}P end-labeled double-stranded oligonucleotide of AP-1, 5′-CGCTTGA**TGACTCA**GCCGGAA-3′ (3′-GCG-AACT**ACTGAGT**CGGCCTT-5′; bold indicates AP-1-binding site) for 15 min at 37° and analyzed by using 6% native polyacrylamide gel. The specificity of binding was examined by competition with unlabeled oligonucleotide. Visualization and quantitation of radioactive bands were done as indicated above.

2.5. Western blot for IκBα, p50, and p65

To determine the levels of IκBα, cytoplasmic extracts were prepared from cells and resolved on SDS–polyacrylamide gel [28]. To determine the levels of NF-κB proteins p65 and p50, cytoplasmic extracts were prepared from ceramide-treated cells and were resolved on 9% SDS–PAGE and Western blot was done using anti-IκBα, -p50, or -p65, and detected by chemiluminescence (Amersham).

2.6. Cytotoxicity assay

The cytotoxicity was assayed by the 3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) dye uptake [29]. Briefly, HeLa cells (10^4 cells/well of 96-well plate) were incubated with test sample in a final volume of 0.1 mL for 72 hr at 37°. Thereafter, 25 μL of MTT solution (5 mg/mL in PBS) was added to each well. After a 2-hr incubation at 37°, 0.1 mL of the extraction buffer (20% SDS, 50% dimethylformamide) was added. After an overnight incubation at 37°, the absorbance was red at 570 nm using a 96-well multiscanner autoreader (BioRad), with the extraction buffer as a blank.

2.7. [^{3}H]Thymidine incorporation assay

The viable and proliferating cell number was detected by [^{3}H]thymidine incorporation assay. Briefly, HeLa cells (10^4 cells/well of 96-well plate) were incubated with test sample in a final volume of 0.2 mL for 72 hr at 37°. Cell proliferation was measured by thymidine incorporation by adding 50 μL of [^{3}H]thymidine (0.5 μCi/well diluted in Hank's buffered salt solution) last 18 hr. Cells were harvested and washed, and thymidine incorporation was measured in a beta counter (Packard).

2.8. Immunoblot analysis of PARP degradation

Ceramide- and oleandrin-induced apoptosis was examined by proteolytic cleavage of PARP. After treatment, cells were extracted and 50 μg extract proteins was used to analyze PARP by anti-PARP Ab [29]. Apoptosis was represented by the cleavage of 116-kDa PARP into an 85-kDa product [30].

2.9. NF-κB-dependent reporter gene transcription

The effect of oleandrin on ceramide-induced NF-κB-dependent reporter gene transcription was measured as previously described [31]. Briefly, HeLa cells were transiently co-transfected by the calcium phosphate method using 0.5 μg plasmid DNA of each *p65* or dominant negative *IκBα* (*IκBα-DN*), lacking either Ser^{32} or Ser^{36} with NF-κB promoter DNA linked to the heat-stable secretory alkaline phosphatase (*SEAP*) gene and *β-galactosidase* expression plasmid (Promega). By adding control plasmid *pCMVFLAG1* DNA, the total amount of DNA was maintained to 3 μg for each transfection. After 12 hr, cells were treated with 0.2 μg/mL oleandrin and after 4 hr ceramide was added. Twelve hours later, cell culture-conditioned medium was harvested, and 25 μL was analyzed for SEAP activity essentially as per the CLONTECH protocol. This reporter system was specific, because ceramide-induced NF-κB SEAP activity was inhibited by overexpression of IκBα-DN mutant. β-Galactosidase activity was measured simultaneously using a β-galactosidase assay kit (Promega). The relative promoter activity was normalized with β-galactosidase activity as the transfection efficiency.

2.10. Determination of lipid peroxidation

Ceramide- and oleandrin-induced lipid peroxidation was determined by detection of thiobarbituric acid (TBA)-reactive substances, which are the end products of polyunsaturated fatty acids and related esters due to peroxidation. HeLa cells (3×10^6/mL), pretreated without or with oleandrin for 4 hr were stimulated with ceramide for 2 hr. Then, cells were washed with PBS and underwent three cycles of freeze–thawing in 200 μL of water. A 20 μL of aliquot was removed, assayed for protein with Bradford protein determination and remaining samples were mixed with 800 μL of assay mix [0.4% (w/v) TBA, 0.5% (w/v) SDS, 9.4% (v/v) acetic acid, pH 3.5]. Samples were incubated for 60 min at 95°, cooled to room temperature, and centrifuged at 14,000 *g* for 10 min, and the absorbance of the supernatants was read at 532 nm against a standard curve prepared using the MDA standard (10 mM 1,1,3,3-tetramethoxypropane in 20 mM Tris–HCl, pH 7.4). Results were calculated as nmol TBA-reactive substance equivalents/mg of protein [32] expressed as a percentage of TBA-reactive substances above control values. Untreated cells showed 0.568 ± 0.08 nmol TBA-reactive substances/mg protein (subtracting the background absorbance obtained by heating 800 μL of assay mix plus 200 μL water).

2.11. Measurement of reactive intermediates

The production of reactive intermediates mainly oxygen and nitrogen species upon treatment of cells with ceramide was determined by flow cytometry as described [33]. Cells were exposed to dihydrorhodamine 123 (5 mM stock in DMSO) and then stimulated with ceramide at 37° for 2 hr, scrapped, washed, and resuspended in 1 mL D-PBS at 0.5×10^6 concentration. Rhodamine 123 fluorescence intensity resulting from dihydrorhodamine 123 oxidation was measured by a FACScan flow cytometer with excitation at 488 nm and was detected between 515 and 550 mm. Data analysis was performed using LYSYSII software (Becton Dickinson).

2.12. Determination of DNA fragmentation and nuclear fragmentation

HeLa cells were treated with oleandrin (0.2 μg/mL) for 4 hr and then stimulated with ceramide (10 μM) for another 24 hr. Cells were then harvested and DNA was extracted following the method described earlier [34]. Extracted DNA (2.0 μg) was analyzed by electrophoresis on a 2% agarose gel. DNA fragments were visualized with ethidium bromide under UV light. The harvested cells were fixed in 80% methanol, stained with propidium iodide (PI), and viewed under fluorescence microscope [35].

3. Results

In this study, we examined the effect of oleandrin on ceramide-induced signal transduction. The chemical structure of oleandrin is shown elsewhere [36]. It is highly soluble in DMSO. For most of the studies, HeLa cells were used because these cells are well characterized in our laboratory. At the concentration of oleandrin and duration of exposure employed in these studies, there was no effect on cell viability (95.52 ± 2.88, 93.26 ± 5.82, and 92.24 ± 4.76 percentage of cell viability was observed at 0.2, 0.5, and 1.0 μg/mL oleandrin, respectively, for 6 hr of incubation).

3.1. Inhibition of ceramide-induced NF-κB activation by oleandrin

HeLa cells were plated in 60 mm petri dish and pretreated at 60% confluent state with different concentrations of oleandrin for 4 hr and then stimulated with 10 μM ceramide for 1 hr. Nuclear extracts were prepared and assayed for NF-κB by EMSA. As shown in Fig. 1A, ceramide induced 10-fold activation of NF-κB, and oleandrin inhibited this activation in a dose-dependent manner; full inhibition occurred at 0.2 μg/mL and at this concentration itself did not activate NF-κB.

To detect the composition and specificity of the retarded band visualized by EMSA, nuclear extracts from ceramide-activated cells were incubated with antibodies (Abs) p50 (NF-κBI), p65 (RelA), or in combination and 50-fold excess of cold NF-κB then conducted EMSA. Abs to either subunit of NF-κB shifted the band to a higher molecular weight (Fig. 1B), thus suggesting that the ceramide-activated complex consisted of p50 and p65 subunits. Neither PIS nor irrelevant Abs, such as anti-c-*Rel* or anti-cyclin D1, had any effect on the mobility of NF-κB. The complex completely disappeared in presence of cold NF-κB indicating the specificity of NF-κB.

We next examined the effect of changes in the length of incubation with oleandrin on NF-κB activation by ceramide. Cells were incubated with 0.2 μg/mL oleandrin for different times and then stimulated with 10 μM ceramide for 1 hr and assayed for NF-κB. The results in Fig. 1C show that oleandrin inhibited ceramide-induced NF-κB activation with increased time of incubation. At 4 hr, complete inhibition was observed.

To determine the effect of oleandrin on NF-κB activation at even higher concentrations of ceramide, both untreated and oleandrin-pretreated cells were incubated with various concentrations of ceramide (0–25 μM) for 1 hr and then assayed for NF-κB by EMSA. Although the activation of NF-κB by 25 μM ceramide was strong (Fig. 1D), oleandrin completely inhibited it as efficiently as it did at higher concentration of ceramide. These results show that oleandrin is a very potent inhibitor of NF-κB activation.

