BJP British Journal of Pharmacology

DOI:10.1111/bph.12493
www.brjpharmacol.org

RESEARCH PAPER

Short-term exposure to oleandrin enhances responses to IL-8 by increasing cell surface IL-8 receptors

Nune Raviprakash and Sunil Kumar Manna

Laboratory of Immunology, Centre for DNA Fingerprinting & Diagnostics (CDFD), Hyderabad 500 001, India

Correspondence
Sunil Kumar Manna, Immunology, Centre for DNA Fingerprinting and Diagnostics, Bldg. 7, Gruhakalpa, 5-4-399/B, Nampally, Hyderabad 500001, India. E-mail address: manna@cdfd.org.in

Keywords
apoptosis; cardiac glycoside; IL-8 receptor; NF-κB; chemotaxis

Received
30 May 2013
Revised
3 October 2013
Accepted
22 October 2013

BACKGROUND AND PURPOSE

One of the first steps in host defence is the migration of leukocytes. IL-8 and its receptors are a chemokine system essential to such migration. Up-regulation of these receptors would be a viable strategy to treat dysfunctional host defence. Here, we studied the effects of the plant glycoside oleandrin on responses to IL-8 in a human monocytic cell line.

EXPERIMENTAL APPROACH

U937 cells were incubated with oleandrin (1-200 ng mL^{-1}) for either 1 h (pulse) or for 24 h (non-pulse). Apoptosis; activation of NF-κB, AP-1 and NFAT; calcineurin activity and IL-8 receptors (CXCR1 and CXCR2) were measured using Western blotting, RT-PCR and reporter gene assays.

KEY RESULTS

Pulse exposure to oleandrin did not induce apoptosis or cytoxicity as observed after non-pulse exposure. Pulse exposure enhanced activation of NF-κB induced by IL-8 but not that induced by TNF-α, IL-1, EGF or LPS. Exposure to other apoptosis-inducing compounds (azadirachtin, resveratrol, thiadiazolidine, or benzofuran) did not enhance activation of NF-κB. Pulse exposure to oleandrin increased expression of IL-8 receptors and chemotaxis, release of enzymes and activation of NF-κB, NFAT and AP-1 along with increased IL-8-mediated calcineurin activation, and wound healing. Pulse exposure increased numbers of cell surface IL-8 receptors.

CONCLUSIONS AND IMPLICATIONS

Short-term (1 h; pulse) exposure to a toxic glycoside oleandrin, enhanced biological responses to IL-8 in monocytic cells, without cytoxicity. Pulse exposure to oleandrin could provide a viable therapy for those conditions where leukocyte migration is defective.

Abbreviations

CsA, cyclosporine A; MTT, (3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide; PI, propidium iodide

Introduction

Chemotaxis of leukocytes, the migration and accumulation of these cells in sites of inflammation, is a critical component of host response to injury or infection. A major chemotactic system is provided by the chemokine IL-8 and its receptors, CXCR1 and CXCR2 (receptor nomenclature follows Alexander *et al.*, 2013). Activation of these chemokine

receptors induces several other biological responses in leukocytes, associated with phagocytosis. Depressed function of leukocytes leads to failure of host defence and increased susceptibility to infection.

Activation of CXCR1 and CXCR2, by IL-8 is transduced by several intracellular signalling pathways, leading to the activation of the transcription factors NFkB, AP-1 and NFAT, and recruitment of the TNF-receptor associated factor 6 (TRAF6) to activate NF-κB and AP-1 (Manna and Ramesh, 2005). NF-κB, a heterodimer of two subunits p50 (NF-κB 1) and p65 (RelA), is normally present in the cytoplasm in an inactive state in complex with an inhibitory subunit of κB (IκBα). Upon phosphorylation and subsequent degradation of IκBα, a nuclear localization signal on the p50–p65 heterodimer is exposed leading to nuclear translocation of NF-κB. The p50–p65 heterodimer binds with a specific sequence in DNA, resulting gene transcription. The expression of several proteins critically involved in the inflammatory process including cytokines, adhesion molecules, cyclins, Bax and Bcl2, is dependent on NF-κB.

The transcription factor NFAT is the target substrate for the phosphatase calcineurin (Kingsbury and Cunningham, 2000; Rusnak and Mertz, 2000; Abbasi *et al.*, 2005). Upon stimulation of calcineurin, several residues in the regulatory domain of NFAT are dephosphorylated and this leads to nuclear translocation of NFAT and, in combination with AP-1, to the activation of target genes of AP-1 (Liu *et al.*, 1997), MEF2 (Srivastava *et al.*, 1999), FasL (Molkentin *et al.*, 1998) and GATA (Zhu *et al.*, 1998) proteins. Calcineurin activity is stimulated by raised intra-cellular Ca^{2+} levels (Klee *et al.*, 1998). Cyclosporine A (CsA) inhibits calcineurin and thus blocks nuclear translocation of NFAT and consequent gene transcription (Molkentin *et al.*, 1998).

The plant glycoside, oleandrin, is a potent inducer of apoptosis in human, but not murine, cells (Raghavendra *et al.*, 2007). Oleandrin activates NF-κB and AP-1, mediated by TNF (Manna *et al.*, 2000)and it interacts with membrane lipids to alter membrane fluidity, thereby decreasing IL-8 binding to its receptors (Manna *et al.*, 2006b; Raghavendra *et al.*, 2007). In earlier work we noted that incubation of cells with oleandrin for 24 h (non-pulse exposure) was clearly cytotoxic (Raghavendra *et al.*, 2007) but incubation for 1 h (pulse exposure) did not induce significant cell death as we show below. Here, we have explored further this time-related difference in exposure to oleandrin in terms of the subsequent responses to inflammatory stimuli and in particular to IL-8. We found that, after pulse exposure to oleandrin, the responses to IL-8 but not those to TNF-α, IL-1, EGF, or other GPCR agonists, in terms of NF-κB activation, were increased. This potentiation of IL-8 action was accompanied by an increased amount of the receptors for IL-8. Thus, although oleandrin was cytotoxic on prolonged exposure, short-term exposure to this glycoside could enhance both leukocyte migration and activity in those conditions where leukocyte functions have been compromised.

Methods

Cells and culture conditions

U-937, A549, HeLa, THP1, Jurkat, HT29, and MCF-7 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C, 95% humidified incubator with 5% CO_2. They were incubated with oleandrin (1–200 ng mL^{-1}) either for 1 h, washed and cultured for 24 h with fresh medium (pulse exposure) or for 24 h (in the continued presence of oleandrin; non-pulse exposure) and then treated with a range of stimuli, as indicated below.

NF-κB, AP-1 and NFAT DNA binding assay

Binding of NF-κB, AP-1 and NFAT to DNA was determined by EMSA (Mahali *et al.*, 2011). Briefly, cytoplasmic and nuclear extracts were prepared from cells (2×10^6 cells), as described by Mahali *et al.* (2011). The nuclear extracts (8 μg) were incubated with ^{32}P end-labelled double-stranded NF-κB oligonucleotide of HIV-LTR, 5′-TTGTTACAAGGGACTTTC CGCTGGGGACTTTCCAGGGAGGCGTGG-3′ for 30 min at 37°C, and the DNA-protein complex was separated from free oligonucleotide on 6.6% native PAGE. Binding of AP-1 and NFAT were assayed similarly, using specific double-stranded labelled oligonucleotides.

NF-κB and NFAT-dependent luciferase gene transcription assay

The expression of NF-κB- and NFAT-dependent luciferase reporter gene was carried out as described previously (Mahali *et al.*, 2011). Cells (1×10^6 cells mL^{-1})were transiently transfected with SuperFect transfection reagent (Qiagen, Hilden, Germany) containing 0.5 μg of each reporter plasmids (*NF-κB-luciferase* or *NFAT-luciferase*) and *GFP* constructs. After treatments, cell pellets were extracted with the lysis buffer, provided in the luciferase assay kit from Promega (Promega Corporation, Madison, WI, USA). Luciferase activity was measured and indicated as fold of activation over vector-transfected value.

Calcineurin activity assay

Calcineurin activity was measured using the synthetic RII phosphopeptide as a substrate and source of inorganic phosphate, followed by Malachite Green as described previously (Mahali *et al.*, 2011).

Determination of nuclear fragmentation

Cells were harvested and fixed in 80% methanol, stained with propidium iodide (PI), and examined by fluorescence microscopy, as described earlier (Manna and Gangadharan, 2009).

Cytotoxicity assay

Cytotoxicity was measured by MTT (3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Mahali *et al.*, 2011). Briefly, cells (1×10^4 cells in 100 μL medium per well of a 96-well plate) were treated with different agents at the indicated concentrations and times and thereafter 25 μL of MTT solution (5 mg mL^{-1} in PBS) was added. After 2 h of incubation, 100 μL extraction buffer (20% SDS in 50% dimethylformamide) was added. After an overnight incubation at 37°C, absorbance was read at 570 nm with the extraction buffer as blank.

