Involvement of c-Jun NH$_2$-Terminal Kinase Activation and c-Jun in the Induction of Apoptosis by the Ether Phospholipid 1-O-Octadecyl-2-O-methyl-rac-glycero-3-phosphocholine

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ABSTRACT

The ether phospholipid 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ET-18-OCH$_3$; edelfosine) is a potent inducer of apoptosis in human tumor cells. We show that ET-18-OCH$_3$-induced apoptosis is associated with activation of the c-Jun NH$_2$-terminal kinase (JNK) signaling. The addition of ET-18-OCH$_3$ to distinct human leukemic cells (HL-60, U937, and Jurkat), which undergo rapid apoptosis on treatment with ET-18-OCH$_3$, induced a dramatic and sustained increase in the level of c-jun mRNA that was associated with activation of activator protein-1 transcription factor. We found that ET-18-OCH$_3$ induced a persistent activation of JNK in HL-60 cells that was detected before the onset of apoptosis, the latter being assessed by DNA fragmentation and by the appearance of phosphatidylserine on the external leaflet of the plasma membrane. The inductions of JNK after HL-60 monocyte/macrophage differentiation and ET-18-OCH$_3$-mediated apoptosis were distinguished by the different activation patterns, transient versus persistent, respectively. ET-18-OCH$_3$ analogues unable to induce apoptosis failed to activate JNK. ET-18-OCH$_3$-dependent JNK activation was not detected in K562 cells, which did not undergo apoptosis on treatment with ET-18-OCH$_3$. Phorbol myristate acetate inhibited both ET-18-OCH$_3$-induced apoptosis and sustained JNK activation; thus, persistent JNK activation by ET-18-OCH$_3$ is associated with the capacity of this ether phospholipid to induce apoptosis. Furthermore, antisense oligonucleotides directed against c-jun blocked ET-18-OCH$_3$-induced apoptosis, indicating a role for c-Jun in this apoptotic response. These data indicate that JNK activation and c-Jun are involved in the induction of apoptosis by ET-18-OCH$_3$.

Synthetic ether phospholipids, characterized by the presence of an ether bond in position sn-1 of the glycerol backbone, are showing promise as a new class of clinical cancer chemotherapeutic drugs (Munder and Westphal, 1990; Houlihan et al., 1995). Some of these lipid molecules are being used as purging agents in autologous bone marrow transplantation (Koenigsmann et al., 1996) due to their antineoplastic activities and high selectivity for tumor cells. Several of these compounds are scheduled for, or currently undergoing, phase I/II clinical evaluation (Houlihan et al., 1993, 1994; Mollinedo et al., 1997). An important finding in the elucidation of the processes involved in the antineoplastic effect of ET-18-OCH$_3$ was its action as a potent inducer of apoptosis in tumor cells (Diomede et al., 1993, 1994; Mollinedo et al., 1993a, 1997), and this apoptotic action seems to account for the previously reported cytotoxic effects exerted by this ether phospholipid (Mollinedo et al., 1997). Although the direct apoptotic action of ET-18-OCH$_3$ on the cancer cell has been established (Diomede et al., 1993, 1994; Mollinedo et al., 1993b, 1997), the molecular mechanisms that initiate the active programmed cell death in ET-18-OCH$_3$-treated tumor cells remain largely unknown. One of the first putative targets for

ABBIWECTIONS: JNK, c-Jun NH$_2$-terminal kinase; AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GSH, glutathione; GST, glutathione S-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TK, thymidine kinase; ITS, insulin/transferrin/sodium selenite; MAPK, mitogen-activated protein kinase; SEK, mitogen-activated protein kinase kinase/c-Jun NH$_2$-terminal kinase kinase; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulfate; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.
the action of ET-18-OCH₃ was considered to be the lipid metabolism, because this ether phospholipid affects lipid biosynthesis and reduces the levels of phosphatidylcholine (Modell et al., 1979; Boggs et al., 1995a). In this regard, it has been recently reported that ET-18-OCH₃ behaves as a negative regulator of de novo phosphatidylcholine synthesis, acting at the CTP:phosphocholine cytidyltransferase step (Boggs et al., 1995b), and it also is a potent inhibitor of coenzyme A-independent transacylase (Winkler et al., 1996). Nevertheless, some studies show a lack of correlation between decreases in phospholipid metabolism and the sensitivity of different cell lines to ET-18-OCH₃, suggesting that the lipid perturbation by ET-18-OCH₃ is unlikely to be the underlying cause for its antineoplastic effect (Lu and Arthur, 1992a, 1992b).