3.2. Blocking of ceramide-induced NF-κB-dependent reporter gene expression by oleandrin

We have shown that oleandrin blocks ceramide-induced NF-κB activation. To detect the role of oleandrin on NF-κB-dependent reporter gene expression, HeLa cells were transiently co-transfected with NF-κB-containing plasmid linked to the *SEAP* and *β-galactosidase* genes with or without dominant negative IκBα plasmids. Cells either pretreated or treated with different concentrations of oleandrin for 4 hr and then stimulated with 10 μM ceramide for 12 hr. The SEAP activity was assayed in culture supernatant and the pellet was taken for β-galactosidase activity assay. Results are expressed as fold activity over the non-transfected control. The results showed in Fig. 1E that oleandrin decreased ceramide-induced SEAP activity in a dose-dependent manner and IκBα-DN-transfected cells showed no induction of SEAP activity by ceramide suggesting oleandrin's inhibitory action of active NF-κB-dependent gene transcription. The β-galactosidase activity from cell extracts showed almost similar reduction of absorbance (as per Promega protocol) at 420 nm (data not shown) suggesting the transfection control for each treatment.

3.3. Inhibition of ceramide-dependent IκBα degradation and nuclear translocation of p65 subunit of NF-κB by oleandrin

The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα. To determine whether the inhibitory action of oleandrin was due to its effect on IκBα degradation, the cytoplasmic levels of IκBα protein were examined by Western blot analysis and nuclear extracts were assayed for NF-κB. Both untreated and oleandrin-pretreated cells were incubated with ceramide (10 μM) for different times and then assayed for NF-κB. In untreated cells, ceramide activated NF-κB in a time-dependent manner (Fig. 2A). In oleandrin-pretreated cells, however, a little activation of NF-κB was detected after ceramide exposure of up to 60 min. Upon treatment with ceramide, the level of IκBα decreased within 15 min and then reappeared slowly from 30 min, indicating degradation followed by re-synthesis of IκBα

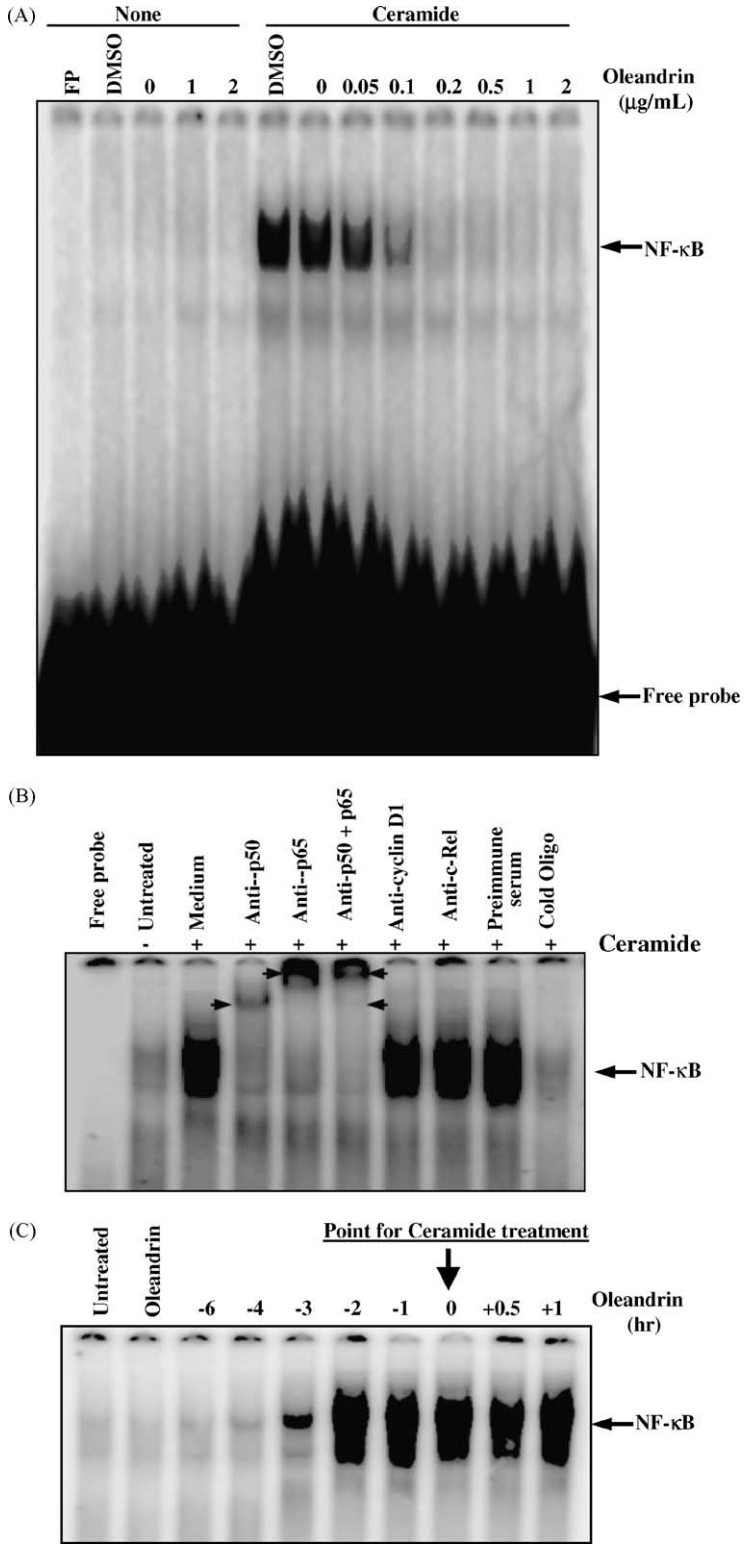

Fig. 1. Effect of oleandrin for the inhibition of ceramide-dependent NF-κB activation. (A) HeLa cells were cultured in 60 mm petri dish at 37° CO_2 incubator. At 60% confluency cells were preincubated for 4 hr with different concentrations (0–2 μg/mL) of oleandrin, followed by 1-hr incubation with 10 μM ceramide. After these treatments, nuclear extracts were prepared and then assayed for NF-κB, as described in Section 2. (B) Nuclear extracts were prepared from untreated or ceramide-treated HeLa cells, incubated for 15 min with different Abs and cold NF-κB oligo, and then assayed for NF-κB, as described in Section 2. (C) Cells were preincubated at 37° with 0.2 μg/mL oleandrin for the indicated times and then stimulated with or without 10 μM ceramide at 37° for 1 hr. After these treatments, nuclear extracts were prepared and then assayed for NF-κB. (D) Cells were preincubated at 37° with 0.2 μg/mL oleandrin for 4 hr and then treated for 1 hr with different concentrations of ceramide at 37° and tested for NF-κB activation. (E) HeLa cells, either pretreated with different concentrations of oleandrin for 4 hr were transiently transfected with NF-κB-containing plasmid linked to the *SEAP* gene with or without dominant negative IκBα plasmid. Cells were then stimulated with 10 μM ceramide for 12 hr. Culture supernatants were assayed for secreted alkaline phosphatase activity as described in Section 2. Results are expressed as fold activity over the vector-transfected control.

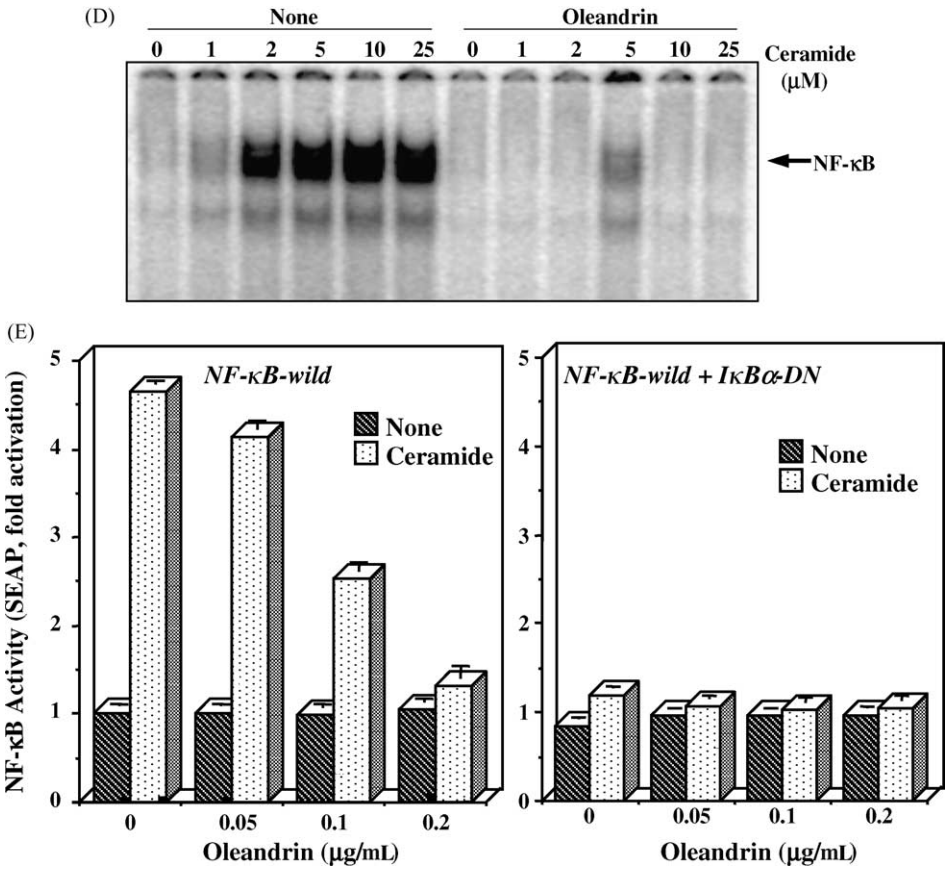

Fig. 1. (*Continued*).

