Oleandrin Suppresses Activation of Nuclear Transcription Factor-κB, Activator Protein-1, and c-Jun NH₂-Terminal Kinase

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ABSTRACT

Agents that can suppress the activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) may be able to block tumorigenesis and inflammation. Oleandrin, a polyphenolic cardiac glycoside derived from the leaves of Nerium oleander, is a candidate NF-κB and AP-1 modulator. We investigated the effect of oleandrin on NF-κB activation induced by inflammatory agents. Oleandrin blocked tumor necrosis factor (TNF)-induced activation of NF-κB in a concentration- and time-dependent manner. This effect was mediated through inhibition of phosphorylation and degradation of IκBα, an inhibitor of NF-κB. A proprietary hot water extract of oleander (Anvirzel) also blocked TNF-induced NF-κB activation; subsequent fractionation of the extract revealed that this activity was attributable to oleandrin. The effects of oleandrin were not cell type specific, because it blocked TNF-induced NF-κB activation in a variety of cells. NF-κB-dependent reporter gene transcription activated by TNF was also suppressed by oleandrin. The TNF-induced NF-κB activation cascade involving TNF receptor 1/TNF receptor-associated death domain/TNF receptor-associated factor 2/NF-κB-inducing kinase/IκBα kinase was interrupted at the TNF receptor-associated factor 2 and NF-κB-inducing kinase sites by oleandrin, thus suppressing NF-κB reporter gene expression. Oleandrin blocked NF-κB activation induced by phorbol ester and lipopolysaccharide. Oleandrin also blocked AP-1 activation induced by TNF and other agents and inhibited the TNF-induced activation of c-Jun NH₂-terminal kinase. Overall, our results indicate that oleandrin inhibits activation of NF-κB and AP-1 and their associated kinases. This may provide a molecular basis for the ability of oleandrin to suppress inflammation and perhaps tumorigenesis.

INTRODUCTION

NF-κB, a nuclear transcription factor, was first identified in 1986 by Sen and Baltimore, as reported by Baldwin (1). Extensive research during the past few years has indicated that this factor regulates the expression of various genes that play critical roles in apoptosis, viral replication, tumorigenesis, various autoimmune diseases, and inflammation (2, 3). Because of its role in the pathogenesis of various diseases, NF-κB is a current target of interest used by various pharmaceutical companies (4). As part of the stress response, NF-κB is activated in response to various inflammatory stimuli including cytokines, mitogens, bacterial products, viral proteins, and apoptosis-inducing agents (2, 3). Under normal conditions, NF-κB is present in the cytoplasm as an inactive heterotrimer consisting of p50, p65 and IκBα subunits. On activation, degradation of IκBα exposes nuclear localization signals on the p50-p65 heterodimer, leading to nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene transcription.

NF-κB is an ideal target for anticancer drug development for several reasons: (a) activation of NF-κB has been shown to block apoptosis and promote proliferation (5, 6); (b) constitutive expression of NF-κB in tumor cells induces proliferation (7, 8); (c) the tumor microenvironment can induce NF-κB activation (9); and (d) NF-κB activation induces resistance to chemotherapeutic agents (10, 11); and (e) several genes involved in tumor initiation, tumor promotion, and metastasis are regulated by NF-κB (12). Thus, because of its role in tumorigenesis, we have attempted to identify specific inhibitors of NF-κB from natural sources.

Most agents that activate NF-κB also activate another transcription factor, AP-1, which is protein heterodimer composed of the basic region leucine zipper protein superfamily, specifically, Fos and Jun, and activating transcription factor proteins (13). AP-1 activation requires phosphorylation of JNK (13). AP-1 activity has been implicated in various cellular functions including proliferation, transformation, and differentiation. High AP-1 has been shown to be involved in tumor promotion and progression of various types of cancers and induction of resistance to chemotherapeutic agents. Therefore, inhibitors of AP-1 have been shown to have potential in blocking carcinogenesis (14).