The JNKs, also known as stress-activated protein kinases, are members of the MAPK-related family (Karlin, 1995; Gupta et al., 1996) and are activated in response to a variety of cellular stresses (Verheij et al., 1996; Zanke et al., 1996). The JNK protein kinases phosphorylate the NH₂-terminal transactivation domain of c-Jun at Ser63 and Ser73, causing increased c-Jun transcriptional activity (Hibi et al., 1993). Thus, JNK can mediate the effect of extracellular stimuli on c-Jun and thereby acts as a physiologically relevant regulator of AP-1 transcriptional activity. JNK activation in turn requires its phosphorylation by mitogen-activated protein kinase kinase 4/c-Jun NH₂-terminal kinase kinase, also referred to as SEK1 (Lin et al., 1995). Mitogen-activated protein kinase kinase 4/c-Jun NH₂-terminal kinase kinase itself is phosphorylated and activated by the upstream kinase MAPK/ERK kinase kinase 1 (Yan et al., 1994). Thus, the stress-activated protein kinase/JNK pathway involves sequential activation of the proteins MAPK/ERK kinase kinase 1, SEK1, JNK, and c-Jun. A number of agents able to induce DNA degradation, including tumor necrosis factor, ceramide, cis-platinum, γ-radiation, or UV radiation, recently have been reported to activate JNK (Sluss et al., 1994; Chen et al., 1996 Verheij et al., 1996; Zanke et al., 1996). Furthermore, transient and stable expression of a dominant-negative kinase-inactive SEK1 construct impairs both activation of JNK and the apoptotic response triggered by various cell stressors (Verheij et al., 1996; Zanke et al., 1996). These observations are consistent with the involvement of JNK in the initiation of apoptosis.

We reported previously that ET-18-OCH₃ is able to induce the expression of fos and jun proto-oncogenes and to activate the transcription factor AP-1 in human leukemic cells (Mollinedo et al., 1994a). In this previous study, we observed that the steady state mRNA level of c-jun was dramatically increased on ET-18-OCH₃ treatment (Mollinedo et al., 1994a). On these grounds, and because JNK activation is involved in the induction of c-jun (Karlin, 1995), we examined whether JNK stimulation and c-Jun could be implicated in the apoptotic response induced by ET-18-OCH₃.

In the current study, we found that ET-18-OCH₃ increases dramatically the steady state levels of c-jun mRNA and induces a persistent JNK activation. The results reported here establish an association between ET-18-OCH₃-induced apoptosis and JNK activation. Furthermore, we found that treatment of cells with antisense oligonucleotides directed against c-jun proto-oncogene protects cells from cell death induced by ET-18-OCH₃, indicating a role for this gene on the induction of apoptosis by ET-18-OCH₃.

Materials and Methods

Chemicals and reagents. ET-18-OCH₃ was from R. Berthold (Biochemisches Labor, Bern, Switzerland) and from Laboratorios INKEYSA (Barcelona, Spain). ET-18-OCH₃ analogues were kindly provided by Dr. P. G. Munder (Max-Planck-Institut für Immunobiologie, Freiburg, Germany). ET-18-OCH₃ and its analogues were dissolved at 500 µg/ml as a stock solution in RPMI-1640 culture medium containing 10% (v/v) heat-inactivated fetal calf serum by heating at 50° for 30 min. The clear solutions were sterilized by filtration through a sterile filter ( pore size, 0.22 µm) and stored at 4°. Human recombinant TNF-α was kindly provided by Dr. G. Adolf (Boehringer Research Institute, Vienna, Austria). RPMI-1640 culture medium, fetal calf serum, and l-glutamine were purchased from Gibco BRL (Gaithersburg, MD). Antibiotics were from Laboratorios Llorente (Madrid, Spain). Annexin-V-FUOS to examine phosphatidylyserine exposure was from Boehringer-Mannheim Biochemica (Mannheim, Germany). The Fluorescein Apoptosis Detection System kit for TUNEL assays was purchased from Promega (Madison, WI). [α-32P]dCTP (3000 Ci/mmol), [γ-32P]ATP (3000 Ci/mmol), and [γ-threo-[dichloroacetetyl-1,1-14C]chloramphenicol (54.6 mCi/mmol) were purchased from Amersham (Buckinghamshire, UK). PMA, GSH-agarose, and pyrithione were from Sigma Chemical (St. Louis, MO). Guanidine thiocyanate was from Fluka (Buchs, Switzerland). Formaldehyde was from J. T. Baker Chemicals B.V. (Deventer, Holland). Acrylamide, bisacrylamide, ammonium persulfate, and N,N,N’,N’-tetramethylethylenediamine were from BioRad (Richmond, CA). All other chemicals were from Sigma or Merck (Darmststadt, Germany).

Cells and culture conditions. The human leucemic cell lines used in this study were promyelocytic HL-60, promonocytic U937, T lymphoid Jurkat, and chronic myelogenous K562. These cells were grown in RPMI-1640 culture medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mm l-glutamine, 100 units/ml penicillin, and 24 µg/ml gentamicin. Cells were incubated at 37° in a humidified atmosphere of 5% CO₂/95% air. Ether phosphorylides were added to the cell cultures at 3 or 5 µg/ml for the times indicated in the respective figures.