(Fig. 2B, first panel). Oleandrin-pretreated cells exhibited a sustained IκBα band at all time points indicating that oleandrin treatment leads to inhibition of ceramide-induced IκBα degradation. Whether oleandrin affects the ceramide-induced nuclear translocation of the p65 subunit of NF-κB was also examined by Western blot analysis. As shown in Fig. 2B (second panel), upon ceramide treatment, p65 disappeared from the cytoplasm, and oleandrin prevented the disappearance. Oleandrin alone had no effect on p65 level. These results indicate that oleandrin block the nuclear translocation of NF-κB. Level of p50 remained the same in ceramide-induced cells at different times both in presence and in absence of oleandrin (Fig. 2B, third panel). All the lanes exhibited equivalent intensities of tubulin, assayed by Western blot as the loading control (Fig. 2B, lower panels).

3.4. Effect of oleandrin on DNA-binding ability of NF-κB proteins in vitro

It has been shown that *N*-tosyl-L-Phe-chloromethylketone, a serine protease inhibitor, and herbimycin A, a protein tyrosine kinase inhibitor, and caffeic acid phenylethyl ester downregulate NF-κB activation by chemical modification of the NF-κB subunits, thus preventing its binding to DNA [37–39]. To determine whether oleandrin also modifies the DNA binding of NF-κB, we incubated the cytoplasmic extracts with 0.8% deoxycholate (DOC) has shown to dissociate IκBα and release NF-κB for 15 min at room temperature. DOC-treated cytoplasmic extracts were then exposed to various concentrations of oleandrin and assayed for DNA binding by EMSA. As shown in Fig. 2C, oleandrin had no effect on the binding of NF-κB to the DNA. Whether oleandrin modifies the nuclear fraction of NF-κB in ceramide-treated cells was also examined. The nuclear extracts from ceramide-induced cells were treated with various concentrations of oleandrin and then examined for DNA-binding activity by EMSA. Our results in Fig. 2D show that oleandrin did not modify the DNA-binding ability of NF-κB proteins prepared from TNF-treated cells either. Therefore, oleandrin inhibits NF-κB activation through a mechanism different from that of TPCK, herbimycin A, and CAPE.

3.5. Inhibition of NF-κB activation by oleandrin is not cell type specific

As NF-κB activation pathways differ in different cell types [40], we therefore studied whether oleandrin affects other cell type as well. It has been demonstrated that distinct signal transduction pathways could mediate induction in epithelial and lymphoid cells. All the effects of oleandrin described above were conducted with HeLa cells, an epithelial cell line. We found that oleandrin blocks

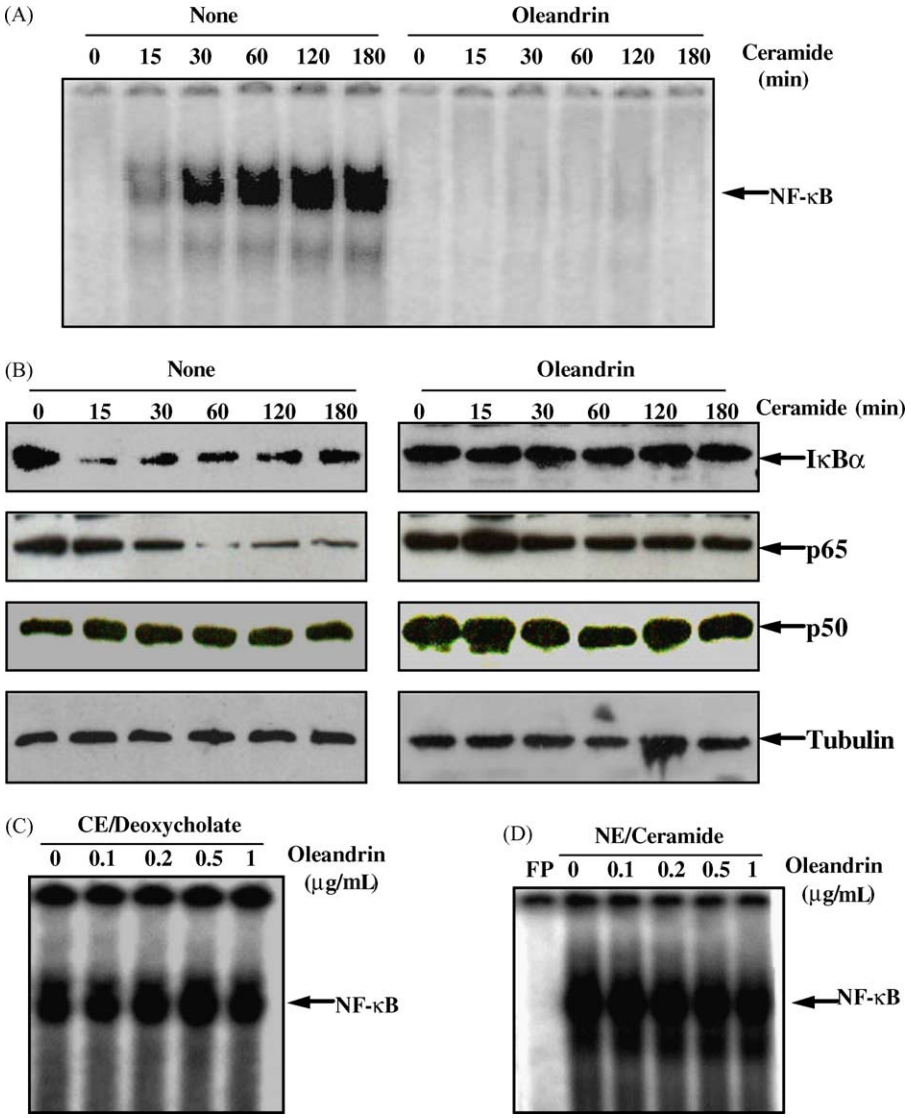

Fig. 2. Effect of oleandrin for the inhibition of ceramide-dependent NF-κB activation and IκBα degradation. Cells were incubated at 37° with 0.2 μg/mL oleandrin for 4 hr and then stimulated with 10 μM ceramide at 37° for different times, as indicated, and then tested for NF-κB activation by EMSA from nuclear extracts (A). Cytoplasmic extracts were assayed for IκBα, p65, p50, and tubulin by Western blot analysis (B). (C) *In vitro* effect of oleandrin on DNA binding of NF-κB protein. Cytoplasmic extracts (CE) from untreated HeLa cells (10 μg protein per sample) were treated with 0.8% DOC for 15 min at room temperature, incubated with different concentrations of oleandrin for 4 hr at room temperature, and then assayed for DNA binding by EMSA. (D) Nuclear extracts (NE) were prepared from 10 μM ceramide-treated HeLa cells; 6 μg per sample NE protein was treated with indicated concentrations of oleandrin for 4 hr at room temperature and then assayed for NF-κB by EMSA.

ceramide-induced NF-κB activation in human T cells, B cells, breast cancer cells, and monkey kidney cells (Fig. 3). An almost complete inhibition of NF-κB in all the cell types suggests that this effect of oleandrin is not restricted to specific cells.

3.6. Inhibition of ceramide-induced AP-1 activation by oleandrin

The activation of JNK causes the activation of AP-1. Ceramide is also a potent activator of AP-1 [41]. Ceramide-induced AP-1 expression was completely inhibited by oleandrin in a dose-dependent manner and complete suppression occurred at 0.5 μg/mL concentration in HeLa cells (Fig. 4A). Oleandrin also suppressed ceramide-induced AP-1 activation in Jurkat and Daudi cells (Fig. 4B). Disappearance of the AP-1 bands by competition with unlabeled nucleotides showed the specificity of the assay procedure employed in this experiment.