Research within the last few years has shown that certain fruits, vegetables, herbs, and plants exhibit chemopreventive effects (Ref. 15 and references therein). As many as 70% of the therapeutic drugs in use today are derived from plants. One potential candidate for preventive and therapeutic use is oleandrin, a polyphenolic cardiac glycoside derived from the leaves of Nerium oleander. Although it has been used to treat congestive heart failure, oleandrin is known to be toxic to a wide variety of tumor cells (16–18). The mechanism of its cytotoxic effects, however, is not understood. Because NF-κB and AP-1 are known to play major roles in cell proliferation, tumor promotion, and drug resistance (11–14), it was hypothesized that the effects of oleandrin were 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 various tumor promoters, including TNF, phorbol ester (PMA), LPSs, H2O2, okadaic acid, and ceramide were investigated. The results demonstrate that oleandrin is a potent inhibitor of NF-κB and AP-1 activated by these inflammatory agents in a wide variety of different cell types. The effect of oleandrin on transcription factors was mediated through inhibition of JNK and MEK activation.

MATERIALS AND METHODS

Materials. Highly purified oleandrin was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in 100% DMSO at 1 mg/ml (M, 576.73). The purity of oleandrin was checked by liquid chromatography attached to mass spectrometer and was found to be >99% pure. All subsequent dilutions were made in media. The sources of all other reagents were as described (19–21).
Expression plasmid encoding FLAG-tagged NIK (22) was kindly provided by D. Wallach (Weizmann Institute of Science, Rehovot, Israel).

**Cell Lines.** The cell lines U937 (human histiocytic lymphoma), CAOV3 (human ovarian cells), HeLa (human epithelial cells), Jurkat (human T cells), and L-929 (murine fibroblast cells) were obtained from the American Type Culture Collection (Rockville, MD). Most of the studies were performed with human histiocytic lymphoma U937 cells because various cellular responses in these cells have been well characterized in our laboratory (20).

**NF-κB Activation Assay.** To assay NF-κB activation, EMSAs were performed essentially as described (23). Briefly, nuclear extracts prepared from treated cells (2 × 10^5/ml) were incubated with 32P end-labeled double-stranded NF-κB oligonucleotide (6 μg of protein with 16 fmol DNA) from the HIV-LTR-5′-TTGTTACAGGGGACTTTGCGGCAGG-3′ (bold indicates NF-κB binding sites) for 15 min at 37°C, and the DNA-nucleoprotein complex formed was resolved from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTAAACGCTTCACTCTTTCCCGTGCTACCTTTCCAGGG GCGGCTG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. The dried gels were visualized, and radioactive bands were quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

**API Activation Assay.** Activation of API was determined as described (20). Briefly, 6 μg of nuclear extract, prepared as indicated above, were incubated with 16 fmol of the 32P-end-labeled API consensus oligonucleotide 5′-CGCTTTTGAGCTGACCTTACCGGCAGGAA GAAA-3′ (bold indicates API binding site) for 15 min at 37°C 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.

**Western Blot for IκBα.** To assay IκBα, postnuclear (cytoplasmic) extracts were prepared from 24-treated cells and resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with rabbit polyclonal antibodies against IκBα, and detected by chemiluminescence (ECL, Amersham-Pharmacia Biotechnology, Arlington Heights, IL).

**c-Jun Kinase Assay.** The c-Jun kinase assay was performed by a modified method as described earlier (20, 25).

**NF-κB-dependent Reporter Gene Transcription.** The effect of oleandrin on TNF-α-TRAF-2, NIK, and p65 (transactivation subunit of NF-κB)-induced NF-κB-dependent reporter gene transcription was measured as described previously (21). Briefly, HeLa cells (0.1 × 10^5/ml) were plated in six-well plates, pretreated with 0.5 μg/ml oleandrin for 1 h, and then cells were transfected by the calcium phosphate method with various plasmids. This reporter assay was therefore specific because TNF-induced NF-κB SEAP activity was inhibited by overexpression of either an IκBα mutant lacking Ser32/36, a kinase-inactive NIK, or a dominant-negative TRAF2 mutant (21).