Analysis of DNA fragmentation in agarose gels. To assess apoptosis, we isolated fragmented DNA as described previously (Mollinedo et al., 1993a). In brief, ~2 × 10⁶ cells were washed with phosphate-buffered saline and then lysed with 200 µl of hypotonic detergent buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% Triton X-100) for 30 min at 4°. Nuclei and cell organelles were centrifuged in a microfuge for 20 min, and the supernatant, containing the DNA released into the cytosol due to DNA fragmentation, was incubated with RNase A (75 µg/ml) for 45 min at 37° and then with proteinase K (200 µg/ml) in the presence of 0.5% SDS for additional 45 min at 37°. The DNA was extracted, precipitated, and analyzed by electrophoresis on 1% agarose gels as described previously (Mollinedo et al., 1993a).

TUNEL assay. Apoptosis was also analyzed in situ by the TUNEL technique using the Fluorescein Apoptosis Detection System Kit (Promega) according to the manufacturer’s instructions, labeling the 3′-OH ends generated by DNA fragmentation through incorporation of fluorescein-12-deUTP (Gavrieli et al., 1992). Fluorescent cells were visualized and scored with a Zeiss LSM 510 laser scan confocal microscope.

Phosphatidylyserine exposure. Phosphatidylyserine exposure at the external surface of the cell was measured by the binding of FITC-labeled annexin V according to the protocol outlined by the manufacturers in the Annexin-V-FLUOS reagent (Boehringer-Mannheim). Then, cells were analyzed with Becton Dickinson (Le Pont de Claix, France) FACScan and FACStar-Plus flow cytometers.
[3H]Thymidine incorporation and TUNEL assay. Cell proliferation was monitored according to [3H]thymidine (Amersham) incorporation as described previously (Mollinedo et al., 1993a).

**Northern blot.** Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method. Samples of 20 μg of RNA were electrophoresed on 0.9% (w/v) agarose-formaldehyde gels and then transferred to Hybond-N nylon membranes (Amer sham) as described previously (Mollinedo et al., 1994a). 32P-labeled cDNA probes were prepared using the random hexanucleotide priming method (oligo-labeling kit; Pharmacia Biotech, Uppsala, Sweden) to a specific radioactivity ≥7 × 10⁶ cpm/mg of DNA. cDNA probes for c-fos and c-jun (Mollinedo and Naranjo, 1991) were kindly provided by Dr. P. Sassone-Corsi (Laboratoire de Genetique Moleculaire des Eucaryotes, Center National de la Recherche Scientifique, Strasbourg, France) and Dr. R. Bravo (Squibb Institute, Princeton, NJ). The plasmid pAC 18.1, used as a probe for β-actin, was used as was a control probe as described previously (Mollinedo and Naranjo, 1991). Conditions for blot hybridization and washing have been described elsewhere (Mollinedo et al., 1994a). Quantitative analysis of the autoradiograms was performed by integration of peak areas after scanning with a PDI computing densitometer (Pharmacia).

**Electrophoration and CAT assay.** Jurkat cells (20 × 10⁶ in the exponential phase of growth were electrophorated as described previ ously (Mollinedo et al., 1994b) with 25 μg of the expression vector AP-1-TK-CAT plasmid (Angel et al., 1987), kindly provided by Dr. M. Karin (University of California, San Diego, CA). The AP-1-TK-CAT plasmid contains a single copy of the phorbol ester-responsive element (AP-1 site) inserted upstream to position 5 of the hypersh simplex virus thymidine kinase promoter and fused to the structural gene coding for the CAT gene, used as reporter gene (Angel et al., 1987). Experimental conditions for CAT assay and thin layer chromatography have been described elsewhere (Mollinedo et al., 1994b). After autoradiogram development, the percentage of acetylation of each treatment was determined by scraping off both the acetylated and nonacetylated chromophenolic and counting radioactivity.

**Solid-phase JNK assay.** Protein kinase assays were carried out using a fusion protein between GST and c-Jun (amino acids 1–223) as a substrate of JNK, as described previously (Hibi et al., 1993) with slight modifications. Cells (3–5 × 10⁶) were resuspended in 200 μl of extract buffer (25 mM HEPES, pH 7.7, 0.3 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na₂VO₃, 0.5 mM phenethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cells were incubated for 30 min in continuous rotation at 4° and then microfuged at 12,000 rpm for 10 min. The pellets were discarded, and the supernatants, representing cell extracts, were diluted with 600 μl of dilution buffer (20 mM HEPES, pH 7.7, 0.1 mM EDTA, 2.5 mM MgCl₂, 0.05% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na₂VO₃, 0.5 mM phenethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Mixtures were incubated for 10 min at 30° in kinase buffer supplemented with 20 μM ATP and 100 μCi [γ-32P]ATP. After 30 min at 37°, the reaction was terminated by washing with dilution buffer containing 50 mM NaCl and microfugation at 12,000 rpm for 1 min. Then, the beads were boiled with 10 μl of 5× SDS-polyacrylamide gel sample buffer to elute the phosphorylated proteins, which were subsequently resolved in an SDS-10% polyacrylamide gel, followed by autoradiography. These conditions have been shown previously to enable specific binding of JNK to c-Jun NH₂-terminal domain (Hibi et al., 1993).