3.7. Potentiation of ceramide-induced reactive intermediates generation, lipid peroxidation, and cytotoxicity by oleandrin

Whether oleandrin mediates its effects through suppression of reactive intermediates (reactive oxygen and nitrogen intermediates), production has been examined by flow cytometry. As shown in Fig. 5A, ceramide treatment led

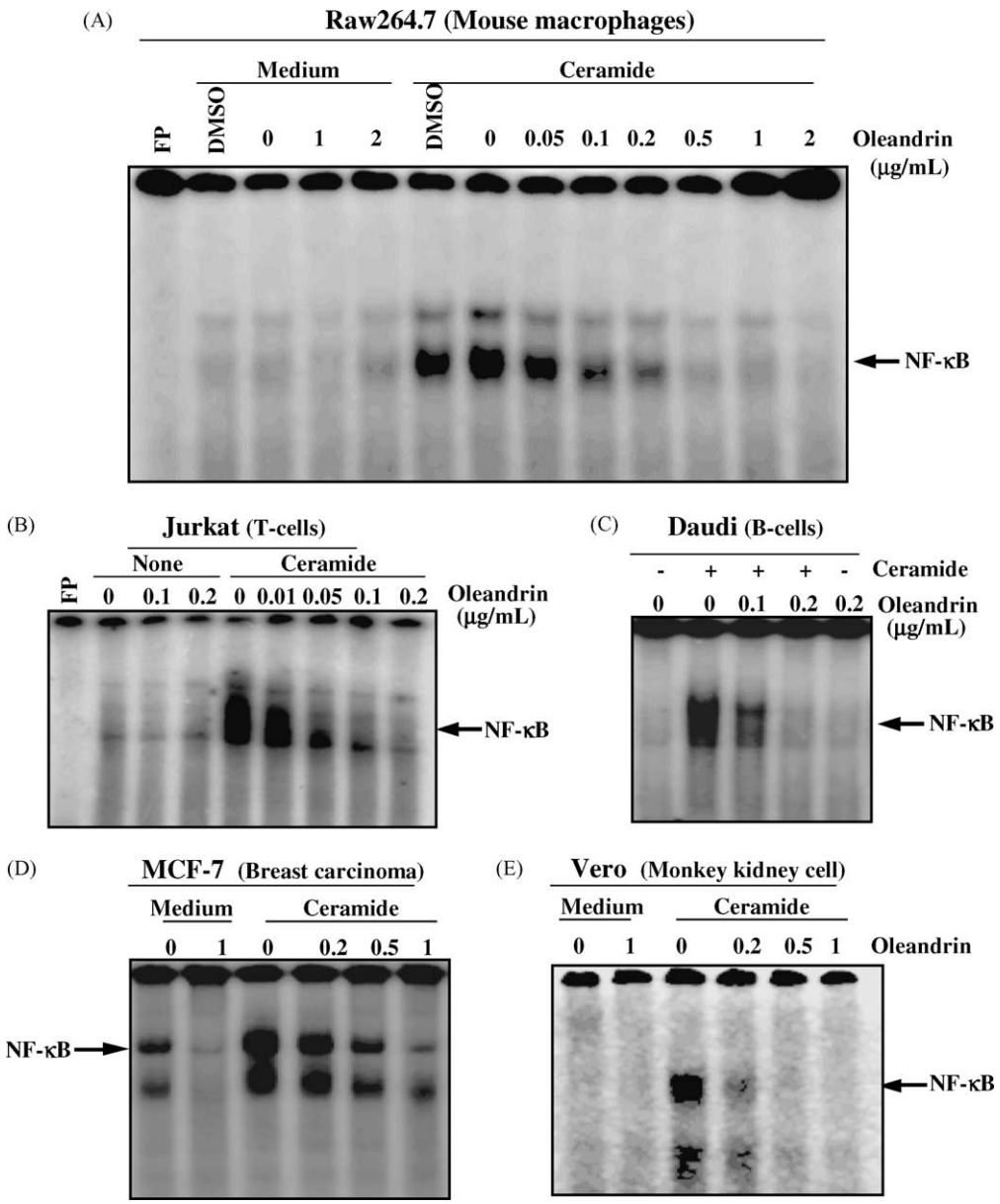

Fig. 3. Effect of oleandrin on activation of NF-κB induced by ceramide in different cell lines. Murine macrophage (A), human Jurkat (B), Daudi (C), MCF-7 (D), and monkey kidney cells (E) were incubated at 37° with 0.2 μg/mL oleandrin for 4 hr and then treated at 37° for 1 hr with 10 μM ceramide. After these treatments, nuclear extracts were prepared and then assayed for NF-κB.

to induction of reactive intermediates generation in a concentration-dependent manner and oleandrin-pretreated cells showed potentiation of reactive intermediates generation. Oleandrin treatment alone at 0.2 μg/mL concentration generated 65% more reactive intermediates than that observed in resting cells. As oleandrin potentiated ceramide-induced reactive intermediates generation, we also examined the effect of oleandrin on ceramide-induced lipid peroxidation in terms of TBA-reactive substances production. Results in Fig. 5B showed that ceramide-induced lipid peroxidation in HeLa cells was in a concentration-dependent manner, and it was potentiated by oleandrin. Oleandrin alone induced 230% lipid peroxidation above unstimulated cells at 0.2 μg/mL concentration (Fig. 5B).

Among all the inducers, ceramide is one of the most potent inducers of apoptosis [1,41]. Whether oleandrin modulates ceramide-induced apoptosis was also investigated. The viable cell number was reflected on cell proliferation and oxidative burst response. The proliferating cell number was assayed by [^{3}H]thymidine incorporation assay. As shown in Fig. 5C, ceramide-induced thymidine incorporation was decreased in a dose-dependent manner. Oleandrin potentiated ceramide-induced thymidine incorporation at any concentration of ceramide. The results indicate that ceramide-mediated cell killing is potentiated by oleandrin. HeLa cells were treated with variable concentrations of ceramide for 72 hr, either in the absence or presence of oleandrin (0.2 μg/mL), and then examined for cytotoxicity by the MTT method. Results in Fig. 5D show

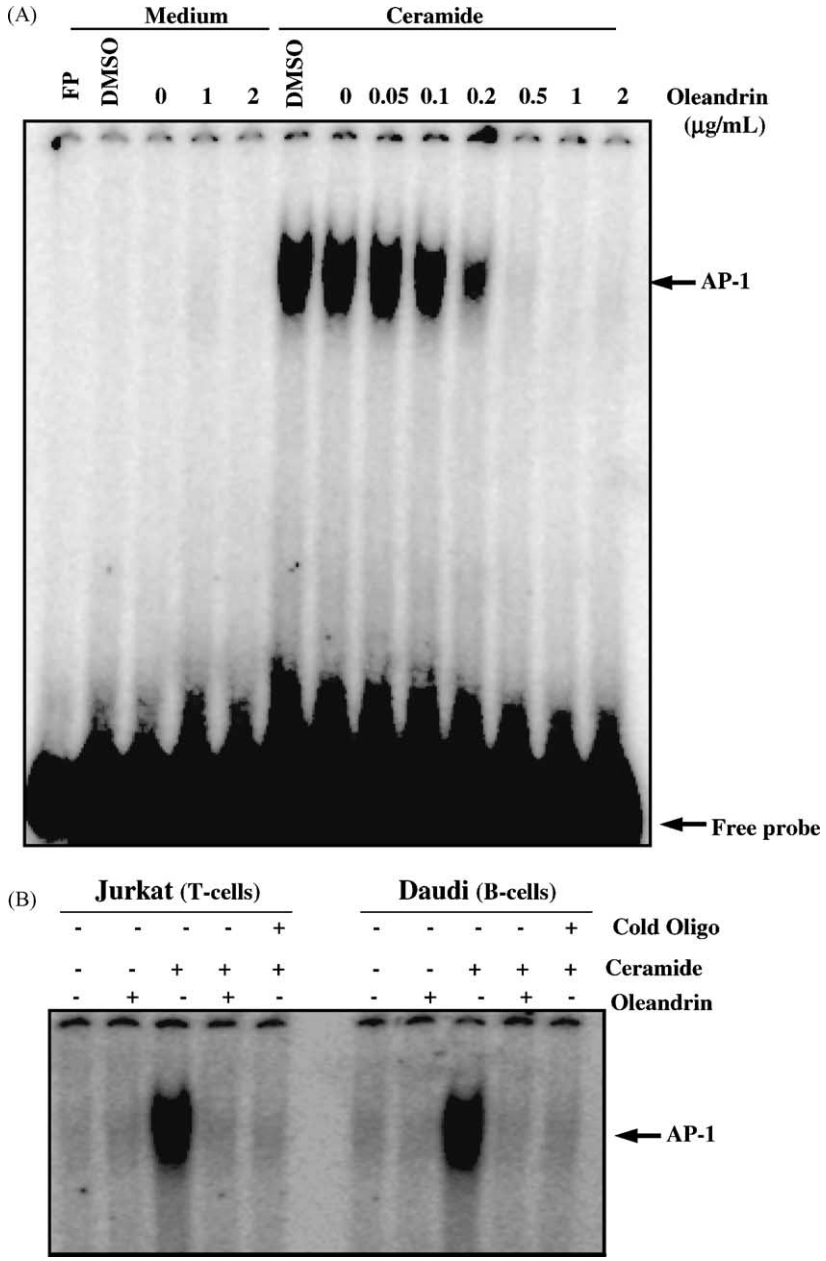

Fig. 4. Oleandrin inhibits ceramide-dependent AP-1 activation. (A) HeLa cells were pretreated with the indicated concentrations of oleandrin for 4 hr at 37°. Then, cells were stimulated with 10 μM ceramide for 1 hr and assayed for AP-1, as described in Section 2. (B) Jurkat and Daudi cells were preincubated at 37° with 0.2 μg/mL oleandrin for 4 hr, and then stimulated with 10 μM ceramide for 1 hr and tested for AP-1 activation with excess 100-fold cold AP-1 oligo in ceramide-stimulated nuclear extract.

that the cytotoxic effects of ceramide in HeLa cells were dose dependent, with almost 45% killing occurred at 2 μM concentration of the ceramide. This cytotoxicity was potentiated by treatment of cells with 0.2 μg/mL oleandrin and this concentration of oleandrin alone induced 60% cell killing. To show that the cell death mediated by oleandrin was not due to necrosis, the cytosolic marker enzyme lactate dehydrogenase (LDH) was assayed from the culture supernatant of oleandrin-treated cells [27]. Culture supernatant from 0.5 μg/mL oleandrin-treated for 0, 24, 36, and 48 hr when incubated with substrate solution (0.23 M sodium pyruvate and 5 mM NADH in 0.1 M phosphate buffer, pH 7.5) did not decrease absorbance at 420 nm significantly (data not shown) indicating cell death was not due to leakage of cytoplasm, i.e necrosis.