Cytolysis was measured by LDH release into the cell medium. Briefly, the culture supernatants were collected after

treatment and incubated with substrate solution (0.23 M sodium pyruvate and 5 mM NADH in 0.1 M phosphate buffer, pH 7.5). The absorbance was recorded at 340 nm after 5 min of incubation at 37°C. The decrease in absorbance indicates the activity of LDH.

Caspase 3 and 8 activities assay

To evaluate caspase 3 and 8 activities, cell lysates from 2×10^6 cells were prepared after their respective treatments. Samples of the cell lysate (50 µg protein) were incubated with 200 µM caspase 3 substrate (Ac-DVED-pNA) or caspase 8 substrate (Ac-ITED-pNA) in 100 µL reaction buffer (1% NP-40, 20 µM Tris-HCl, pH 7.5, 137 mM NaCl, and 10% glycerol) and incubated for 2 h at 37°C. The release of the chromophore *p*-nitroaniline (pNA) was monitored photometrically at 405 nm (Mahali *et al*., 2011).

Enzyme release assay

U-937 cells (1×10^7 cells mL^{-1}), in phenol-red free RPMI-1640 medium with 10% FBS, were stimulated with different concentrations of IL-8 for 4 h The supernatant was collected and used for assay of three different enzymes (Manna *et al*., 2006a), myeloperoxidase, alkaline phosphatase and β-D-glucuronidase. Enzyme activities were measured using *o*-phenylenediamine, *p*-nitrophenyl phosphate and *p*-nitrophenyl β-D-glucuronide as substrate respectively.

Reverse transcriptase (RT)-PCR

Total RNA was isolated using the standard TRIzol method (Life Technologies Inc., Carlsbad, California, USA). Samples of total RNA (1µg) were used to reverse transcribe into cDNA by One step Access RT-PCR kit (Promega), followed by the amplification of the gene of interest using gene specific primers for CXCR1, CXCR2, CXCR4, and actin using specific primers (see below) and visualized by ethidium bromide stained agarose gel (2%).

Primers used were as follows: *CXCR1*: 190 bp {forward} 5′-CAGACAGAGCTCTCTTCCAT-3′, {reverse}5′-GCAGCTCTG TGTGAAGGTGCA-3′; *CXCR2*: 781 bp {forward}5′-CTATAGT GGCATCCTGCTAC-3′, {reverse}5′-CCAAGAAGAACCAGTGG ACA-3′; *CXCR4*: 610 bp {forward}5′-TGGTCTATGTTGGCGT CTGGA′-3′, {reverse}5′-‘CTT TTACATCTGTGTTAGCTGG-3′; *Actin*: 616 bp {forward}5′-CCAACCGTGAAAAGATGACC-3′, {reverse}5′-GCAGTAATCTCCTTCTGCATCC-3′.

Data analysis

Results are expressed as mean ± SEM of the number of independent experiments as shown. Differences between means were analysed with one way ANOVA or with unpaired *t*-test . $P < 0.05$ was considered to be significant.

Materials

Unless otherwise indicated, general chemicals, inhibitors and substrate peptides were obtained from Sigma (St Louis, MO, USA). DMEM and FBS were obtained from Life Technologies (Grand Island, NY, USA). Antibodies and gel shift oligonucleotides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The plasmid constructs for *p65*, consensus and dominant negative *TRAF6*, *NF-κB-luciferase*, and *NFAT-luciferase* were kind gifts from Prof. B. B. Aggarwal (University of Texas, M. D. Anderson Cancer Center, Houston, TX, USA).

Results

No significant increase in the LDH activity of culture supernatants was observed upon treatment of cells with different agents used for this study.

Continuous (non-pulse) exposure to oleandrin but not pulse exposure, induces apoptosis

To explore further the different effects of pulse exposure (1 h) and non-pulse exposure (24 h) to oleandrin, we incubated U-937 cells with different concentrations of oleandrin. As shown in Figure 1A, non-pulse exposure caused concentration-dependent cytotoxicity, rising to a maximum of about 60% cell death at 200 ng mL^{-1}, as measured by the MTT assay. By contrast, pulse exposures to oleandrin showed no significant cytotoxicity, over the same range of concentrations. This difference, between pulse and non-pulse exposures. in cytotoxic effects was also evident when assessed by PI staining of the cells (Figure 1B). Similarly, other measures of apoptosis, such as PARP cleavage (Figure 1C) and caspase 3 and 8 activities (Figure 1D), demonstrated that the pulse exposure did not induce apoptosis, in contrast to that induced by the non-pulse exposure.

We then studied the time course of this cytotoxicity by sampling at different times after exposure to oleandrin (100 ng mL^{-1}). After pulse exposure for 1 h, all subsequent samples taken for up to 48 h incubation, showed very little, if any, cell death by PI-staining (Figure 1F) or MTT assays (Figure 1G). However, continuous, non-pulse, exposure induced time-related cell death, from 3 h onwards. Using the same experimental design, the data from EMSA assays (Figure 1E; summary data in Supporting Information, Fig S1A), showed marginal time-dependent effects on binding of NF-κB to DNA. The last trace in this Figure shows that, 24 h after the standard 1 h pulse with oleandrin, the activation of NF-κB by IL-8 was markedly increased, compared with the activation by IL-8, without exposure to oleandrin.

Pulse exposure to oleandrin, but not to azadirachtin, benzofuran, P_3-25 (dichlorophenyl thiadiazolidine) or resveratrol increases IL-8, but not TNF-induced NF-κB activation

We next tested four other compounds known to induce apoptosis, azadirachtin, benzofuran, P_3-25 (dichlorophenyl thiadiazolidine) or resveratrol, for their effects on (1) the responses to IL-8 or TNF-α, in U-937 cells and (2) cell viability. 24 h after a 1 h pulse with these compounds, cells were stimulated with IL-8 (100 ng mL^{-1}) or TNF-α (100 pM). for 2 h and NF-κB binding to DNA measured in nuclear extracts, as an assay of NF-κB activation. As shown in Figure 2A, pulse exposure to these compounds inhibited the NF-κB activation induced by TNF-α or IL-8 (summary data in Fig S2A). Increased activation of NF-κB was seen only after pulse

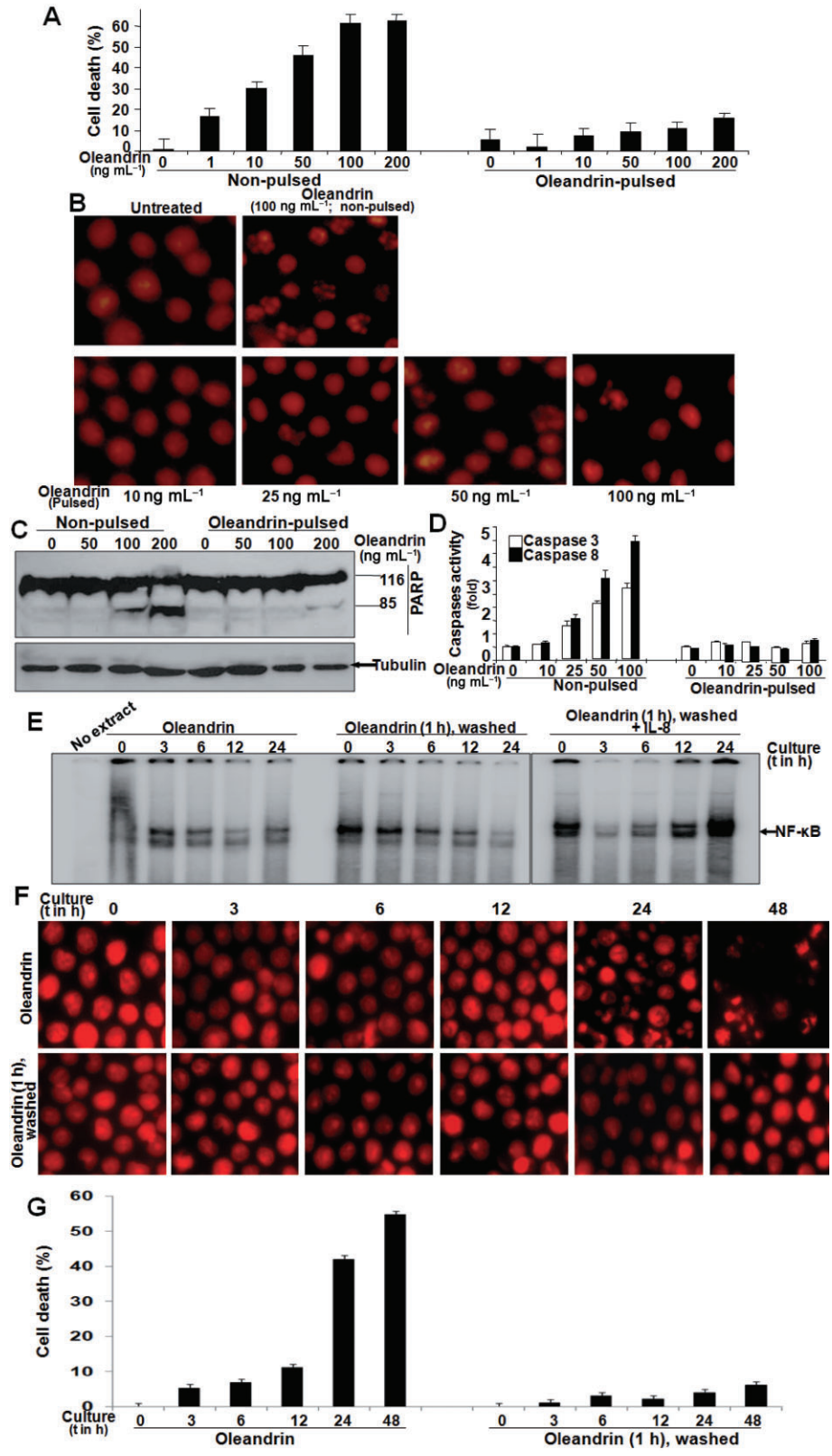
A
Cell death (%)
Oleandrin (ng mL⁻¹)
Non-pulsed
Oleandrin-pulsed
B
Untreated
Oleandrin (100 ng mL⁻¹; non-pulsed)
Oleandrin (Pulsed)
10 ng mL⁻¹
25 ng mL⁻¹
50 ng mL⁻¹
100 ng mL⁻¹
C
Non-pulsed
Oleandrin-pulsed
PARP
Tubulin
D
Caspase 3
Caspase 8
Caspases activity (fold)
E
No extract
Oleandrin
Oleandrin (1 h), washed
Oleandrin (1 h), washed +IL-8
Culture (t in h)
NF-κB
F
Oleandrin
Oleandrin (1 h), washed
G
Cell death (%)