**In-gel kinase assay for JNK activation.** This assay was performed according to Hibi et al. (1993) with slight modifications. Cells were lysed with extract buffer as above and centrifuged at 12,000 rpm for 10 min, and the supernatants, representing the cell extracts, were saved. Protein content was determined in these extracts using a BioRad protein assay kit. Approximately 60 μg of protein was loaded onto an SDS-10% polyacrylamide gel, which was polymerized in the presence of GST-c-Jun (~100 μg/ml). After electrophoresis, the gel was washed twice for 15 min each with 100 ml of 50 mM HEPES, pH 7.6, and 20% (v/v) 2-propanol to remove SDS and then twice for 15 min each with 50 mM HEPES, pH 7.6, 5 mM β-mercaptoethanol, and 0.05% Tween 20, supplemented first with 3 μl urea and then with 15 μl urea and finally with 0.75 μl urea. The proteins were renatured by washing the gel in 50 mM HEPES, pH 7.6, and 0.05% Tween 20 at 4°. The kinase reaction was performed by incubating the gel for 1 hr at 30° in kinase buffer supplemented with 20 μM ATP and 100 μCi [γ-32P]ATP. Finally, the gel was washed with 100 ml of 5% trichloroacetic and 1% sodium pyrophosphate at room temperature, followed by drying and autoradiography.

**Western blot.** Mininuclear extracts were obtained from 3 × 10⁶ cells as described previously (Mollinedo et al., 1993b), and proteins (30 μg) were separated through an SDS-12% polyacrylamide gel under reducing conditions, transferred to nitrocellulose filters, and subjected to immunological detection. Low range prestained protein molecular mass standards (BioRad) also were run in the same gel. After electroblotting and blocking for 1 hr in 2% powdered nonfat dry milk in TBS buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl), the nitrocellulose filters were incubated overnight with sheep anti-human c-Jun antibody (Cambridge Research Biochemicals, Cheshire, UK) at a dilution of 1:250 in TBS containing 0.05% Tween 20. Signal was developed after incubation with a rabbit anti-sheep immunoglobulin coupled to peroxidase and using 1:700 3,3′-diaminobenziidine with 0.03% (v/v) H₂O₂ in 50 mM Tris-HCl, pH 7.4, as a substrate solution. A rabbit antisem to the Fos M peptide, kindly provided by Dr. J. Jain (Dana-Farber Cancer Institute, Boston, MA), was used for Fos detection.

**Oligonucleotides.** c-fos sense (5′-TCTCGGCGCTCTAAGCGCA-3′), c-fos antisense (5′-TGCGTGAAGCCGGAGAAGA-3′), c-jun sense (5′-CTGCTTTCACTTCTTCTGATG-3′), and a random oligonucleotide (5′-ACCCGTTGTCGGTTATCCT-3′) were synthesized by using phosphorothioate linkages. The antisense nucleotide sequences were complementary to the first 18 bases after the AUG sequences of human mRNAs for c-fos (accession no. K00650) and c-jun (accession no. J04111) obtained from the GenBank EMBL database. The corresponding sense oligonucleotides and the random oligonucleotide were used as a control. Oligonucleotides penetrated into the cells without any treat ment, as described by Loke et al. (1989). HL-60 cells at an initial concentration of 1.2 × 10⁶ cells/ml were either untreated or treated with specific oligonucleotides in serum-free ITS medium composed of 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite in RPMI-1640, containing 0.4% (w/v) bovine serum albumin, 2 mM glutamine, 100 units/ml penicillin, and 24 μg/ml gentamicin. Oligonucleotides were added at a final concentration of 50 μg/ml and incubated for 48 hr in serum-free ITS medium. Then, cells were incubated in the absence or presence of 5 μg/ml ET-18-OCH₃ for 4 hr in serum-free ITS medium and assayed for DNA fragmentation and Western blot analysis. In addition, cells pretreated for 48 hr with random, sense, and antisense c-jun oligonucleotides also were assayed for ET-18-OCH₃-induced JNK activation and [3H]thymidine incorporation as indicated above.