As oleandrin induces reactive oxygen intermediate (ROI) generation and also apoptosis, the role of ROI was detected in manganese superoxide dismutase (Mn-SOD) overexpressed MCF-7 cells. Both MCF-7 *neo* (control vector) and *Mn-SOD* cells were treated with different concentrations of oleandrin for 4 hr followed by stimulation with ceramide (10 μM) for 72 hr, subsequently cell viability was assayed by MTT uptake. Oleandrin potentiated ceramide-induced cell viability in MCF-7 (*neo*) cells (Fig. 5E1).

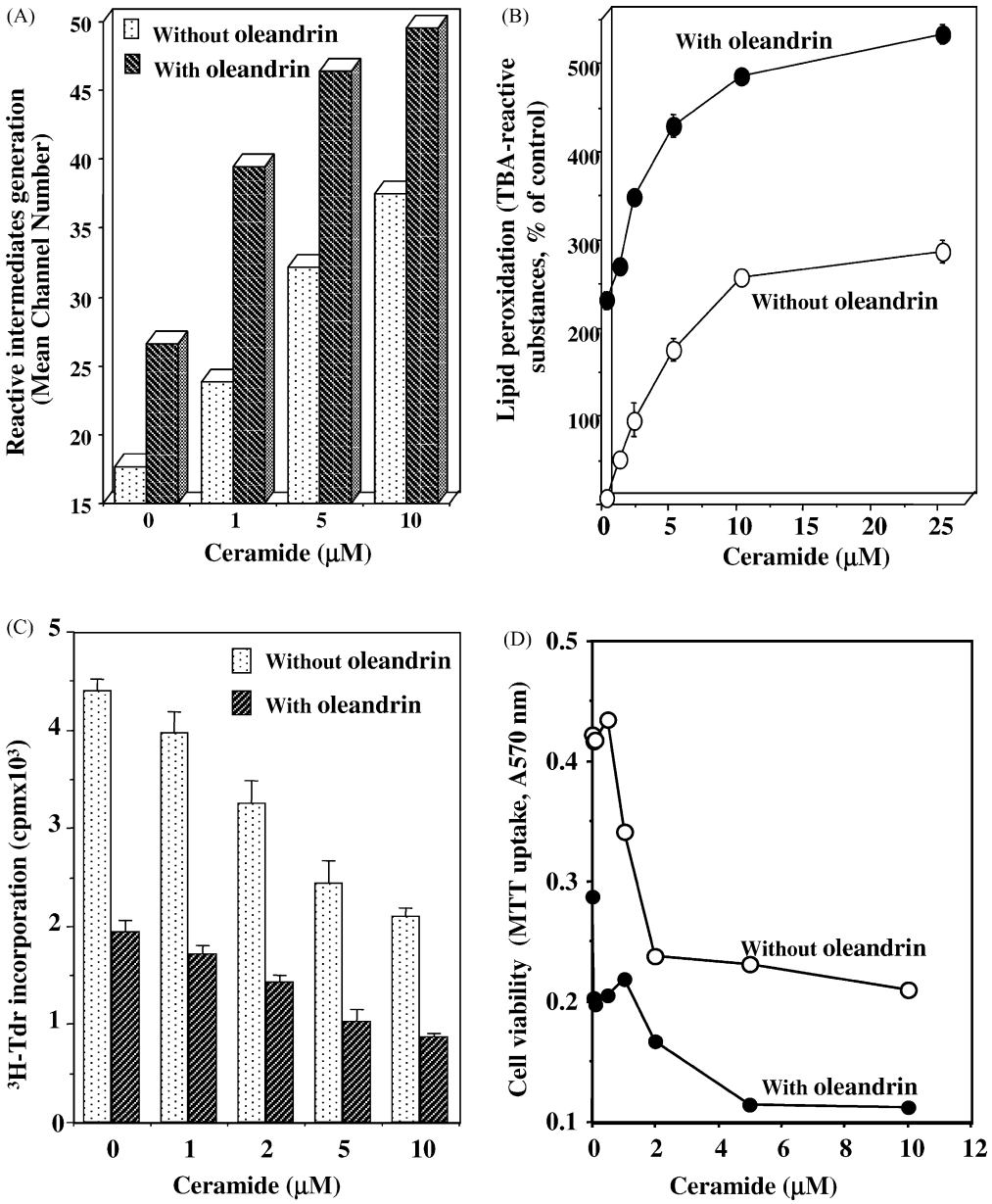

Fig. 5. (A) Effect of oleandrin on ceramide-induced reactive intermediates generation. HeLa cells were treated with 0.2 μg/mL oleandrin for 4 hr and then exposed to different concentration of ceramide for 2 hr in a CO_2 incubator. Reactive intermediates production was then determined by the flow cytometry method, as described in Section 2. The results shown are representative of two independent experiments. (B) Effect of oleandrin on ceramide-induced lipid peroxidation. HeLa cells were treated with 0.2 μg/mL oleandrin for 4 hr and then exposed to different concentration of ceramide for 6 hr in a CO_2 incubator. Cell pellets were extracted by three times freeze–thaw method with the addition of 200 μL of water and 180 μL sample was used to measure malondialdehyde by TBA–SDS buffer as described in Section 2. The results shown are representative of two independent experiments. (C) Effect of oleandrin and ceramide in [^{3}H]thimidine incorporation. HeLa cells treated with 0.2 μg/mL oleandrin for 4 hr and then stimulated with different concentrations of ceramide for 72 hr at 37°, in a CO_2 incubator. Last 18 hr cells were incubated with 0.5 μCi ([^{3}H]thimidine) and then assayed for [^{3}H]thymidine incorporation assay. Results were mean cpm ± SD of triplicate assays. (D) Effect of oleandrin on ceramide-induced cytotoxicity. HeLa cells, untreated or pretreated with 0.2 μg/mL oleandrin for 4 hr at 37° were incubated with the indicated concentrations of ceramide for 72 hr at 37°, in a CO_2 incubator. Then, MTT assayed and absorbance was taken at 570 nm. The result indicated was mean O.D. of triplicate assays. (E) Effect of oleandrin on ceramide-induced cytotoxicity. MCF-7 (*neo* and *Mn-SOD*) cells were treated with different concentrations of oleandrin for 4 hr and then stimulated with ceramide (10 μM) for 72 hr. Cell viability was assayed by MTT uptake. Results were mean absorbance of triplicate assays. (F) Effect of oleandrin on ceramide-induced PARP cleavage. HeLa cells, untreated or pretreated with 0.2 μg/mL oleandrin for 4 hr at 37°, were incubated with the indicated concentrations of ceramide for 24 hr at 37°, in a CO_2 incubator. Then, cell extracts were prepared and 50 μg protein was analyzed by Western blot using anti-PARP mAb. The bands were located at 116 and 85 kDa. (G) Effect of oleandrin on ceramide-induced DNA fragmentation. HeLa cells, untreated or pretreated with 0.2 μg/mL oleandrin for 4 hr at 37°, were incubated with 10 μM ceramide for 24 hr at 37°, in a CO_2 incubator. Then, cells were scrapped, washed, DNA was prepared, and 2.0 μg DNA was analyzed by 2% agarose gel. (H) Effect of oleandrin on ceramide-induced nuclear fragmentation. HeLa cells, untreated or pretreated with 0.2 μg/mL oleandrin for 4 hr at 37°, were incubated with 10 μM ceramide for 24 hr at 37°, in a CO_2 incubator. Then, cells were scrapped, washed, fixed with methanol, and stained with PI. Cells were then taken in slide and visualized in fluorescence microscope. The picture showed the phase contrast view of untreated (a), ceramide-treated (b), oleandrin-treated (c), and oleandrin-pretreated followed by ceramide-treated (d) cells. Same cells were shown as untreated (a1), ceramide-treated (b1), oleandrin-treated (c1), and oleandrin-pretreated followed by ceramide-treated (d1) under fluorescence microscope view.

In MCF-7 (*Mn-SOD*) cells, oleandrin induced cell death by 10–25% at 0.2–1 μg/mL concentrations (Fig. 5E2). Ceramide partially induced cell death in MCF-7 (*Mn-SOD*) cells and about 0–10% potentiation of cell death was observed at varying concentrations of oleandrin-pretreated MCF-7 (*Mn-SOD*) cells. The data indicate that detoxification of ROI partially block oleandrin-mediated cell death.