Figure 1

Effect of pulsed and non-pulsed exposure to oleandrin on cell viability. U-937 cells were treated with different concentrations of oleandrin for 1 h and then cells were washed and cultured for a further 24 h; this is referred to as 'pulse exposure'. In another set, U-937 cells were incubated with the same concentrations of oleandrin for 24 h (non-pulse exposure). In A, the MTT assay was used to measure cell viability; data from one of three experiments is shown. In B, cell death was assessed by nuclear fragmentation assay as detected by PI staining, following the same pulse and non-pulse exposures,. In (C), another measure of apoptosis, cleaved PARP, in whole cell extracts of U-937 cells after pulse and non-pulse exposures to oleandrin, was detected by Western blot. (D) Activities of caspase 3 and 8 were measured in whole cell extracts. Results are expressed as fold activation, relative to values in cells without oleandrin exposure. U-937 cells were incubated with oleandrin (100 ng mL^{-1}) under pulsed or non-pulsed conditions, followed by washing and culturing for different times. They were then stimulated with IL-8 (100 ng mL^{-1}) for 2 h. Nuclear extracts were prepared and assayed for NF-κB DNA binding (E). Cell death was detected by PI staining of cell nuclei (F) and viability (G) by the MTT assay.

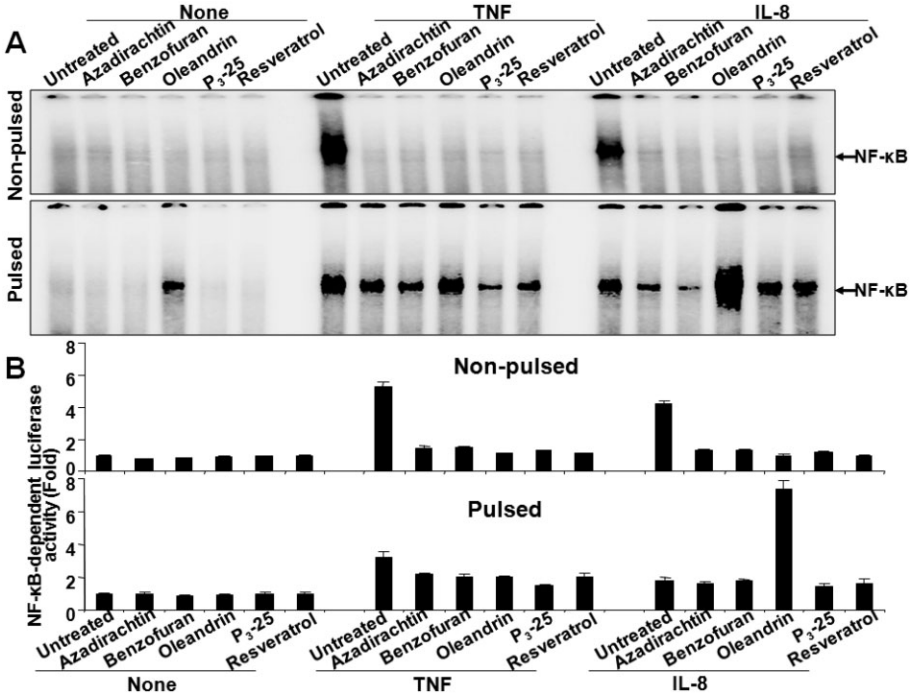

Figure 2

Effect of pulse exposure to a range of apoptosis–inducing compounds on IL-8- or TNF-induced NF-κB activation. In (A), U-937 cells were pulsed with azadirachtin (10 μM), benzofuran (100 nM), oleandrin (100 ng mL^{-1}), P_3-25 (100 nM), or resveratrol (10 μM) for 1h then washed and cultured in drug-free media for another 24h. Cells were then were stimulated with TNF (100 pM) or IL-8 (100 ng mL^{-1}) for 2 h. As an indication of cell activation, binding of NF-κB to DNA was measured in nuclear extracts. In (B), U-937 cells were transfected with NF-κB-luciferase and GFP constructs; 45% of the cells were GFP positive. Cells were treated with the different compounds for 1 h, washed and cultured for 24 h. Whole cell extracts were prepared and luciferase activity was measured and expressed as fold of values in untreated cells (set to unity).

treatment with oleandrin and stimulation by IL-8. Similar results were obtained for in the reporter gene assay using NF-κB-dependent luciferase activity in NF-κB-luciferase transfected cells (Figure 2B). Measurement of cell viability after incubation with these compounds showed that only oleandrin exhibited low toxicity after pulse treatment (Figure S2B).

Pulse exposure to oleandrin increases activation of NF-κB, stimulated by IL-8 but not that stimulated by TNF, LPS, IL-1 or EGF

Oleandrin-pulsed U-937 cells were stimulated with TNF-α (10 pM), LPS (100 ng mL^{-1}), IL-1 (50 nM), IL-8 (50 ng mL^{-1}) and EGF (100 pM) for 6 h. EMSA assays showed that activation of NF-κB was increased by all these stimuli in cells not exposed to oleandrin. In oleandrin-pulsed cells, NF-κB activation was increased only with IL-8 as the stimulus (Figure 3A; summary data in Fig S3A).

Pulse exposure to oleandrin increases IL-8-induced NF-κB activation and involves TRAF6

Other human cell lines, MCF-7, HeLa, THP1, Jurkat, HT29 and A549 cells, were treated with the pulse exposure to oleandrin and the activation of NF-κB following IL-8 stimulation was increased in these cells also (Figure 3B; summary data in Fig S3B), suggesting that the potentiation of this response to IL-8 was independent of cell type.