**Results**

c-Jun expression induced by ET-18-OCH₃. We reported previously that ET-18-OCH₃ induces jun and fos proto-oncopenes and activates AP-1 transcription factor in
This activation of AP-1 enhancer activity by ET-18-OCH₃ is able to induce apoptosis, as assessed by CAT assays (Fig. 1B). AP-1 transcriptional activity when used at a concentration of 3 μg/ml ET-18-OCH₃, whereas the relative increase in the steadystate mRNA levels of both c-jun and c-fos in three ET-18-OCH₃-sensitive human leukemic cell lines (U937, HL-60, and Jurkat) (Mollinedo et al., 1993a, 1994a, 1997) on treatment with ET-18-OCH₃. We corroborated our previous findings (Mollinedo et al., 1994a), showing that ET-18-OCH₃ induces a dramatic and sustained increase in the c-jun mRNA level, whereas the c-fos mRNA level was weakly or hardly increased (Mollinedo et al., 1994a). Because U937, HL-60, and Jurkat cells undergo apoptosis after only 6 hr of treatment with 3 μg/ml ET-18-OCH₃ (Mollinedo et al., 1993a, 1994a, 1997), we compared the relative increases in the steady state levels of c-jun and c-fos mRNA on treatment with ET-18-OCH₃ before the onset of apoptosis (Fig. 1A). After 4 hr of treatment with ET-18-OCH₃ (3 μg/ml), we observed a maximal induction in c-jun and c-fos expression occurring before the onset of apoptosis (Mollinedo et al., 1994a; data not shown). At this incubation time, we found relative inductions of 27-fold (U937), 38-fold (HL-60), and 17-fold (Jurkat) in the c-jun mRNA steady state levels on treatment with ET-18-OCH₃, whereas the relative increase in the c-fos mRNA steady state level was <5-fold in the three distinct human leukemic cell lines (Fig. 1A). Thus, we found that ET-18-OCH₃ is an extremely potent inducer of c-jun expression. We also found, corroborating our previous findings (Mollinedo et al., 1994a), that ET-18-OCH₃ activated AP-1 transcriptional activity when used at a concentration able to induce apoptosis, as assessed by CAT assays (Fig. 1B). This activation of AP-1 enhancer activity by ET-18-OCH₃ resulted in lower than that observed when cells were incubated with PMA (Fig. 1B), a strong activator of AP-1 transcription factor. PMA, unlike ET-18-OCH₃, induces a strong increase in the c-fos mRNA steady state level in these cell lines (Mollindedo et al., 1993b; data not shown). The relative weaker activation of AP-1 transcriptional activity in relation to the extremely high induction of c-jun proto-oncogene observed on ET-18-OCH₃ treatment (Fig. 1) could be explained by the very weak induction of c-fos in ET-18-OCH₃-treated cells (Fig. 1), because the AP-1 transcription factor containing c-Jun/c-Fos shows a higher DNA binding capacity and transcriptional activity than the c-Jun homodimers (Karim, 1995).

**ET-18-OCH₃-induced apoptosis is associated with JNK activation.** The c-jun proto-oncogene is positively autoregulated by its own gene product, once it is properly phosphorylated (Karim, 1995). Because JNK activates c-Jun transcriptional activity (Hibi et al., 1993), candidate inducers of the JNK pathway include agents that cause sustained induction of c-Jun, such as the ether phospholipid ET-18-OCH₃ (Mollinedo et al., 1994a). On the other hand, several studies suggest that JNK activation may be related to apoptosis (Sluss et al., 1994; Chen et al., 1996; Verheij et al., 1996; Zanke et al., 1996). Fig. 2A shows that 5 μg/ml ET-18-OCH₃ induced a very rapid DNA internucleosomal fragmentation in HL-60 cells after only a 3-hr treatment. On these grounds, we examined JNK activation in HL-60 cells treated with ET-18-OCH₃. To this aim, we used a GST fusion protein containing amino acids 1–223 of c-Jun, GST-c-Jun(1–223). This fusion protein was bound through its GST moiety to GSH-agarose beads to generate an affinity matrix to precipitate JNK activities from HL-60 cell lysates. The precipitated complexes were washed and subjected to solid-phase kinase assay. As shown in Fig. 2B, GST-c-Jun phosphorylation was observed after 30–60 min of ET-18-OCH₃ incubation, and the response was further increased with the incubation time and was found to be persistent (Fig. 2B). A strong JNK activation was obtained after 2 hr of treatment with ET-18-OCH₃ (Fig. 2B).
2B), before DNA fragmentation, occurring after 3 hr of treatment (Fig. 2A). Identical results were obtained using a GST-c-Jun-(1–79) fusion protein (data not shown). Both the apoptotic response and the JNK activation induced by ET-18-OCH₃ were found to be dose dependent and were well correlated (Fig. 3). Thus, HL-60 underwent apoptosis on treatment with 3 μg/ml ET-18-OCH₃, and this response was dramatically increased at 5 μg/ml ET-18-OCH₃ (Fig. 3A). ET-18-OCH₃-induced JNK activation was observed after cell incubation with a concentration of 1 μg/ml of the ether phospholipid, and it was particularly strong after treatment with 3 and 5 μg/ml ET-18-OCH₃ (Fig. 3B). In this regard, it is interesting to note that 1 μg/ml ET-18-OCH₃ has been reported as the threshold concentration in the inhibitory effect on DNA synthesis shown by this ether phospholipid (Mollinedo et al., 1993a). As shown in Figs. 2 and 3, ET-18-OCH₃-induced apoptosis as well as JNK activation were time and dose dependent, and JNK activation always preceded the induction of internucleosomal DNA fragmentation.