3.8. Potentiation of ceramide-induced PARP cleavage and DNA fragmentation by oleandrin

Because of the cytotoxic effects of ceramide and oleandrin or in combination, we also examined the effect of oleandrin on ceramide-induced caspase activation in the form of PARP protein cleavage. As shown in Fig. 5F, ceramide induced partial cleavage of PARP at 10 μM, and oleandrin-pretreated cells showed potentiation of PARP cleavage, even at 1 μM concentration of ceramide complete cleavage of PARP was observed. Oleandrin and ceramide each induced DNA fragmentation in HeLa cells, and in combination they potentiated DNA fragmentation (Fig. 5G).

DNA fragmentation was also detected in oleandrin- and ceramide-induced cell nuclei as detected by PI staining, and oleandrin enhanced ceramide-induced DNA fragmentation (Fig. 5H).

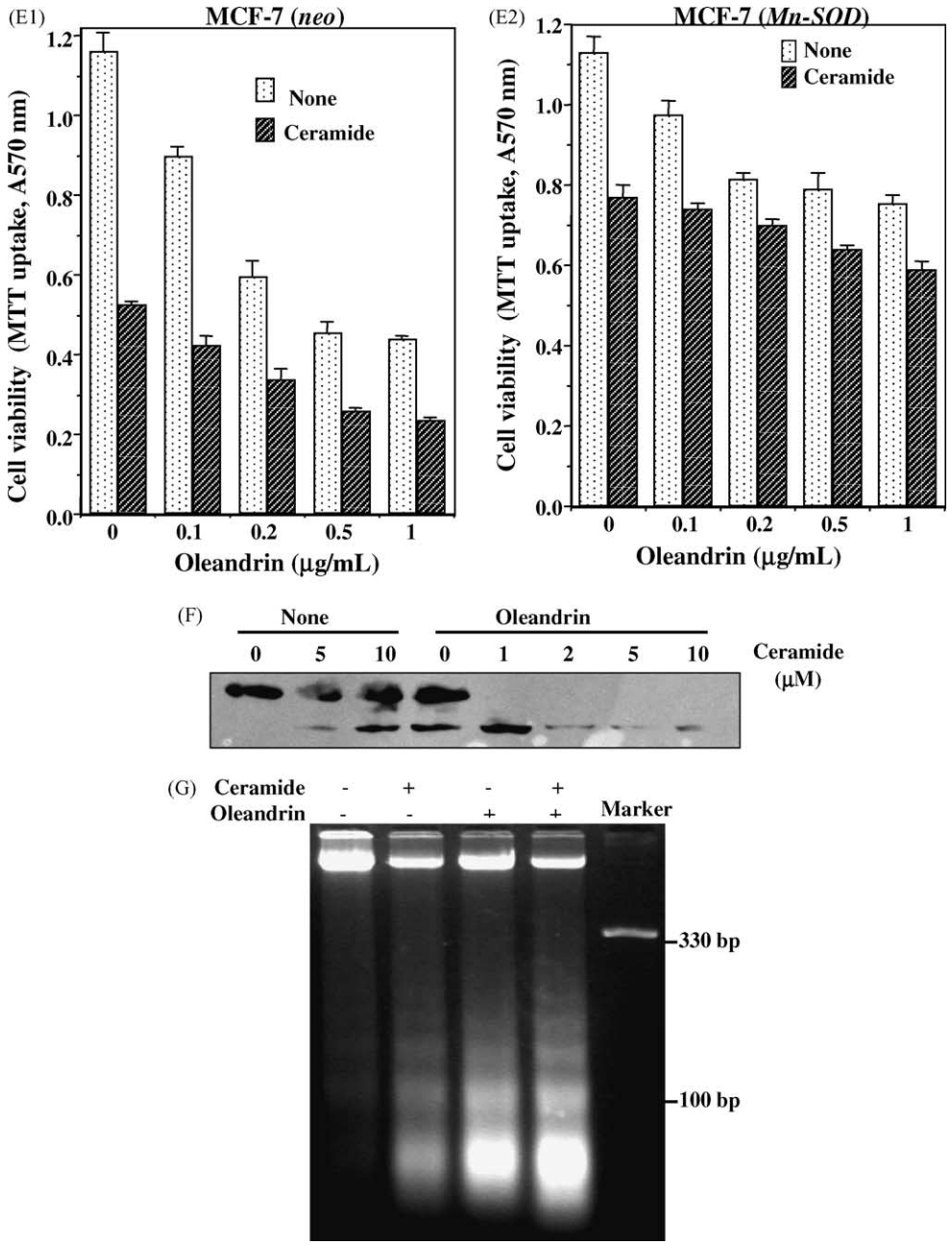

Fig. 5. (*Continued*)

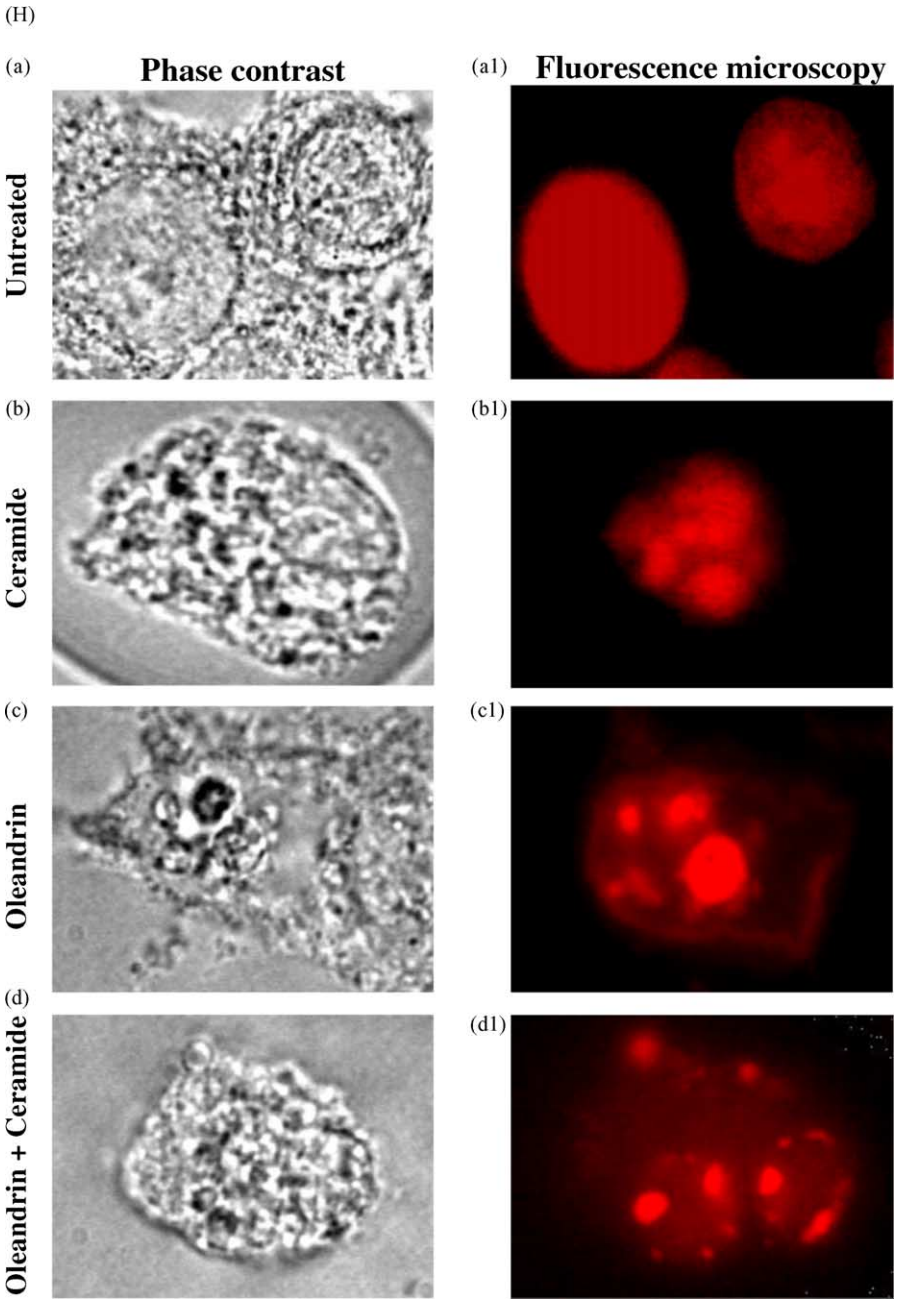

Fig. 5. (*Continued*).

3.9. Potentiation of cell death by oleandrin in NF-κB overexpressed cells

In order to detect the role of NF-κB on oleandrin-mediated cell death, HeLa cells were co-transfected either vector or p65 plasmids with β-galactosidase and SEAP reporter DNA. As shown in Fig. 6A, p65-transfected HeLa cells showed NF-κB activation and oleandrin downregulated NF-κB activation. The SEAP activity was observed in p65-transfected cells about 7-fold compared to vector control. Oleandrin considerably downregulated the SEAP activity (Fig. 6B) similar to NF-κB activation. The activity of β-galactosidase in all sets were equal (data not shown). Vector control and p65-transfected cells were incubated with oleandrin (0.2 μg/mL) for 4 hr and then stimulated with ceramide (10 μM) for 36 hr and the cytotoxicity was assayed by MTT uptake. The cell viability was decreased by oleandrin, ceramide, or in combination in vector-transfected cells (Fig. 6C) which was similar to non-transfected HeLa cells as shown previously. In NF-κB overexpressed cells, ceramide was unable to induce cell death but oleandrin alone or in combination of oleandrin and ceramide cell death was observed (Fig. 6C). The data indicate that oleandrin-mediated cytotoxicity is due to downregulation of NF-κB.