**Determination of Lipid Peroxidation.** TNF-induced lipid peroxidation was determined by detection of thiobarbituric acid-reactive MDA, an end product of the peroxidation of polyunsaturated fatty acids and related esters, as described (26). U937 (3 × 10^5/ml) cells pretreated with either media or different concentrations of oleandrin for 1 h were stimulated with TNF (1 nM) for 30 min. Then cells were washed with PBS and subjected to three cycles of freeze-thawing in 200 μl of water. After protein determination, we added 300 μg of protein (in 0.1 ml) in 800 μl of assay mix containing 0.4% (w/v) thiobarbituric acid, 0.5% (w/v) SDS, and 9.4% (v/v) acetic acid (pH 3.5). After incubation for 1 h at 95°C, samples were cooled to room temperature and centrifuged at 14,000 × g for 10 min, and the absorbance of the supernatants was read at 532 nm. Results were normalized with the amount of MDA equivalents/mg of protein and expressed as a percentage of thiobarbituric acid-reactive substances above control values. Untreated cells showed 0.571 ± 0.126 nmol of MDA equivalents/mg of protein.

**Measurement of ROIs.** The production of ROIs after treatment of cells with TNF was determined by flow cytometry as described (20).

**RESULTS**

The chemical structure of oleandrin is shown in Fig. 1. It is an enolide that is soluble in alcohol, chloroform, and DMSO. In preliminary experiments (data not shown), the concentration and duration of treatment with oleandrin used in these studies had no effect on either the TNF receptors or cell viability.

**Oleandrin Inhibits TNF-induced NF-κB Activation.** U937 cells were pretreated with the indicated concentrations of oleandrin for 1 h and then stimulated with 100 pm TNF for 30 min; nuclear extracts were then prepared and assayed for NF-κB by EMSA. As shown in Fig. 2A, TNF activated NF-κB by almost 12-fold, and oleandrin inhibited TNF-mediated NF-κB activation in a concentration-dependent manner, with almost 90% inhibition occurring at 1 μg/ml. Oleandrin (even up to 10 μg/ml) or the DMSO solvent (0.4% v/v) alone did not activate NF-κB.

We next tested the length of incubation required for oleandrin to block TNF-induced NF-κB activation. The cells were incubated with oleandrin for 240, 120, 60, and 30 min before the addition of TNF, at the same time as the addition of TNF, or 5, 15, and 30 min after the addition of TNF. The cells were treated with TNF for 30 min. Only when the cells were pretreated for 60 min with oleandrin was maximum inhibition of NF-κB activation observed, and the inhibition decreased with shorter preincubation time (Fig. 2B). Cotreatment or posttreatment with oleandrin did not inhibit NF-κB activation (Fig. 2B). TNF-induced NF-κB activation could be seen as early as 5 min, reached maximum after 15 min, and was maintained until 60 min (Fig. 2C). Pretreatment of cells with oleandrin suppressed TNF-induced NF-κB activation at all time points. These results suggest that oleandrin is an NF-κB-dependent inhibitor of NF-κB activation.

Various combinations of Rel/NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA. To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated nuclear extracts from TNF-activated cells with antibody to either p50 (NF-κB) or p65 (Rel A) subunits and then conducted EMSA. Antibodies to either subunit of NF-κB shifted the band to a higher molecular weight (Fig. 2D), suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor such irrelevant antibodies as anti-cRel or anti-cyclin DI had any effect on the mobility of NF-κB. Excess unlabeled NF-κB (100-fold) caused complete disappearance of the band, indicating the specificity of NF-κB. The oligonucleotide with mutated NF-κB site failed to compete with the binding of the NF-κB protein.
Inhibition of NF-κB Activation by Oleandrin Is Not Cell Type Specific. Recent reports indicate that the NF-κB activation pathway may differ in different cell types (27). Therefore, we also studied whether oleandrin affects other cell types. Specifically, we studied the ability of oleandrin to block TNF-induced NF-κB activation in ovarian (CaCOV3) cells, epithelial (HeLa) cells, and T-cells (Jurkat). The results of these experiments (Fig. 3, A–C) indicate that oleandrin inhibited TNF-induced NF-κB activation in all cell types. These results suggest that the effect of oleandrin is not restricted to myeloid cells but also suppresses NF-κB activation in other cell types.