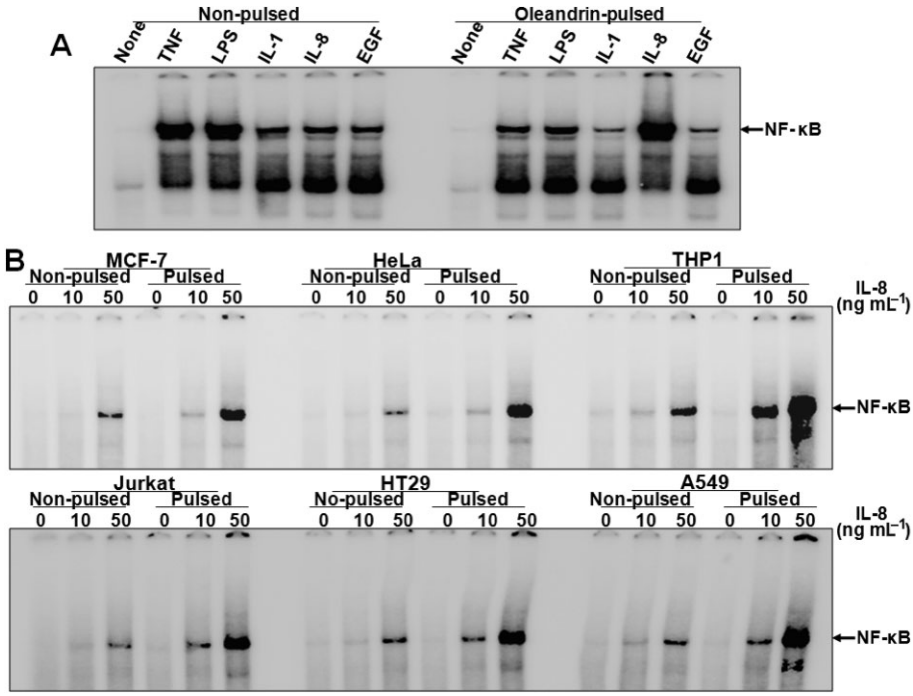

Figure 3

Effect of oleandrin pulse on NF-κB activation mediated by different agonists and in different cells. In (A), oleandrin-pulsed U-937 cells were stimulated with TNF (10 pM), LPS (100 ng mL^{-1}), IL-1 (50 nM), IL-8 (50 ng mL^{-1}) or EGF (100 pM) for 6 h. NF-κB DNA binding was assayed in nuclear extracts (A). In (B), a range of different cell types was pulsed with oleandrin and then stimulated with 10 and 50 ng mL^{-1} IL-8 for 4 h and NF-κB DNA binding was assayed.

Oleandrin-pulsed U937 cells were incubated with antibodies against CXCR1 and CXCR2 (1 μg each per 2×10^6 cells) for 2 h and then stimulated with 100 ng mL^{-1} IL-8 for 4 h. Assays of NF-κB activation showed that the pre-incubation with receptor antibodies completely inhibited IL-8-induced NF-κB activation in both oleandrin-pulsed and non-pulsed cells (Figure 4A; summary data in Fig S4A). Similarly, in cells transfected with the dominant negative mutant of TRAF6 (*TRAF6-DN*), activation of NF-κB by IL-8 was completely inhibited in both oleandrin-pulsed and non-pulsed cells (Figure 4B; summary data in Fig S4B). In cells transfected with TRAF6, pulse exposure to oleandrin increased IL-8-induced activation of NF-κB more than in the non-pulsed cells (Figure 4C; summary data in Fig S4C). Pre-incubation of U937 cells with another inhibitor of this signalling pathway, the TRAF6 binding protein (TRAF6-BP), but not the TRAF6-BP mutant peptide, also completely inhibited the IL-8-induced NF-κB activation in both non-pulsed and oleandrin-pulsed cells (Figure 4D; summary data in Fig S4D). Taken together, these results confirmed that IL-8 was acting via its receptors and via the intracellular signalling pathway involving TRAF6, as outlined previously (Manna and Ramesh, 2005).

Pulse exposure to oleandrin increases IL-8-, but not FMLP-, NGF-, MSH-, 5-HT- or vasopressin-mediated cell signalling

Oleandrin-pulsed cells were stimulated with NGF (100 nM), FMLP (100 nM), α-MSH (1 μM), arginine-vasopressin (100 nM) or 5-HT (100 nM) for 6 h. All these agents, except α-MSH, increased NF-κB and AP-1 binding to DNA in cells without any exposure to oleandrin. After pulse exposure to oleandrin, only IL-8 increased, almost threefold, the activation of NF-κB and AP-1 (Figure 4E; summary data in Fig S4E). These data suggest that the pulse exposure to oleandrin affected only the cell response to IL-8 and not those to other bioactive peptides or agonists of GPCRs.

Pulse exposure to oleandrin increases expression of CXCR1 and CXCR2 by increasing synthesis of the receptors

The amount of mRNA for CXCR1, CXCR2 and CXCR4 increased in oleandrin-pulsed cells, as shown by RT-PCR (Figure 5A). The amount of immunoreactive CXCR1 in oleandrin-pulsed cells was estimated by staining whole cells with a specific anti-CXCR1 antibody, followed by Alexa-Fluor-labelled secondary antibodies (Figure 5B). Western blot analysis of whole cell extracts of similarly treated cells, for CXCR1 and CXCR2 together (IL-8 receptors) showed a clear increase in these receptors (Fig S5A) in pulsed cells, compared with non-pulsed cells. Cells, pulsed with oleandrin for 1 h, washed and treated with cycloheximide (CHX) (1 μM) and/or cystamine (10 μM) for 1 h, were cultured for a further 24 h. Oleandrin-pulsed cells showed an increased amount of IL-8 receptors in whole cell extracts and in the isolated membrane fraction. Cells pretreated with CHX showed almost 40% decrease in the amount of IL-8 receptors in whole cell extracts

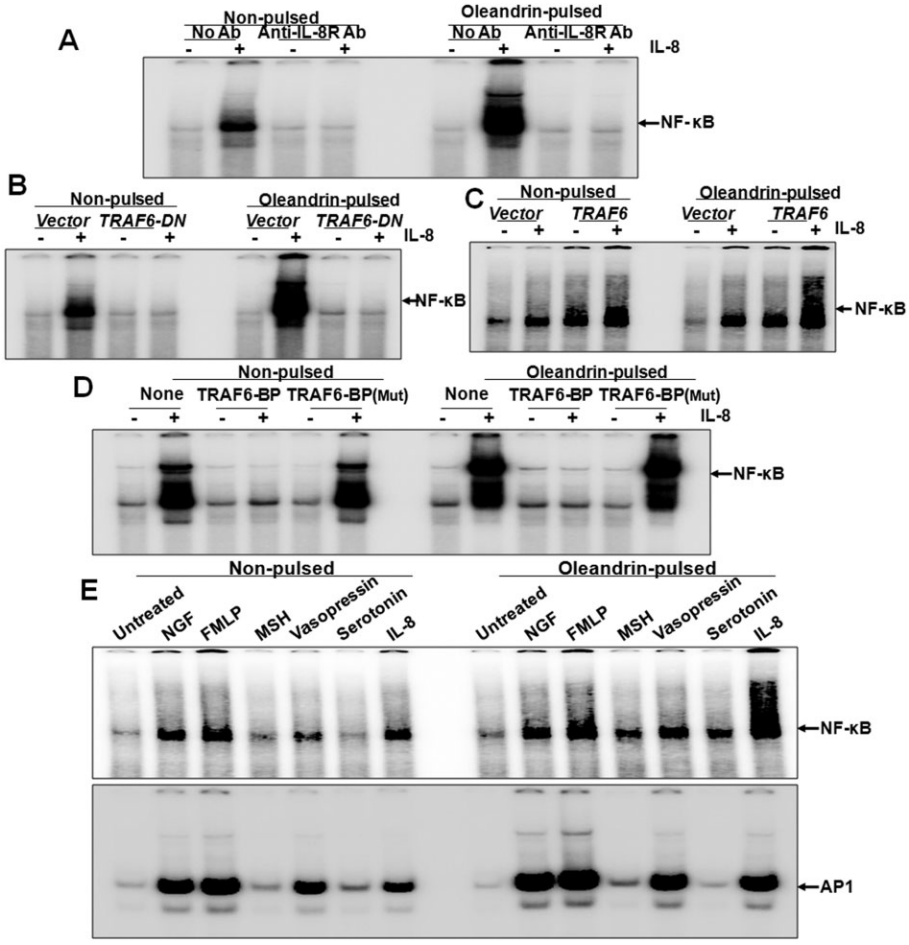

Figure 4

Effect of oleandrin pulse on IL-8-mediated signalling pathway. In (A), Oleandrin-pulsed U-937 cells, incubated with antibodies to the two IL-8 receptors (1 μg mL^{-1} each) for 2 h, were stimulated with IL-8 for 4 h. NF-κB DNA binding was assayed in nuclear extracts. In (B), Oleandrin-pulsed cells were cultured for 12 h, transfected with 1 μg of *TRAF6-DN* or (in C) the *TRAF6* construct for 3 h, washed and cultured for 12 h. Cells were then stimulated with 100 ng mL^{-1} IL-8 for 4 h. NF-κB DNA binding was measured. In (D), oleandrin-pulsed U-937 cells were incubated with 200 μM of TRAF6-BP or TRAF6-BP (Mut) for 4 h and then stimulated with IL-8 for 4 h. NF-κB DNA binding was determined in nuclear extracts. In (E), oleandrin-pulsed cells were stimulated with NGF (100 nM), FMLP (100 nM), α-MSH (1 μM), vasopressin (100 nM), serotonin (100 nM) or IL-8 (100 ng mL^{-1}) for 6 h. NF-κB DNA binding was measured in nuclear extracts.

and the membrane fraction. Cystamine-pretreated cells showed almost 30% decrease in the amount of IL-8 receptors in the membrane extract, but no decrease in the whole cell extracts (Figure 5C). Cells treated with both, showed complete inhibition of IL-8 receptors in the membrane, but 50% inhibition in the whole cell extracts.