3.10. Effect of oleandrin on ceramide-induced NF-κB activation, cytotoxicity, and nuclear fragmentation in isolated PBMC and neutrophils

To detect the effect of oleandrin in primary cells, PBMC and neutrophils were isolated from whole human blood. The cells were treated with different concentrations of oleandrin for 4 hr followed by incubation of ceramide (10 μM) for 24 hr. Then, cells were examined for cytotoxicity by MTT method. The U937 cells showed the cell killing by oleandrin in a dose-dependent manner and ceramide potentiated the cell killing (Fig. 7A). In PBMC and neutrophils oleandrin unable to induce cell killing at any concentrations, and surprisingly, ceramide or in combination with oleandrin were unable to kill these primary cells as shown by MTT assay (Fig. 7A).

To detect the effect of oleandrin in ceramide-induced NF-κB activation in primary cells, PBMC and neutrophils were treated with different concentrations of oleandrin for 4 hr and then stimulated with 10 μM ceramide for 2 hr. Nuclear extracts were prepared and analyzed for NF-κB by EMSA. The results showed in Fig. 7B that ceramide potentially induced NF-κB in these cells but oleandrin was unable to block NF-κB activation at any concentration.

To detect the oleandrin-mediated nuclear fragmentation neutrophils, pretreated with oleandrin (0.2 μg/mL) for 4 hr were stimulated with ceramide (10 μM) for 24 hr. Nuclear fragmentation was assayed by PI staining method. As shown in Fig. 7C, the nuclear fragmentation was not observed upon treatments of oleandrin, ceramide, or in combination in neutrophils indicating oleandrin or ceramide alone or in combination are unable to induce cell death.

4. Discussion

Because several *in vitro* and *in vivo* activities assigned to oleandrin require suppression of NF-κB activation, we tested the hypothesis that oleandrin directly blocks NF-κB activation and potentiates apoptosis. In the present report, we demonstrated that oleandrin can block NF-κB activation, as determined by consensus DNA binding and dependent reporter gene transcription. We found that oleandrin is indeed a potent inhibitor of ceramide-induced activation of NF-κB, and this inhibition is not cell line specific. Beside NF-κB, oleandrin blocked AP-1 activation. Oleandrin's ability is not only to increase but also potentiates ceramide-induced ROI generation, lipid peroxidation, and DNA fragmentation. In NF-κB overexpressed cells oleandrin was able to inhibit NF-κB activation and induce apoptosis. Surprisingly, in primary cells oleandrin was unable to block NF-κB activation as well as apoptosis.

Recent evidence indicates that different inflammatory agents may activate NF-κB through mechanisms that consist of some overlapping and some non-overlapping steps [40,42,43]. Ceramide is produced by the induction of those inflammatory agents, activates NF-κB and AP-1 and potentiates inflammation. Ceramide-induced NF-κB and AP-1 activation was inhibited by oleandrin, suggesting its anti-inflammatory property. Manna *et al*. previously

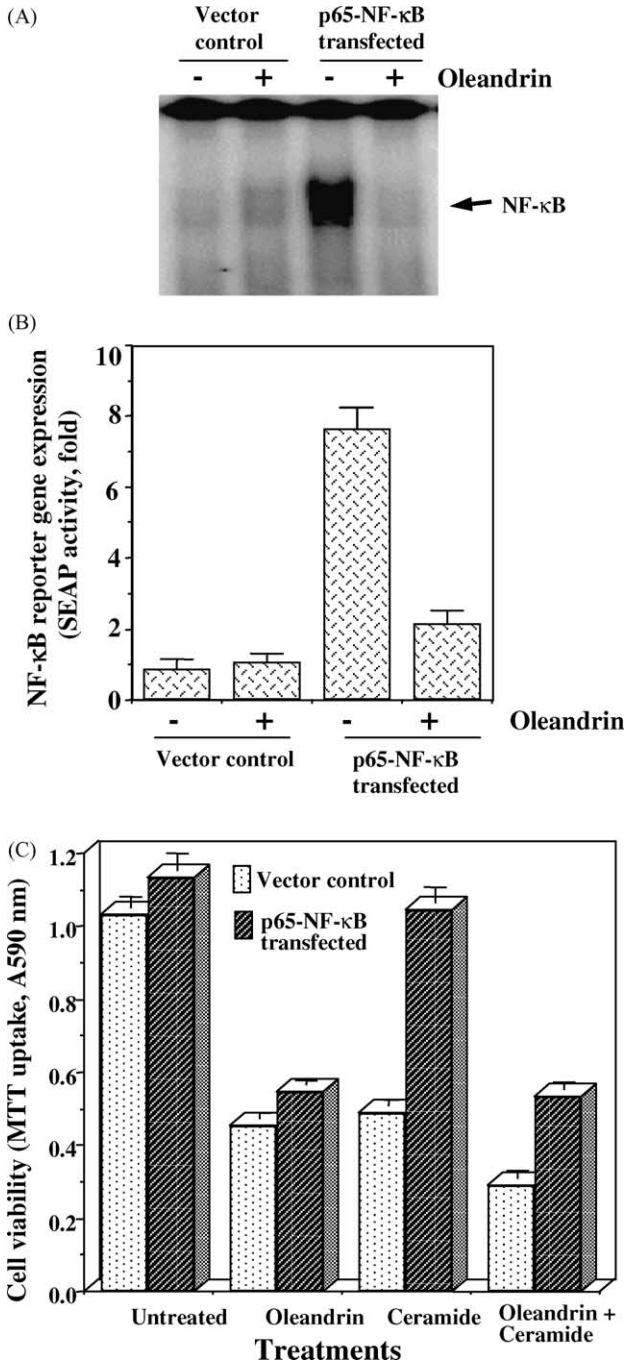

Fig. 6. Effect of oleandrin on NF-κB activation and cell viability in NF-κB overexpressed cells. HeLa cells were transfected with control plasmid *pCMVFLAG1* or *p65-NF-κB* DNA with *β-galactosidase* and *SEAP* reporter genes for 12 hr and then treated with oleandrin (0.2 μg/mL) for 4 hr. Then, NF-κB was assayed from nuclear extracts (A), SEAP was assayed from culture supernatant (B). Vector control and p65-transfected cells were treated with oleandrin (0.2 μg/mL) for 4 hr and then stimulated with ceramide (10 μM) for 36 hr. Cell viability was assayed by MTT dye uptake (C). Results are mean absorbance of triplicate assays.