There are reports that oleandrin may exert species-specific effects (17). All of the cell lines we tested thus far are of human origin. We also examined the effect of oleandrin on TNF-dependent NF-κB activation in ovarian (CaCOV3) cells, epithelial (HeLa) cells, and T-cells (Jurkat). The results of these experiments (Fig. 3, A–C) indicate that oleandrin inhibited TNF-induced NF-κB activation in all cell types. These results suggest that the effect of oleandrin is not restricted to myeloid cells but also suppresses NF-κB activation in other cell types.

Inhibition of TNF-mediated NF-κB Activation by Oleandrin Is Not through Inhibition of Na/K ATPase. Most glycosides exert their effects through inhibition of Na/K-ATPase (28). To determine whether oleandrin blocks NF-κB activation by inhibiting this ATPase, we tested the ability of ouabain, a potent inhibitor of Na+/K-ATPase (29, 30), to block TNF-induced NF-κB activation. Ouabain had no effect on TNF-induced activation of this transcription factor (Fig. 4). These results suggest that oleandrin blocks NF-κB activation through some other mechanism.

Oleandrin Blocks Phorbol Ester-, LPS-, Okadaic Acid- and Ceramide-mediated Activation of NF-κB. Besides TNF, NF-κB is also activated by a wide variety of other agents including phorbol ester, LPS, okadaic acid, and ceramide. However, the signal transduction pathway induced by various activators may differ (26, 31). We therefore examined the effect of oleandrin on the activation of NF-κB by these various agents. The results shown in Fig. 5 indicate that oleandrin completely blocked the activation of NF-κB induced by phorbol ester, LPS, okadaic acid, or ceramide. These results suggest that oleandrin may act at a step where TNF, PMA, and LPS converge in the signal transduction pathway leading to NF-κB activation. Because oleandrin had no effect on NF-κB induced by H2O2, OA, or ceramide, the data suggest that a decrease of TNF-induced NF-κB by oleandrin is specific and not attributable to cell death or quantitative loss of nuclear protein or degradation.

Fig. 2. Effect of oleandrin on TNF-dependent NF-κB activation. U937 cells (2 × 10⁶/ml) were preincubated at 37°C for 1 h with different concentrations (0–10 μg/ml) of oleandrin, followed by 30-min incubation with 0.1 nM TNF. After these treatments, nuclear extracts were prepared and then assayed for NF-κB as described in “Materials and Methods” (A). Cells were preincubated at 37°C with 1 μg/ml oleandrin for the indicated times and then tested for NF-κB activation at 37°C for 30 min either with or without 0.1 nM TNF. –, time oleandrin was present before the addition of TNF; +, coincubation with TNF; +, time oleandrin was added after TNF. After these treatments, nuclear extracts were prepared and then assayed for NF-κB (B). Cells were preincubated at 37°C with 1 μg/ml oleandrin for 1 h and then treated with TNF (0.1 nM) for the indicated times. After these treatments, nuclear extracts were prepared and then assayed for NF-κB (C). Nuclear extracts were prepared from untreated or TNF-treated (0.1 nM) U937 cells (2 × 10⁶/ml), incubated for 15 min with different antibodies and unlabeled NF-κB probe, and then assayed for NF-κB as described in “Materials and Methods” (D).
Oleandrin Inhibits TNF-dependent Phosphorylation and Degradation of IκBα. The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα (32). To determine whether the inhibitory action of oleandrin was attributable to an effect on IκBα degradation, the cytoplasmic levels of IκBα proteins were examined by Western blot analysis. IκBα began to degrade 5 min after TNF treatment of U937 cells and disappeared completely within 15 min. The band reappeared by 30 min. The pretreatment of cells with oleandrin completely abolished the TNF-induced degradation of IκBα (Fig. 6A).

To determine whether inhibition of TNF-induced IκBα degradation by oleandrin is attributable to suppression of IκBα phosphorylation, cells were treated with the proteosome inhibitor ALLN (33) for 1 h, and then the hyperphosphorylated form of IκBα was assayed by Western blot. The hyperphosphorylated form of IκBα appears as a slow-migrating band on SDS-PAGE (Fig. 6B). The lack of appearance of a slow-migrating band in oleandrin-treated cells indicates that oleandrin blocked TNF-induced IκBα phosphorylation (see Lane 4 versus Lane 8). These results were further confirmed by using antibodies that detect only the serine-phosphorylated form of IκBα. These results also clearly show that TNF induces the phosphorylation of IκBα, and oleandrin suppresses it (Fig. 6C).