Cells, pulsed with oleandrin for 1 h, washed and treated with CHX and/or cystamine for 1 h were cultured for 24 h. Cells were then stimulated with IL-8 for 4 h. In non-pulsed cells, IL-8-induced NF-κB activation was not altered by treatment with CHX or cystamine alone or in combination. In oleandrin-pulsed cells, IL-8 increased the activation of NF-κB more than in non-pulsed cells and this activation was decreased by CHX or cystamine, by almost 50% when used alone and totally inhibited when used in combination (Figure 5D; summary data for CHX and cystamine effects are shown in Fig S5B)

Oleandrin-pulsed cells increase IL-8-mediated biological responses

IL-8 increased NF-κB and AP-1 DNA binding with increasing concentrations in oleandrin-pulsed cells, compared to non-pulsed cells (Figure 6A; summary data in Fig S6A). IL-8-inducedThe oxidative burst induced in response to IL-8 was measured by the nitroblue tetrazolium assay and the number of cells staining positively increased twofold in oleandrin-pulsed cells at any concentration of IL-8 (Figure 6B; summary data for 100 ng mL^{-1} IL-8 is shown in Fig S6B). The chemotactic index increased twofold at any concentration of IL-8 as determined from the number of migrated cells by oleandrin-pulsed cells (Figure 6C). The release of enzymes induced by IL-8 from oleandrin-pulsed cells was almost doubled, at any concentrations of IL-8 (Figure 6D; summary data for 100 ng mL^{-1} IL-8 is shown in Fig S6C). IL-8 increased calcineurin

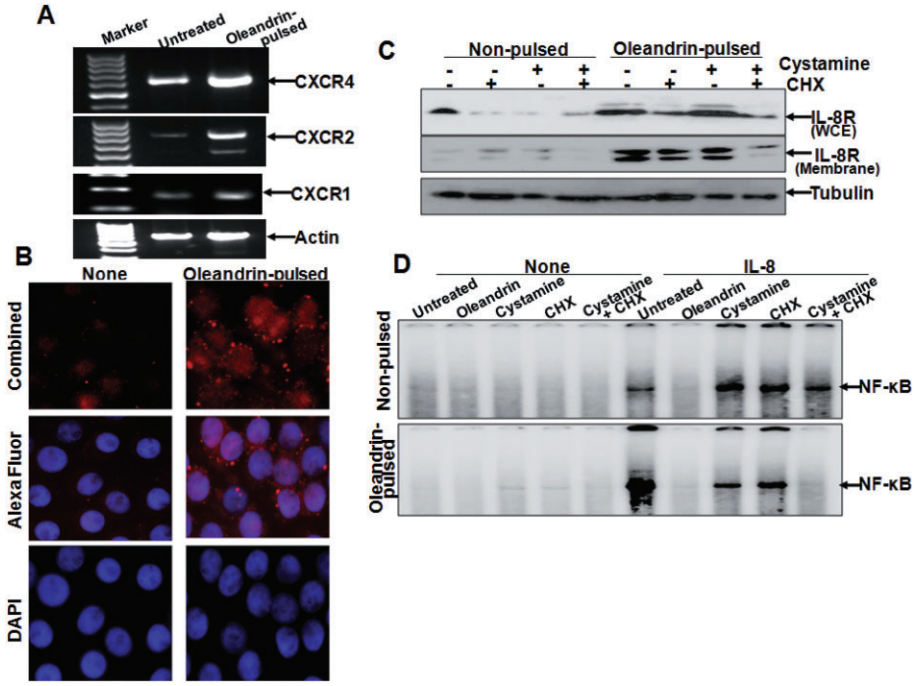

Figure 5

Effect of oleandrin pulse on expression of IL-8 receptors. In (A), total RNA was isolated from cells pulsed with oleandrin (1h) followed by culture in oleandrin-free medium for 24 h. The amounts of mRNA for CXCR4, CXCR2, CXCR1 and actin were measured by RT-PCR. In (B), the amount of CXCR1 on U 937 cells was detected by immunofluorescence using an appropriate antibody and visualised with goat anti-rabbit IgG conjugated with Alexa fluor 594. (C) Cells were pulsed with oleandrin for 1 h, then washed and treated with 1 μM cycloheximide (CHX) or 10 μM cystamine for 1 h and then cultured for 24 h. The amount of IL-8 receptors were determined in 100 μg of whole cell extracts (WCE) and 50 μg of membrane extract by Western blot. In (D), U-937 cells, either non-pulsed or pulsed with oleandrin, treated with CHX, cystamine or oleandrin for 2 h were cultured for 24 h. Cells were stimulated with 100 ng mL^{-1} IL-8 for 4 h. NF-κB activation was assayed in nuclear extracts.

activity (Figure 6E; summary data for 100 ng mL^{-1} IL-8 is shown in Fig S6D), NFAT DNA binding (Figure 6F) and NFAT-dependent luciferase activity (Figure 6G) in oleandrin-pulsed cells, compared to the response in non-pulsed cells. All these data suggest that oleandrin-pulsed cells exhibited an increased biological response to IL-8.

As we found that the amount of IL-8 receptors was increased in oleandrin-pulsed cells, we measured IL-8-mediated angiogenesis, using a wound-healing assay in cultured cells. Monolayers of A549 cells, either non-pulsed or oleandrin-pulsed, were scratched at 50% confluence and incubated with 50 or 100 ng mL^{-1} of IL-8. Cells were visualized under phase contrast microscope at different times. The scratch was covered more rapidly in cultures of oleandrin-pulsed cells, compared to that in non-pulsed cells (Figure 7)

Lipids block the expression of IL-8 receptors in oleandrin-pulsed cells

Cells were pre-incubated with different lipid molecules followed by oleandrin-pulsing and exposure to IL-8. Assay of NF-κB DNA binding (Figure 8A; summary data in Fig S8A) in these cells showed inhibition of the increase mediated by IL-8. These lipid molecules had no effect on IL-8-mediated NF-κB DNA binding in non-pulsed cells. Pre-treatment with a combination of all the lipid molecules almost completely inhibited the responses to IL-8 in oleandrin-pulsed cells (Figure 8B; summary data in Fig S8B).

Activity of the transcription factor NFAT is increased in oleandrin-pulsed cells

The activity of NFAT, measured by DNA binding, was increased with increasing concentrations of oleandrin used to pulse the cells (Figure 8C, upper panel). As a positive control, nuclear extracts were prepared from cells stimulated with phorbol myristate acetate (25 ng mL^{-1}) for 2 h. The amount of total IL-8 receptors was increased with increasing oleandrin concentration (Figure 8C, lower panel). In cells pre-incubated with the combination of lipids (see above), the increase in NFAT DNA binding in oleandrin-pulsed cells was almost completely inhibited (Figure 8D, lower panel) as was the expression of IL-8 receptors (Figure 8D, upper panels). NFAT activation was also inhibited by BAPTA-AM or CsA, but not by pyrrolidine dithiocarbamate, in oleandrin-pulsed cells (Figure 8E).

Discussion

Oleandrin, a glycoside found in extracts, fruits and/or seeds of *Nerium oleander*, shares several features with two other, better studied, plant glycosides, ouabain and digoxin, including the inhibition of the Na^+ K^+ ATPase and consequent cardio-toxicity. It is also toxic to other types of human cells including several tumour cell lines, but short-term exposure