Apoptosis also is accompanied by a loss of membrane phospholipid asymmetry, resulting in the exposure of phosphatidylserine on the outer leaflet of the plasma membrane. This phosphatidylserine externalization is considered to be an early process in the apoptotic response, and it seems to play an important role in the recognition and removal of the apoptotic cells by macrophages (Koopman et al., 1994). Expression of phosphatidylserine at the surface of the cell can be monitored by flow cytometry using the binding of FITC-labeled annexin V to phosphatidylserine (Koopman et al., 1994). We found that the appearance of internucleosomal DNA degradation (Fig. 2A) preceded the exposure of phosphatidylserine to the cell outer leaflet (Fig. 4) in ET-18-OCH₃-treated HL-60 cells. In these cells, we observed an intense DNA degradation, a hallmark in the apoptotic response, after 3 hr of ET-18-OCH₃ treatment (Fig. 2A), whereas at this incubation time, only 1.5% of the cells were positive for phosphatidylserine exposure, as assessed by FITC-annexin V binding (Fig. 4). A time course of phosphatidylserine translocation in ET-18-OCH₃-treated HL-60 cells indicated that ∼19% and ∼42% of cells expressed phosphatidylserine after 6 and 9 hr of treatment, respectively (Fig. 4). Interestingly, ∼19% and ∼57% of HL-60 cells underwent DNA fragmentation after 3 and 6 hr of treatment with ET-18-OCH₃, respectively, as assessed by TUNEL analysis.

The above results clearly indicate that JNK activation is an early process in the apoptotic response and precedes DNA degradation and phosphatidylserine exposure (compare Fig. 2B with Figs. 2A and 4), two well known features of the apoptotic response.

We used an in-gel kinase assay to determine the size of the JNK protein kinase or kinases that resulted activated by ET-18-OCH₃. Crude extracts of unstimulated and ET-18-OCH₃-treated HL-60 cells were run on 12% SDS-PAGE gels, and positions of phosphorylated GST-c-Jun-(1–223) were determined by autoradiography.

**Fig. 2.** ET-18-OCH₃ induces rapid apoptosis and persistent JNK activation in HL-60 cells. HL-60 cells were treated with 5 μg/ml ET-18-OCH₃ for the indicated times and assayed for DNA fragmentation (A) or for JNK activation (B) as described in Materials and Methods. Control untreated cells were run in parallel in the same gels. Fragmented DNA from 6 × 10⁵ cells was loaded in each lane of the agarose gel. A, 123-base pair DNA ladder was used as standard (Std). B, Position of phosphorylated GST-c-Jun-(1–223) is indicated. Left, molecular masses (in kDa) of protein markers. Values are representative of three experiments.

**Fig. 3.** Dose response of ET-18-OCH₃-induced DNA fragmentation and JNK activation. HL-60 cells were incubated for 4 hr with increasing concentrations of ET-18-OCH₃ and assayed for DNA fragmentation (A) or JNK activation (B) as described in Materials and Methods. Control untreated cells were run in parallel in the same gels. Fragmented DNA from 6 × 10⁵ cells was loaded in each lane of the agarose gel. A, 123-base pair DNA ladder was used as standard. B, Position of phosphorylated GST-c-Jun-(1–223) (GST-c-Jun) is indicated. Values are representative of three experiments.
OCH₃-stimulated HL-60 cells were fractionated on the same gel, and after electrophoresis, the proteins were renatured in the gel and incubated with [γ-³²P]ATP. As shown in Fig. 5, in-gel kinase assays revealed activation of the 46- and 55-kDa forms of JNK in extracts of HL-60 cells treated with ET-18-OCH₃. This result further demonstrates the JNK activation by ET-18-OCH₃ and indicates that two JNK protein kinases are stimulated by ET-18-OCH₃. In good correlation with the above solid-phase kinase data, we observed a persistent activation of JNK (compare Figs. 2B and 5), which was maintained for ≥6 hr (data not shown).