Oleandrin Represses TNF-induced NF-κB-dependent Reporter Gene Expression. Thus far, we have shown that oleandrin blocks the DNA binding of NF-κB protein to its consensus sequence. DNA-binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting the role of additional regulatory steps (34). To determine the effect of oleandrin on TNF-induced NF-κB-dependent reporter gene expression, the oleandrin pretreated or untreated
NF-κB site of action of oleandrin in the TNF-signaling pathway leading to interaction of the TNF receptor with TRADD, TRAF2, NIK, and block the activity of IKK-β completely inhibited by oleandrin in a concentration-dependent manner, with maximum suppression occurring at 0.50 μg/ml (Fig. 8A). Supershift analysis with specific antibodies against c-fos and c-jun indicate that TNF-induced AP-1 consists of fos and jun (data not shown). Lack of supershift by unrelated antibodies and disappearance of the AP-1 band with competition with cold oligonucleotide shows the specificity.

Most agents that activate NF-κB also activate AP-1 (37). Therefore, we also investigated the effect of oleandrin on AP-1 activation by PMA, LPS, H₂O₂, okadaic acid, and ceramide. Similar to NF-κB, all of the agents activated AP-1, but only PMA- and LPS-induced AP-1 was blocked by oleandrin (Fig. 8B). Thus, these results indicate that oleandrin blocks both NF-κB and AP-1 induced by TNF, PMA, and LPS.

**Oleandrin Inhibits TNF-induced JNK and MEK Activation.** TNF is a potent activator of MEKK, which activates MEK, which in turn activates JNK. These kinases are known to be involved in the activation of both NF-κB and AP-1 (37–39). Whether these kinases are modulated by oleandrin was also examined. U937 cells were pretreated with different concentrations of oleandrin for 1 h and then stimulated with TNF (1 ng/ml) for 10 min; activation of JNK was then measured. TNF activated JNK by ∼14-fold, an activation that gradually decreased with increasing concentration of oleandrin. A 1 μg/ml concentration of oleandrin inhibited more of the JNK induced by TNF (Fig. 9A). We also found that TNF activated MEK in a concentration-

HeLa cells were transiently transfected with the NF-κB SEAP reporter construct and then stimulated with TNF. An almost 3-fold increase in SEAP activity over the vector control was noted upon stimulation with TNF (Fig. 7). However, TNF-induced SEAP activity was almost completely abolished when the cells were pretreated with oleandrin. These results demonstrate that oleandrin also represses NF-κB-dependent reporter gene expression induced by TNF.

TNF-induced NF-κB activation is mediated through sequential interaction of the TNF receptor with TRADD, TRAF2, NIK, and IKK-β, resulting in phosphorylation of IκBα (35, 36). To delineate the site of action of oleandrin in the TNF-signaling pathway leading to NF-κB activation, cells were transfected with TRAF2, NIK, and p65 plasmids, and then NF-κB-dependent SEAP expression was monitored in oleandrin-untreated and -treated cells. As shown in Fig. 7, TRAF2, NIK, and p65 plasmids induced gene expression, and oleandrin suppressed TRAF-2 and NIK-induced but had little effect on p65-induced NF-κB reporter expression. RANK, another NF-κB-inducing receptor, was minimally affected by oleandrin, indicating the specificity. Specificity of the assay results are also indicated by the suppression of the TNF-induced NF-κB reporter activity by the dominant negative-IκBα plasmid. Thus, oleandrin must act at a step downstream from NIK. Because NIK is known to activate IKK-β, which in turn phosphorylates IκBα, it appears that oleandrin must block the activity of IKK-β, a kinase that phosphorylates IκBα directly.

**Oleandrin Inhibits TNF-induced AP-1 Activation.** TNF is also a potent activator of AP-1 (37). TNF induced AP-1 expression by 5-fold in myeloid cells at 1 nm concentration. The activation of AP-1 was completely inhibited by oleandrin in a concentration-dependent manner, with maximum suppression occurring at 0.50 μg/ml (Fig. 8A). Supershift analysis with specific antibodies against c-fos and c-jun indicate that TNF-induced AP-1 consists of fos and jun (data not shown). Lack of supershift by unrelated antibodies and disappearance of the AP-1 band with competition with cold oligonucleotide shows the specificity.