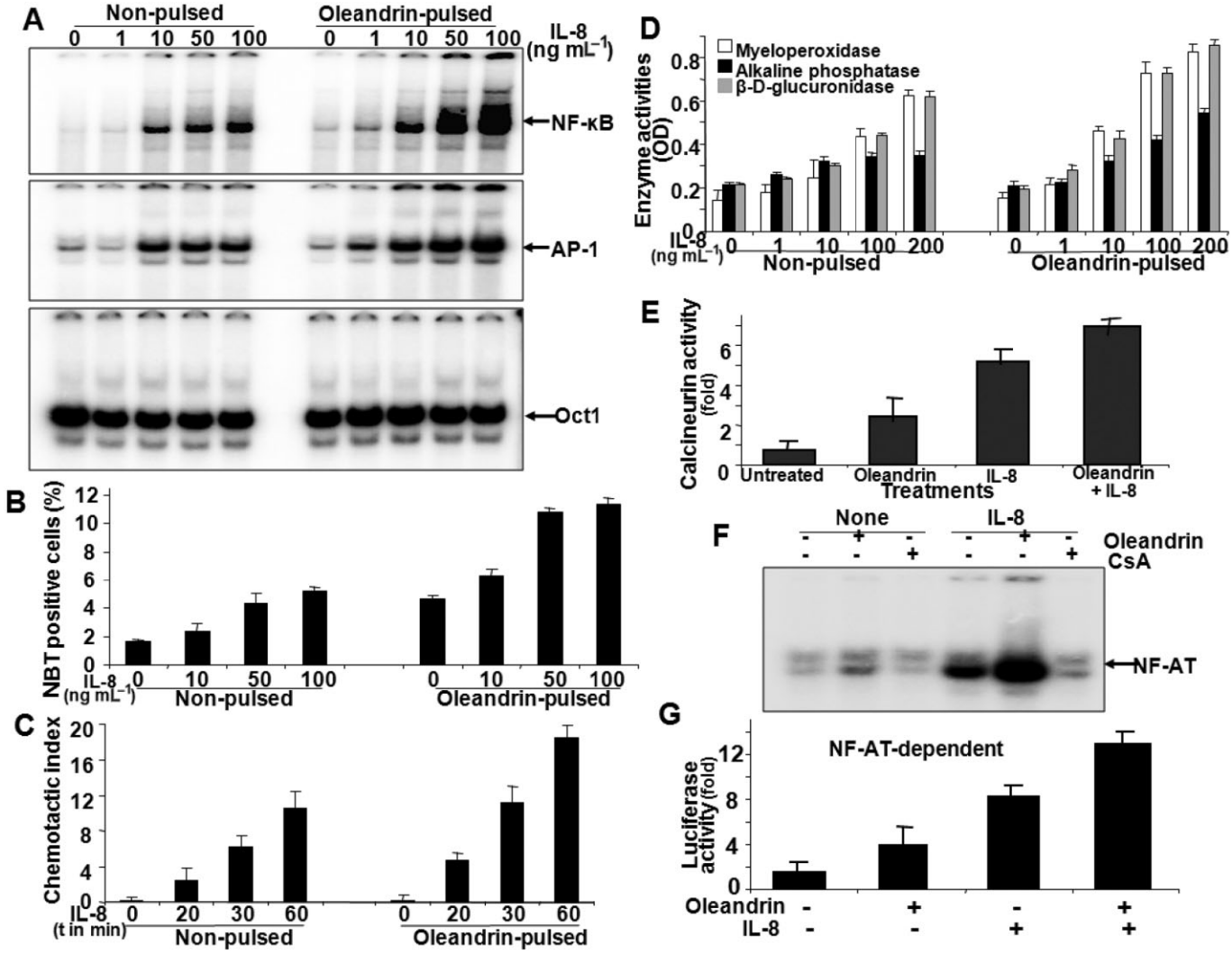

Figure 6

Effect of oleandrin pulse on IL-8-mediated biological responses in U-937 cells. (A) Oleandrin-pulsed and non-pulsed cells were stimulated with different concentrations of IL-8 for 4 h. Activation of NF-κB, AP-1 and Oct1 was determined in nuclear extracts. (B) Oxidative burst in cells stimulated by IL-8 was assessed with nitroblue tetrazolium (NBT) dye. The proportion of NBT positive cells was determined and shown as fold of of values in untreated cells (set to unity). (C) Migration of U-937 cells to IL-8 was determined in Boyden chambers and the chemotactic index (ratio of cell migration with and without IL-8) is shown. (D) Activities of myeloperoxidase, β-D-glucuronidase and alkaline phosphatase were measured in the culture supernatant of oleandrin-pulsed and non-pulsed cells, stimulated with different concentrations of IL-8 for 4 h. Activities are expressed as the OD values corresponding to each assay. In (E), the calcineurin activity in oleandrin-pulsed cells stimulated with IL-8 (100 ng mL^{-1}) for 2 h is shown. (F) Oleandrin-pulsed cells, incubated with cyclosporine A (CsA; 2.5 μM) for 2 h were stimulated with IL-8 and NFAT activation was measured in nuclear extracts. In (G), cells were pulsed with oleandrin for 1 h, cultured for 12 h, and then transfected with NFAT-luciferase construct (1 μg) for 3 h and cultured for another 12 h. Cells were then stimulated with IL-8 (100 ng mL^{-1}) for 12 h and the luciferase activity was measured.

(1–3 h) to oleandrin is much less cytotoxic. Such 'pulse' exposure has allowed us to investigate the effects of oleandrin on a crucial component of inflammation, the chemotaxis and cell migration of leukocytes, without inducing cell death. Pulse treatment of U937 cells, a human monocytic cell line, with oleandrin, but not with other apoptosis-inducing agents like azadirachtin, benzofuran, thiadiazolidine or resveratrol, increased the expression of IL-8 receptors and responses to exogenous IL-8 such as the oxidative burst, secretion of enzymes and intracellular Ca^{2+} release. All these responses would strengthen host defence against invading pathogens.

Oleandrin-pulsed cells increased their responses to IL-8, but not to other cytokines or growth factors. Furthermore, responses to agonists of other GPCRs such as NGF, FMLP, MSH, vasopressin or serotonin were also unaffected. The mechanism underlying this selective effect of oleandrin on IL-8 induced responses is not fully explored. Although a 3 h incubation with oleandrin down-regulated cell surface IL-8 receptors in macrophages by changing the microviscosity of the plasma membrane , by 10–15% (Manna *et al.*, 2006b), other GPCRs (for FMLP) and growth factor receptors (for EGF and NGF) were also affected. The loss of cell surface receptors

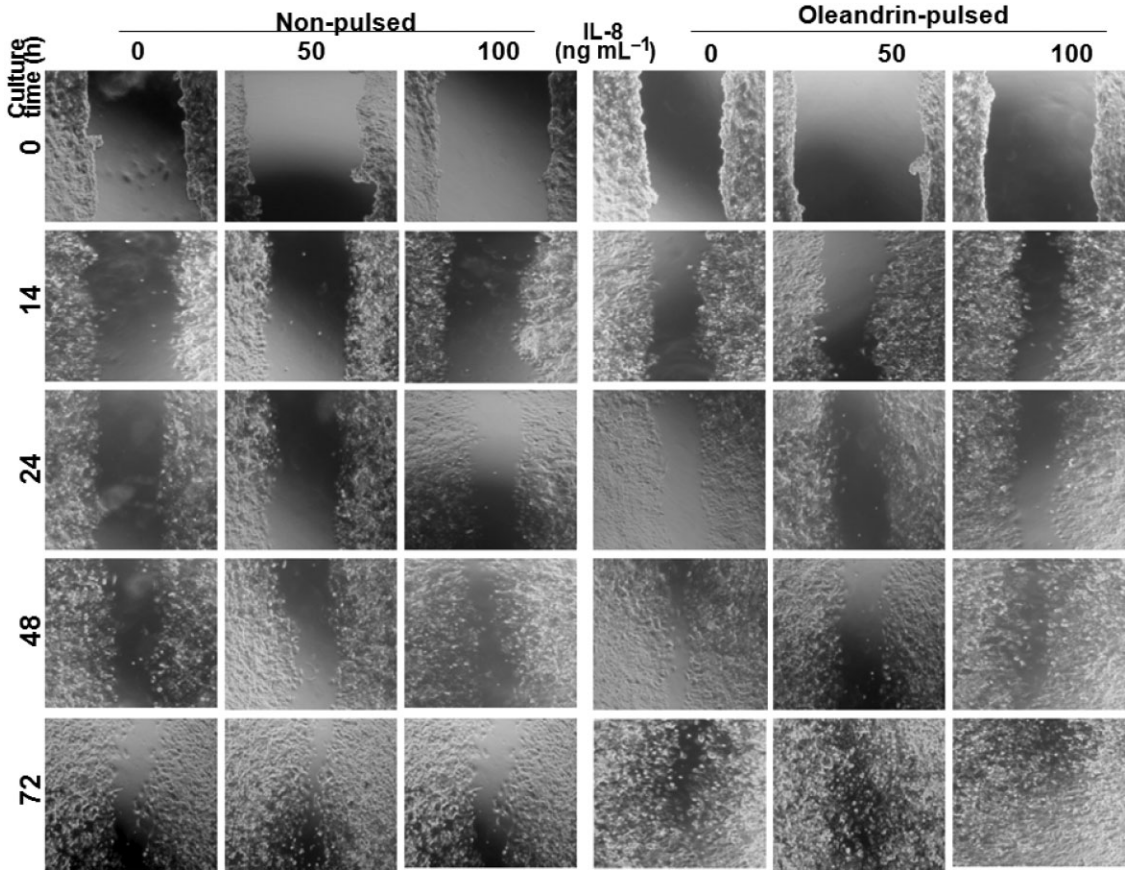

Figure 7

Effect of oleandrin exposure on a model of wound healing. Cultures of A549 cells at 50% confluency were pulsed or non-pulsed with oleandrin. The cell monolayer was then scratched with a sterile needle to simulate the wound and the cultures stimulated with 50 or 100 ng mL^{-1} of IL-8. Phase contrast images were taken at different times after wounding, as indicated in the Figure.

for IL-8 could induce a compensatory increase in the synthesis of IL-8 receptors. In oleandrin-pulsed cells, such nascent IL-8 receptors may not be able to stay in the cell membrane and thus be unable to mediate responses to IL-8, such as NF-κB activation. In the present experiments, pulsing with oleandrin increased myeloperoxidase release in response IL-8, suggesting increased degranulation of azurophilic granules. IL-8 receptors have been found in the secretory vesicles, as well as in the plasma membrane fraction of neutrophils (Uriarte *et al.*, 2008).