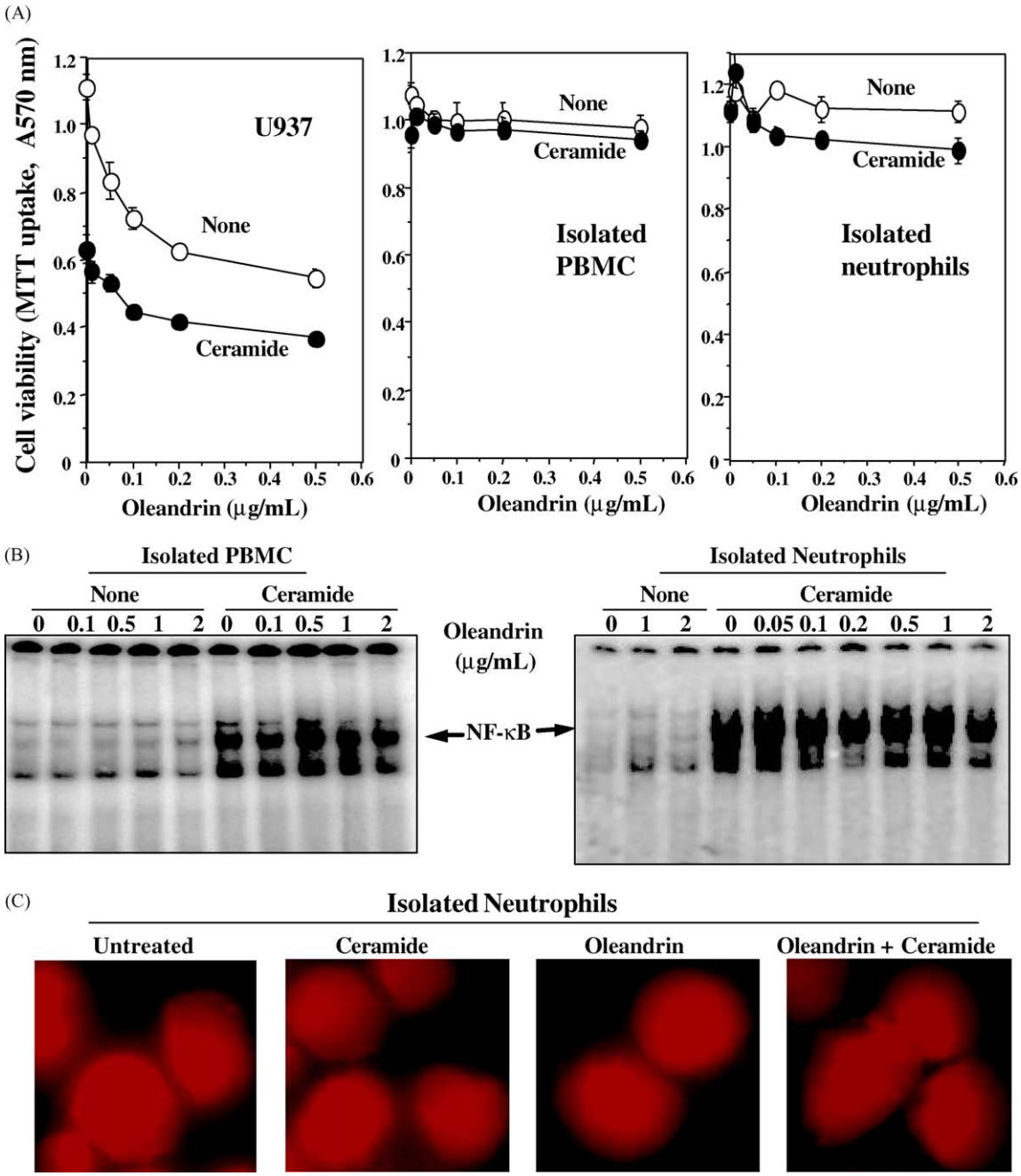

Fig. 7. (A) Effect of oleandrin on ceramide-induced cytotoxicity in U937 cells, PBMC, and neutrophils. Isolated PBMC and neutrophils, incubated with different concentrations of oleandrin for 4 hr were stimulated with 10 μM ceramide for 24 hr. Then, MTT was assayed. (B) Effect of oleandrin on ceramide-induced NF-κB activation in PBMC and neutrophils. Cells, incubated with different concentrations of oleandrin, were stimulated with ceramide (10 μM) for 2 hr. The nuclear extracts were prepared and assayed for NF-κB. (C) Effect of oleandrin on ceramide-induced nuclear fragmentation in neutrophils. Isolated neutrophils incubated with oleandrin (0.2 μg/mL) for 4 hr and then stimulated with ceramide (10 μM) for 24 hr. The cells were then fixed in 70% methanol, stained with PI, and analyzed under fluorescence microscope to detect fragmented nuclei.

reported that oleandrin, when pretreated for 1 hr was unable to protect ceramide-induced NF-κB activation [36]. When cells were pretreated with oleandrin for 4 hr, then ceramide-induced NF-κB activation was completely blocked (Fig. 1C). How oleandrin blocks ceramide-induced NF-κB activation is at present unclear. Most inhibitors of NF-κB activation mediate their effects through suppression of phosphorylation and degradation of IκBα (e.g. curcumin and silymarin) [33,44,45]. Caffeic acid phenethyl ester and resveratrol block NF-κB activation without any effect on IκBα phosphorylation or degradation [39,46] but by inhibiting p65 phosphorylation followed by translocation to nucleus [47]. We showed that oleandrin does not interfere with the NF-κB consensus DNA-binding site, but that it did block the ceramide-induced translocation of NF-κB to nucleus and reporter gene transcription. It has been reported

that oleandrin blocks TNF-induced NF-κB activation through inhibition of IκBα phosphorylation, degradation, and nuclear translocation of NF-κB [36]. Similarly, oleandrin blocked ceramide-induced IκBα degradation. Phosphorylation of IκBα is regulated by different kinases (IκB kinase α, β, or γ, NIK, and mitogen-activated protein kinase kinase-1) [48–53]. Therefore, it is possible that some of these kinases may be involved in oleandrin-mediated inhibition of IκBα phosphorylation. Oleandrin also blocked ceramide-induced AP-1 activation. Most agents that activate NF-κB also activate AP-1 [23,24].

Ceramide-induced cytoxicity and caspase activation was also potentiated by oleandrin. Because NF-κB activation has been shown to play an anti-apoptotic role [5], the suppression of NF-κB by oleandrin may seem to be the cause of potentiation of apoptosis, suggesting NF-κB's role in apoptosis. Previously, it was reported that oleandrin alone did not induce reactive intermediates generation or lipid peroxidation in 1-hr incubation time [36] but 4-hr treatment with oleandrin alone showed generation of reactive intermediates and lipid peroxidation (Fig. 5A and B). Though ROI generation causes NF-κB and AP-1 activation, but we did not find any NF-κB activation by oleandrin alone, still it induced reactive intermediates generation. Long time exposure with these toxic agents induced by oleandrin even by ceramide might have deleterious effect to induce cell death as shown by different apoptosis assay. MCF-7 cells, overexpressed with Mn-SOD protected almost 50% oleandrin-mediated cytotoxicity (Fig. 5E) indicating that oleandrin-induced ROI generation might cause apoptosis partially. Overexpression of other ROI detoxifying enzymes may be helpful to address the actual role of oleandrin-mediated apoptosis. Our discovery that oleandrin potentiates ceramide-induced reactive intermediates generation and lipid peroxidation explains the mechanism by which oleandrin exerts its effects.

Several reports indicate that constitutively or induced NF-κB induces resistance to apoptosis stimulated by a wide variety of agents [9,12,23,54]. Because oleandrin is known to be cytotoxic to various tumor cells [55,56], it is possible that this toxicity is mediated through the suppression of NF-κB. HeLa cells, when overexpressed with p65-NF-κB showed downregulation of NF-κB by oleandrin and induction of cell death (Fig. 6), indicating the involvement of NF-κB for oleandrin-mediated apoptosis. Ceramide was unable to induce cell death in NF-κB overexpressed cells indicating the different mechanism involved for ceramide- and oleandrin-mediated apoptosis. Surprisingly, oleandrin was unable to downregulate NF-κB and thereby to induce cell death in primary cells. How the primary cells are protected from oleandrin-mediated cell signaling and cell death needs to be elucidated. Oleandrin potentiates ceramide-induced cytotoxicity as shown by MTT assay, PARP cleavage, DNA fragmentation, and PI-stained cells possibly by inhibiting NF-κB and AP-1 activation. Oleandrin also potentiates ceramide-induced lipid peroxidation and reactive intermediates generation influencing cells towards apoptosis as indicated through DNA fragmentation.

Several genes, such as cytokines, cyclooxygenase-2, metalloproteases, urinary plasminogen activator, and cell surface adhesion molecules, are involved in tumor promotion those are regulated by NF-κB [57–61]. As oleandrin blocked NF-κB-dependent reporter gene expression, so it may play a critical role in carcinogenesis and inflammation exhibiting anticarcinogenic and anti-inflammatory effects. Inhibiting NF-κB by adenoviral IκBα or proteosome inhibitors are currently being tested to overcome chemotherapy-induced resistance [9]. Therefore, NF-κB suppressive ability of oleandrin could be exploited by combination with chemotherapy. Evidence suggests that ceramide generated inside the cells by the action of sphingomyelinases in response to a variety of cytotoxic agents may be used by cells to propagate apoptotic signals [62]. It is possible that the intracellular pool of sphingomyelin used for signaling is decreased leading the cells towards an anti-apoptotic situation. An additional justification for the use of oleandrin may come from its ability to suppress AP-1, which is known to play a critical role in tumorigenesis.

Because replication of certain viruses, such as human immunodeficiency virus-1, is also dependent on NF-κB [2–5], oleandrin may also abolish viral replication. Due to its ability to suppress COX-2 through NF-κB, aspirin is beneficial for preventing colon cancer [63]. This suggests that oleandrin may also prove to be beneficial for colon cancer. Oleandrin's ability to suppress ceramide-induced NF-κB, AP-1, and other cellular responses may provide the molecular basis for the anticarcinogenic properties of oleandrin. Cardiac glycosides oleandrin, ouabain, and digoxin induce apoptosis in androgen-independent human prostate cancer cell lines *in vitro* by sustaining intracellular Ca^{2+} for long time [56]. Recently, oleandrin was also found to inhibit the fibroblast growth factor-2 export through membrane interaction with the Na^{+},K^{+}-ATPase pump in human prostate cancer cell lines and arresting cell growth [20]. In addition, adenovirus-enforced overexpression of mitochondrial superoxide dismutase gene therapy has been used to treat ischemia/reperfusion injury of the liver through the downregulation of NF-κB and AP-1 activation [64]. Our results indicate that suppressive effects of oleandrin on NF-κB and AP-1 activation and on other cellular responses may also explain its protective effects on liver and against cardiovascular diseases. Oleandrin's inhibitory activity on NF-κB activation and stimulatory activities on apoptosis were not observed in primary cells, indicating its less or almost no side effects if it is designed for therapy. Our results suggest that oleandrin may also have applications for various other diseases, including inflammation and arthritis, where NF-κB activation has been shown to mediate pathogenesis. These possibilities require further investigation in detail. The apoptotic activity of oleandrin in tumor, but not in primary cells, indicates its potential anticancer property with less side effects.

Acknowledgments

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