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Fig. 7. Oleandrin inhibits the NF-κB-dependent reporter gene expression induced by TNF, TRAF-2, and NIK. HeLa cells were either untreated or treated with oleandrin (0.5 μg/ml) for 1 h and then transiently transfected with indicated plasmids along with an NF-κB containing plasmid linked to the SEAP gene. Where indicated, cells were exposed to 1 nM TNF for 12 h. Cells were assayed for secreted alkaline phosphatase activity as described in “Materials and Methods.” Results are expressed as fold activity over the nontransfected control; bars, SD.

OLEANDRIN BLOCKS NF-κB, AP-1, AND JNK ACTIVATION

NF-κB Inhibiting Species in Oleander Extracts Is Oleandrin. Oleandrin is derived from oleander extracts. These extracts have been shown to be active in other systems. We investigated whether oleander extracts have NF-κB suppression activity and whether this activity is attributable to oleandrin. Thus, cells were pretreated with different concentrations of crude oleander extracts and then examined for TNF-induced NF-κB activation. As shown in Fig. 10A, the extracts inhibited NF-κB activation in a concentration-dependent manner with maximum suppression occurring at 100 μg/ml. TNF activated NF-κB in a time-dependent manner, and oleander extracts inhibited the activation (Fig. 10B). TNF also activated AP-1 in a time-dependent manner, and oleander extracts inhibited this activation (Fig. 10C). To further investigate whether oleander extracts also blocked NF-κB and AP-1 activated by other inflammatory agents, cells were treated with these agents as described for oleandrin and tested for NF-κB (Fig. 10D). Like oleandrin, only TNF-, PMA-, and LPS-induced NF-κB activation was blocked by oleander extracts, suggesting that the active moiety in the extracts could be oleandrin. To further confirm that oleandrin is responsible for suppression of NF-κB, the plant extracts were fractionated by high pressure liquid chromatography, and various fractions were examined for suppression of NF-κB. Only fractions B2 and C blocked TNF-induced NF-κB activation. Analysis indicated that fraction B2 is an oleander extract without oleandriogenin and fraction C has the complete nonpolar compound content including oleandrin, oleandriogenin, and other cardiac glycosides. Lyophilized fractions A (without oleandrin and oleandriogenin), B1 (without oleandrin), or D (polysaccharides only) were inactive in suppressing NF-κB activation. Thus, these results clearly indicate that the active principle in the oleander extracts is oleandrin, which inhibited NF-κB and AP-1 activation.

Oleandrin Blocks TNF-induced ROI Generation and Lipid Peroxidation. Previous reports have shown that TNF activates NF-κB through generation of ROI (40–42). Whether oleandrin suppresses NF-κB activation through suppression of ROI generation was examined by flow cytometry. As shown in Fig. 11A, TNF induced ROI generation in a time-dependent manner, and this was suppressed on pretreatment of cells with oleandrin. Because lipid peroxidation has also been implicated in TNF-induced NF-κB activation (26), we also examined the effect of oleandrin on TNF-induced lipid peroxidation. Results in Fig. 11B show that TNF induced lipid peroxidation in U937 cells, and this was completely suppressed by oleandrin. Thus, it is quite likely that oleandrin blocks TNF signaling through suppression of ROI generation and lipid peroxidation.

DISCUSSION

Agents that can suppress activation of the transcription factors NF-κB and AP-1 have potential in blocking tumor initiation, promotion, and metastasis. In the present report, we demonstrate that oleandrin can block NF-κB activation, as determined by consensus DNA binding, IκBα degradation, and NF-κB-dependent reporter gene expression. Oleandrin blocked NF-κB activation induced by various inflammatory stimuli including TNF, PMA, and LPS. The effect of oleandrin was not cell type specific. Besides NF-κB activation, oleandrin also blocked AP-1 activation induced by various inflammatory stimuli, and it inhibited the TNF-induced activation of JNK and MEK. The generation of both ROI and lipid peroxidation induced by TNF was strongly inhibited by oleandrin.