One function mediated by IL-8 receptors expressed on the cell surface is chemotaxis, the rapid and directed migration of cells towards a site of inflammation and this was increased, in our experiments, in oleandrin-pulsed cells. The oleandrin-pulsed cells also showed an increased respiratory burst, another important host defence process, in which NADPH oxidase transfers electrons from NADPH to molecular oxygen to form superoxide anions (McPhail and Snyderman, 1983; Manna *et al.*, 1997). Thus, these oleandrin-pulsed cells, on exposure to IL-8, increased their production of superoxide and other ROS. This change would not only increase the killing of invading microorganisms but would also activate several redox-sensitive cellular kinases activating transcription factors such as NF-κB and AP-1, Thus, pulsing with oleandrin would increase both the number of leukocytes and their response to an inflammatory stimulus. The oleandrin pulse also increased activity of calcineurin, possibly by increasing intracellular Ca^{2+}. This increased calcineurin activity would increase dephosphorylation of NFAT, thereby increasing its nuclear translocation and allowing this transcription factor to increase expression of genes, including those for the IL-8 receptors. Inhibition of Ca^{2+} or calcineurin inhibited NFAT-dependent expression of IL-8 receptors and almost 50% the expression of these receptors on the cell surface, following the oleandrin pulse. Blockade of protein synthesis, inhibited the remaining 50% of IL-8 receptor expression. We have already shown oleandrin to interact with membrane lipids and to down-regulate cell surface receptors including those for IL-8 (Manna *et al.*, 2006b). In our present experiments, pre-incubation of cells with a range of lipids prevented potentiation by the oleandrin-pulse, of responses to IL-8. This negative interaction of oleandrin with lipids further supports our previous observation (Manna *et al.*, 2006; Raghavendra *et al.*, 2007).

Mortality is high, due to pyrogenic infections, in conditions of defective chemotaxis, especially in case of infants, juvenile periodontitis and Chediak-Higashi syndrome (Repo *et al.*, 1990). Phagocytic dysfunction leads to a wide range of

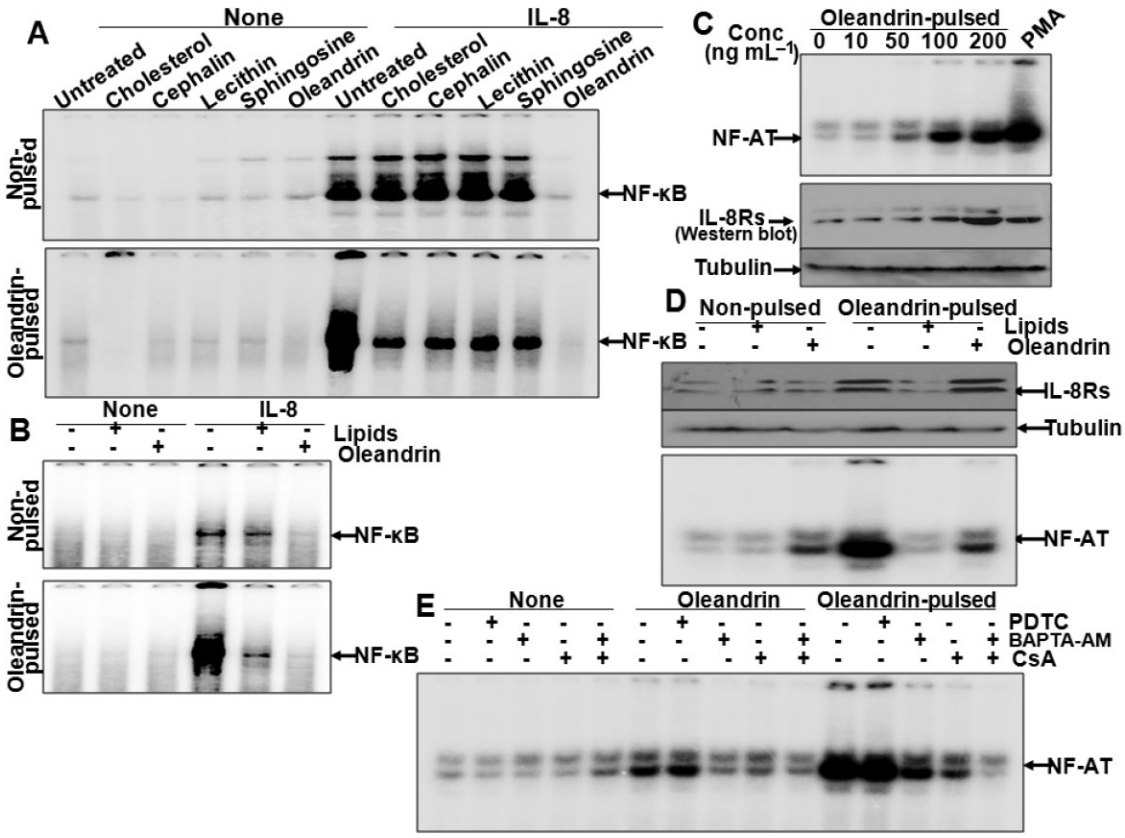

Figure 8

Effect of lipid compounds on the effects of pulse exposure to oleandrin. (A) U-937 cells were incubated with cholesterol, cephalin, sphingosine or lecithin (500 ng mL^{-1} each) for 4 h and then pulsed with oleandrin (1h), washed and incubated for a further 24h. Non-pulsed cells were similarly exposed to the lipids and then incubated with oleandrin for 24h. Cells were then stimulated with IL-8 for 4 h and NF-κB activation was assessed in nuclear extracts. (B) Cells, incubated with lipids for 4 h were pulsed or non-pulsed with oleandrin. For the last 2 h, cells were treated with oleandrin (100 ng mL^{-1}) in one set of samples, followed by stimulation with IL-8 for 4 h and NF-κB activation assayed. (C) Cells were pulsed with a range of concentrations of oleandrin (10–200 ng mL^{-1}). They were then stimulated with 50 ng mL^{-1} phorbol myristate acetate (PMA) for 2 h. Activation of NFAT was determined in nuclear extracts and IL-8 receptors were measured in whole cell extracts (WCE). (D) Cells, incubated for 4 h with the combination of lipids were pulsed or non-pulsed with oleandrin. NFAT activation was measured. IL-8 receptors were measured by Western blot in whole cell extracts. (E) Cells pulsed with oleandrin for 1 h and then cultured with pyrrolidine dithiocarbamate (PDTC) (100 μM), BAPTA-AM (2.5 μM), or cyclosporine A (CsA) (2.5 μM) for 24 h. In one set, cells were treated with 100 ng mL^{-1} oleandrin for 4 h. NFAT activation was assayed in nuclear extracts.

pathologies, from mild recurrent skin infections to fatal systemic infection, because the affected patients are more susceptible to bacterial and fungal infections. Most are diagnosed in infancy due to the severity of the infection or the unusual presentation of the organism. Our work has provided evidence that short-term treatment with low doses of oleandrin could enhance the response to chemokines, particularly the leukocyte migration involved in early host defence. Several NF-κB-dependent genes, such as those for adhesion molecules, are involved in wound healing (Nagaoka *et al.*, 2000). As the oleandrin-pulsed cells increased NF-κB activation mediated by IL-8, the wound healing ability could also be increased through expression of those NF-κB-dependent adhesion molecules.

In summary, we have here provided evidence that oleandrin, a compound that failed clinical trials for tumour therapy because of its high toxicity, could nevertheless be therapeutically beneficial, if given for only short periods, to potentiate the immune responses in the first line of host defence. Such activity may be useful in those patients, where defects in chemotaxis, phagocytosis, secretion of proteolytic enzymes and wound healing are apparent.

Acknowledgements

This work is supported by the core grant of Centre for DNA Fingerprinting and Diagnostics (CDFD). We acknowledge the Indian Council of Medical Research (ICMR), India for providing fellowship to N. R.