Because JNK activation also has been found during cell treatment with nonapoptotic stimuli, including mitogenic ones, we analyzed the JNK activation pattern during HL-60 differentiation by TNF-α and compared it with that derived from ET-18-OCH₃ treatment. TNF-α, at a concentration of 100 units/ml, induces HL-60 cells to differentiate toward the monocyte/macrophage lineage (Collins, 1987; data not shown). JNK activation by incubation with 100 units/ml TNF-α displayed a rapid and transient induction pattern (Fig. 6). The addition of TNF-α to HL-60 cells rendered an intense band of phosphorylated GST-c-Jun after 15-min treatment; then, this GST-c-Jun phosphorylation decreased gradually until reaching almost basal levels after 120 min of TNF-α treatment (Fig. 6). Thus, this TNF-α-induced JNK activation time course pattern (transient JNK induction) differed from that obtained when HL-60 cells were incubated with ET-18-OCH₃ (persistent JNK induction) (compare Figs. 2B and 6). These data indicate that duration of JNK induction is regulated differentially in HL-60-monocytic differentiation and apoptosis.

We demonstrated recently that the induction of apoptosis by ET-18-OCH₃ was critically dependent on the molecular structure of the ether phospholipid (Mollinedo et al., 1997). In this regard, subtle changes in positions sn-2 and sn-3 of the glycerol backbone in the ET-18-OCH₃ molecule resulted in a complete loss of its capacity to induce apoptosis (Mollinedo et al., 1997). Thus, ET-18-OCH₃ analogues, in which the methoxy group in the sn-2 position was replaced by an OH (1-O-octadecyl-rac-glycero-3-phosphocholine) or H (1-O-octadecyl-propanediol-3-phosphocholine) (Fig. 7), were unable to induce an apoptotic response in HL-60 cells (Fig. 8A) (Mollinedo et al., 1997). To determine whether the JNK activation by ET-18-OCH₃ was due to its apoptotic effect, we analyzed the effects on JNK activation of the two inactive analogues 1-O-octadecyl-rac-glycero-3-phosphocholine and 1-O-octadecyl-propanediol-3-phosphocholine (Fig. 7). Interestingly, these inactive analogues also were unable to induce JNK activation (Fig. 8B).

Although several human leukemic cell lines have been reported to be sensitive to the action of ET-18-OCH₃ (Diomede et al., 1993, 1994; Mollinedo et al., 1993a, 1997), we found that ET-18-OCH₃ was unable to elicit an apoptotic response in the human chronic myelogenous K562 leukemic cell line, even after a prolonged incubation time of up to 48 hr (data not shown). This result is in agreement with previous data showing lack of a cytotoxic effect of this ether phospholipid in these cells (Tidwell et al., 1981; Diomede et al., 1993). We also found that ET-18-OCH₃ failed to activate JNK in K562 cells (Fig. 9), whereas parallel studies indicated that this ether phospholipid promoted a remarkable JNK activation in the drug-sensitive HL-60 cells (Fig. 9).

Phorbol esters have been reported to be inhibitors of apoptosis induced by different agents (McConkey et al., 1989; Pérez-Sala et al., 1995). We found that PMA prevented ET-18-OCH₃-induced DNA fragmentation in HL-60 cells (Fig. 10A). In good correlation with this protective effect, we found that PMA also abrogated JNK activation induced by ET-18-OCH₃ (Fig. 10B).

PMA, another differentiating agent of HL-60 cells toward the monococyte/macrophage lineage (Collins, 1987), induced by itself a rather small, rapid, and transient increase in JNK activation in HL-60 cells (Fig. 11), but it inhibited the persistent JNK activation promoted by ET-18-OCH₃ (Fig. 11). Thus, when HL-60 cells were incubated in the presence of both PMA and ET-18-OCH₃, the induction of JNK activity was inhibited drastically compared with the JNK activation exerted by ET-18-OCH₃ alone (Figs. 10B and 11). This inhib-

![Fig. 4. Flow cytometric analysis of the binding of FITC-labeled annexin V to HL-60 cells treated with ET-18-OCH₃. Untreated (a) and treated HL-60 cells with 5 μg/ml ET-18-OCH₃ for 1 (b), 3 (c), 4 (d), 6 (e), or 9 (f) hr were stained with FITC-labeled annexin V and analyzed by fluorescence flow cytometry as described in Materials and Methods. Numbers at top right, percentage of cells that are positive for FITC-annexin V staining. Values are representative of three experiments.](https://molpharm.aspetjournals.org/doi/abs/10.1093/molpharm/607.5.607)
itory effect of PMA, observed when PMA and ET-18-OCH₃ were added simultaneously to the cell culture (Fig. 11), was potentiated further if the cells were pretreated with PMA 15 min before the addition of ET-18-OCH₃ (Fig. 10B).