There are several explanations of how oleandrin might inhibit TNF-induced NF-κB activation. We showed that oleandrin does not interfere with the binding of NF-κB to the consensus DNA binding site. NF-κB activation requires sequential phosphorylation, ubiquitination, and degradation of IκBα. Because oleandrin blocks IκBα degradation, it suggests that the effects of oleandrin on NF-κB is through inhibition of either phosphorylation or IκBα proteolysis. The phosphorylated form of IκBα is known to appear on the gel as a band with retarded mobility (32, 43). The lack of a retarded IκBα band after treatment of cells with oleandrin suggests that oleandrin blocked the
phosphorylation of IκBα. By using antibodies that specifically detect the phosphorylated form of IκBα, it was shown that oleandrin blocks TNF-induced phosphorylation of IκBα. The phosphorylation of IκBα is regulated by a large number of kinases including IKK-α, IKK-β, IKK-γ, NIK, TGF-β-activated kinase-1, AKT, and MEKK1 (44–55). In addition to MEKK1, MEKK2 and MEKK3 have been implicated in NF-κB activation, whereas MEKK4 activates JNK (56). AKT and NIK are primarily known to activate IKK-α, whereas MEKK1 and atypical protein kinase C activate IKK-β (55). MEKK is known to induce the phosphorylation of MEK, which oleandrin inhibited. Thus, it is possible that oleandrin inhibited IκBα phosphorylation by inhibiting the activity of MEKK1 or other kinases.

We found that oleandrin blocked NF-κB activation induced by a wide variety of agents including TNF, PMA, and LPS, but had no effect on okadaic acid, ceramide, and H2O2-induced NF-κB activation, suggesting a difference in the pathway leading to NF-κB activation by different activators. This is consistent with a recent report that different inducers activate NF-κB by different mechanisms (26, 31). Compared with most other agents, NF-κB activation induced by H2O2 does not require IκBα degradation, again suggesting a difference in the signaling pathway (31).

Our results also indicate that oleandrin is a potent inhibitor of AP-1. This is not too surprising because most agents that activate NF-κB also activate AP-1. Activation of AP-1 requires JNK, another kinase of the mitogen-activated protein kinase family. It is possible that AP-1 is suppressed through inhibition of JNK. Recent studies from our laboratory showed that overexpression of cells with either superoxide dismutase (40) or with γ-glutamylcysteine synthetase, a rate-limiting enzyme in the glutathione biosynthesis pathway (41), blocked both NF-κB and AP-1 activation induced by TNF, indicating a similar mechanism of activation of both transcription factors. These results also suggest that oleandrin may suppress these factors by regulating the redox status of the cells.

We did indeed find that oleandrin blocks TNF-induced ROI production and lipid peroxidation. Our results, however, do not

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**Fig. 8.** A, oleandrin inhibits TNF-dependent AP-1 activation. U937 cells (2 × 10^6) were pretreated with different concentrations of oleandrin for 1 h at 37°C. Cells were then stimulated with 0.1 nM TNF for 1 h and assayed for AP-1 as described in “Materials and Methods.” B, effect of oleandrin on AP-1 activation induced by PMA, serum-activated LPS, H2O2, okadaic acid, ceramide, and TNF. U937 cells (2 × 10^6/ml) were preincubated for 2 h at 37°C with 1 μg/ml oleandrin, followed by PMA (25 ng/ml), SA-LPS (1 μg/ml), H2O2 (250 μM), okadaic acid (500 nM), ceramide (10 μM), and TNF (0.1 nM) for 30 min and then tested for AP-1 activation as described in “Materials and Methods.”

**Fig. 9.** Effect of oleandrin on TNF-induced JNK (A) and MEK (B) activation. U937 cells were pretreated with different concentrations of oleandrin as indicated and then stimulated with 1 nM TNF at 37°C for 10 min. The cells were then washed, and pellets were extracted and assayed for JNK. For MEK, U937 cells were pretreated with oleandrin (1 μg/ml) and then stimulated with different concentrations of TNF at 37°C for 30 min. The cells were then washed, and pellets were extracted and assayed for MEK activation as described in “Materials and Methods.”
OLEANDRIN BLOCKS NF-κB, AP-1, AND JNK ACTIVATION