Conflict of interest

Authors declare that they have not any conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12493

Figure S1A Effect of oleandrin pulse treatment for different times on IL-8-induced NF-κB activation. U-937 cells were treated with 100 ng/ml oleandrin for 1 h. One set of cells were incubated with oleandrin without washing, and the other two sets washed and then incubated in fresh medium. All these sets were cultured for different times and then one washed set was stimulated with 100 ng/ml IL-8 for 2 h. NF-κB DNA binding was assayed in nuclear extracts and the intensity of the bands were represented as fold change relative to the values in untreated cells, set to unity. Data are means ± SEM (N = 3).

Fig.S1B Effect of oleandrin pulse on NF-κB DNA binding activity. NF-κB DNA binding was assayed by EMSA from non-pulsed and pulsed cells (100 ng/ml oleandrin for 1 h, followed by culture for 24 h). The values were obtained from densitometry analysis of the respective bands taken from four independent experiments and are expressed as fold increase over levels in non-pulsed cells Data shown are means ± SD (N = 4). ****$P < 0.05$, unpaired *t*-test.

Figure S2A Effect of pulse treatment with different inhibitors on IL-8- or TNF-induced NF-κB activation. U-937 cells were pulsed with azadirachtin (10 μM), benzofuran (100 nM), oleandrin (100 ng/ml), P_3-25 (100 nM), or resveratrol (10 μM) were stimulated with TNF (100 pM) or IL-8 (100 ng/ml) for 2 h. NF-κB DNA binding was assayed in nuclear extracts and the intensity of the bands are represented as fold change relative to the values in untreated cells, set to unity. Data are means ± SEM (N = 3).

Figure S2B Effect of pulse treatment with different inhibitors on cell viability. U-937 cells were either pulsed for 1 h

with azadirachtin (10 μM), benzofuran (100 nM), oleandrin (100 ng/ml), P_3-25 (100 nM), or resveratrol (10 μM), followed by washing and further incubation in medium alone for 72 h, in triplicate. Cell viability was assayed by MTT assay. Cell viability is shown as % dead cells in the cultures. Data shown are means ± SD (N = 2).

Figure S3A Effect of oleandrin pulse on NF-κB activation mediated by different inducers. Oleandrin-pulsed or non-pulsed U-937 cells were stimulated with TNF (10 pM), LPS (100 ng/ml), IL-1 (50 nM), IL-8 (50 ng/ml), or EGF (100 pM) for 6 h. NF-κB DNA binding was assayed in nuclear extracts and the intensity of the bands are represented as fold change relative to the values in untreated cells, set to unity. Data are means ± SEM (N = 3).

Figure S3B Effect of oleandrin pulse on NF-κB activation mediated by IL-8 in different cells. A range of cell types were pulsed with oleandrin (OL) and stimulated with 10 or 50 ng/ml IL-8 for 4 h. NF-κB DNA binding was assayed in nuclear extracts and the intensity of the bands are represented as fold change relative to the values in untreated cells, set to unity. Data are means ± SEM (N = 3)

Figure S4A & B Effect of oleandrin pulse on IL-8-mediated signaling pathway. In A, oleandrin-pulsed U-937 cells were incubated with antibodies to IL-8 receptors (1 μg/ml each) for 2 h and then stimulated with IL-8 for 4 h. NF-κB DNA binding was assayed in nuclear extracts. In B, oleandrin-pulsed cells were cultured for 12 h, transfected with 1 μg of *TRAF6-DN* construct for 3 h, washed and cultured for 12 h. Cells were then stimulated with 100 ng/ml IL-8 for 4 h. NF-κB DNA binding was measured in nuclear extracts . The intensity of the bands are represented as fold change rletive to values in untreated cells (Non-pulsed, no antibodies and without IL-8 in case of A; Non-pulsed, vector and without IL-8 in case of B) set to unity. Data are means ± SEM (N = 3).

Figure S4C & D Effect of oleandrin pulse on IL-8-mediated signaling pathway. In C, oleandrin-pulsed cells were cultured for 12 h, transfected with 1 μg of *TRAF6* construct for 3 h, washed and cultured for 12 h. Cells were then stimulated with 100 ng/ml IL-8 for 4 h. NF-κB DNA binding was measured. Oleandrin-pulsed U-937 cells were incubated with 200 μM of TRAF6-BP or TRAF6-BP (Mut) for 4 h and then stimulated with IL-8 for 4 h. NF-κB DNA binding was determined in nuclear extracts and the intensity of the bands were represented as fold change relative to the values in untreated cells (vector, without IL-8 in C; Non-pulsed, without IL-8 in D), set to unity. Data are means ± SEM (N = 3).

Figure S4E Effect of oleandrin pulse on IL-8-mediated signaling pathway. Oleandrin-pulsed cells were stimulated with NGF (100 nM), FMLP (100 nM), α-MSH (1 μM), vasopressin (100 nM), serotonin (100 nM), or IL-8 (100 ng/ml) for 6 h. NF-κB DNA binding was measured in nuclear extracts and the intensity of the bands were represented as fold change relative to the values in untreated cells (None), set to unity. Data are means ± SEM (N = 3).

Figure S5A Effect of oleandrin pulse on IL-8 receptor expression. The amount of IL-8 receptors was determined by Western blot from non-pulsed and oleandrin-pulsed (100 ng/ml for 1 h, followed by culture for 24 h) whole cell extracts. The data represent fold change relative to the values in non-pulsed cells, set to unity. Data are means ± SEM (N = 3). $P < 0.05$; unpaired *t*-test.

Figure S5B Effect of oleandrin pulse on IL-8-mediated NF-κB activation. U-937 cells, either non-pulsed or pulsed, were treated with CHX, cystamine, or oleandrin for 2 h were cultured for 24 h. Cells were stimulated with 100 ng/ml IL-8 for 4 h. NF-κB DNA binding was assayed in nuclear extracts and the intensity of the bands were represented as fold change relative to the values in untreated cells (without IL-8), set to unity. Data are means ± SEM (N = 3).

Figure S6A Effect of oleandrin pulse on IL-8-mediated biological responses. Oleandrin-pulsed and non-pulsed cells were stimulated with different concentrations of IL-8 for 4 h. NF-κB DNA binding was determined in nuclear extracts and the intensity of the bands were represented as fold change relative to the values in untreated cells (Non-pulsed, 0 ng/ml IL-8), set to unity. Data are means ± SEM (N = 3).

Figure S6B Effect of IL-8 on oxidative burst (as NBT positive staining) in U937 cells exposed to oleandrin pulsing. Data shown represent the increase of NBT positive cells as fold change, relative to the basal level (non-pulsed, no IL-8) set to unity. Data are means ± SEM (N = 2); $*P < 0.05$, unpaired *t*-test.

Figure S6C Effect of oleandrin pulse on enzyme release from U937 cells in response to IL-8. Oleandrin-pulsed cells were suspended in phenol-free DMEM and stimulated with 100 ng/ml IL-8 for 4 h. Activities of myeloperoxidase, β-D-glucuronidase and alkaline phosphatase were measured in the culture supernatant and are shown as OD in the Figure. Data are means ± SEM (N = 2). $*P < 0.05$, unpaired *t*-test.

Figure S6D Effect of oleandrin pulse on IL-8-mediated calcineurin activity. Oleandrin-pulsed and non-pulsed cells were stimulated with IL-8 (100 ng/ml) for 2 h and the calcineurin activity was determined by measuring inorganic phosphate released from the phospho-peptide substrate (RII peptide). Data are means ± SEM (N = 2). $**P < 0.005$, one-way ANOVA.

Figure S8A Effect of lipid compounds on NF-κB activation after oleandrin pulse and IL-8. U-937 cells, incubated with 500 ng/ml each of cholesterol, cephalin, sphingosine, or lecithin for 4 h were pulsed with oleandrin. Cells were stimulated with IL-8 for 4 h and NF-κB DNA binding was assayed in nuclear extracts and the intensity of the bands were represented as fold change relative to the values in untreated cells (without IL-8), set to unity. Data are means ± SEM (N = 3).

Figure S8B Effect of a combination of lipid molecules on oleandrin-pulse-mediated NF-κB activation. U-937 cells, incubated with a combination of lipids (500 ng/ml each of cholesterol, cephalin, sphingosine, and lecithin) for 4 h were pulsed with oleandrin. For the last 2 h, cells were treated with 100 ng/ml oleandrin in one set of samples, followed by stimulation with IL-8 (100 ng/ml) for 4 h. NF-κB was assayed in nuclear extracts. The intensity of the bands are represented as fold change relative to the values in untreated cells (Non-pulsed, without IL-8), set to unity. Data are means ± SEM (N = 3).