![Fig. 10. Effect of oleander extracts on TNF-dependent NF-κB activation. A, U937 cells (2 × 10^6/ml) were preincubated at 37°C for 1 h with different concentrations of oleander extracts, followed by 30 min incubation with 0.1 nM TNF. After these treatments, nuclear extracts were prepared and then assayed for NF-κB as described in “Materials and Methods.” B, cells were preincubated at 37°C with 50 μg/ml oleander extracts for 1 h and then treated for different times with 0.1 nM TNF as indicated. After these treatments, nuclear extracts were prepared and then assayed for NF-κB. C, cells were preincubated at 37°C with 50 μg/ml oleander extracts for 1 h and then treated for different times with 0.1 nM TNF as indicated. After these treatments, nuclear extracts were prepared and then assayed for NF-κB. D, effect of oleander extracts on NF-κB activation induced by PMA, serum-activated LPS, H₂O₂, okadaic acid, ceramide, and TNF. U937 cells (2 × 10^6/ml) were preincubated for 1 h at 37°C with 50 μg/ml oleander extracts, followed by PMA (25 ng/ml), SA-LPS (1 μg/ml), H₂O₂ (250 μM), okadaic acid (500 μM), ceramide (10 μM), and TNF (0.1 nM) for 30 min and then tested for NF-κB activation. E, effect of different fractions of oleander extract on NF-κB activation induced by TNF. U937 cells (2 × 10^6/ml) were preincubated for 1 h at 37°C with 50 μg/ml of different fractions of oleander extract, activated by TNF (0.1 nM) for 30 min, and then tested for NF-κB activation.

rule out redox-independent mechanism of suppression of NF-κB by oleandrin.

Our results indicate that oleandrin blocks TNF-induced NF-κB, JNK, and AP-1 activation. TRAF2, which is known to bind to TNF receptor through TRADD, is required for NF-κB, AP-1, and JNK activation (35). The suppression of TRAF2 activity may explain how oleandrin inhibits NF-κB, AP-1, and JNK. The activity of NIK, which interacts with TRAF2, was also blocked by oleandrin. This suggests that oleandrin must inhibit the IKK that phosphorylates IκBα.

Several recent reports indicate that NF-κB activation induces resistance to apoptosis stimulated by a wide variety of agents (5–7, 10). Because oleandrin is known to be cytotoxic to various tumor cells (17, 18), it is possible that this toxicity is mediated through the suppression of NF-κB. There are several different tumor cell types that express constitutive NF-κB and thus are resistant to apoptosis induced by various agents (8–10, 40). Oleandrin should induce apoptosis in these cells by suppression of NF-κB. The mechanism by which oleandrin induces apoptosis in tumor cells was recently investigated in detail (57). AP-1 is also known to be involved in cell proliferation (14), and thus suppression of AP-1 by oleandrin may explain its cytotoxic effects. Suppression of AP-1 may also suggest the role of oleandrin in inhibition of tumor promotion and progression (12–15).

We found that oleandrin blocked NF-κB-dependent reporter gene expression. Several genes are involved in tumor promotion that are regulated by NF-κB. These include cytokines, cyclooxygenase-2, metalloproteases, urinary plasminogen activator, and cell surface adhesion molecules (1–3). Because NF-κB-regulated genes also play a critical role in carcinogenesis and inflammation, oleandrin may also exhibit anticarcinogenic and anti-inflammatory effects. Adenoviral IκBα, an NF-κB inhibitor, and proteosome inhibitors that block IκBα degradation are currently being tested to overcome chemotherapy-induced resistance (10). Therefore, NF-κB suppressive ability of oleandrin could be exploited by combination with chemotherapy. An additional justification for the use of oleandrin may come from its ability to suppress AP-1, a transcription factor also 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 (1–3), oleandrin may also abolish viral replication.

Similar to oleandrin, the anti-inflammatory drugs sodium salicylate and aspirin are also known to block the activation of NF-κB by preventing the degradation of IκBα (58). The effects of salicylate on NF-κB activation were observed, however, at suprapharmacological concentrations (>5 mM). In contrast, oleandrin was effective at 2000-fold lower concentration, suggesting that it is a potent inhibitor. 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.

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REFERENCES