We also found that Zn²⁺, acting as an endonuclease inhibitor (Giannakis et al., 1991), blocked ET-18-OCH₃-induced apoptosis (Fig. 10A), indicating that this DNA degradation induced by ET-18-OCH₃ was due to the activation of an endogenous endonuclease. Thus, no internucleosomal DNA degradation was observed when HL-60 cells were treated with ET-18-OCH₃ in the presence of micromolecular concentrations of Zn²⁺ plus a Zn²⁺ ionophore, pyrithione, to facilitate cellular uptake of Zn²⁺ (Fig. 10A).

Requirement of c-Jun in ET-18-OCH₃-induced apoptosis. To examine the role of c-jun in the induction of apoptosis by ET-18-OCH₃, we used antisense c-jun oligonucleotides. In addition, because c-fos has been implicated previously in the induction of apoptosis (Colotta et al., 1992), we used antisense c-fos oligonucleotides. Incubation of HL-60 cells in serum-free ITS medium for 48 hr with antisense oligonucleotides directed against c-jun or c-fos drastically reduced the respective c-Jun and c-Fos protein levels, whereas sense oligonucleotide-treated cells expressed the same protein levels found in untreated cells (Fig. 12D; data not shown). Thus, when HL-60 cells were treated with antisense c-jun oligonucleotides for 48 hr before the addition of ET-18-OCH₃, no c-Jun protein was detected (Fig. 12D). The treatment of HL-60 cells with ET-18-OCH₃ slightly increased the protein level of c-Jun, which was not affected by the
presence of sense c-jun oligonucleotides (Fig. 12D). However, no c-Jun protein was detected when antisense c-jun oligonucleotide-treated HL-60 cells were incubated with ET-18-OCH₃ (Fig. 12D). Under these experimental conditions, we found that antisense c-jun oligonucleotides blocked the apoptotic response induced by ET-18-OCH₃ (Fig. 12A), whereas ET-18-OCH₃-induced apoptosis was not affected by the presence of antisense c-fos oligonucleotides (Fig. 12C). An additional scrambled and random oligonucleotide, with the same base composition as that used for antisense-c-jun oligonucleotide (see Materials and Methods), had no effect on ET-18-OCH₃-induced apoptosis or on c-Jun protein level (Fig. 12B; data not shown). These results indicate a role for c-Jun in ET-18-OCH₃-induced apoptosis.

Incubation of HL-60 cells in serum-free ITS medium for 48 hr with antisense c-jun oligonucleotides hardly reduced their proliferative capacity (~12% inhibition), as determined by [³H]thymidine incorporation. Treatment of HL-60 cells with 5 µg/ml ET-18-OCH₃ for 16 hr inhibited cell proliferation by ~77%. This ET-18-OCH₃ inhibitory effect on cell proliferation was not affected by the presence of sense c-jun or random oligonucleotides (~76% inhibition), and it was slightly diminished (~57% inhibition) by the presence of antisense c-jun oligonucleotides. These results suggest that ET-18-OCH₃ is able to induce two signaling routes leading to apoptosis and inhibition of cell proliferation and that c-Jun would be mainly involved in the apoptotic response. In agreement with this, Boggs et al. (1995a) reported that restoration of phosphatidylcholine synthesis through supplementation with lysophosphatidylcholine overrode the cytotoxic but not the cytostatic activity of ET-18-OCH₃.

Treatment of HL-60 cells with antisense c-jun oligonucleotides did not block JNK activation (Fig. 13), suggesting that inhibition of the apoptotic response to ET-18-OCH₃ by antisense c-jun oligonucleotides occurs at a level downstream of

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**Fig. 10.** Suppression of ET-18-OCH₃-induced DNA degradation by PMA and Zn²⁺ and inhibition of ET-18-OCH₃-induced JNK activation by PMA. A, HL-60 cells were incubated for 4 hr in the absence (Control) or presence of the indicated agents: 5 µg/ml ET-18-OCH₃, 100 ng/ml PMA, 100 ng/ml PMA plus 5 µg/ml ET-18-OCH₃, 20 µM Zn²⁺ plus 100 µM pyridoxine, and 20 µM Zn²⁺ plus 100 µM pyridoxine plus 5 µg/ml ET-18-OCH₃. The Zn²⁺ ionophore pyridoxine was added to facilitate cellular uptake of Zn²⁺. PMA, pyridoxine, and Zn²⁺ were added 15 min before ET-18-OCH₃. Fragmented DNA was obtained as described in Materials and Methods. Fragmented DNA from 6 × 10⁵ cells was loaded in each lane. A 123-base pair DNA ladder was used as standard (Std). B, HL-60 cells were incubated for 2 hr in the absence (Control) or presence of the indicated agents (5 µg/ml ET-18-OCH₃, 100 ng/ml PMA, or 100 ng/ml PMA plus 5 µg/ml ET-18-OCH₃) and then assayed for JNK activation as described in Materials and Methods. PMA was added 15 min before ET-18-OCH₃. The position of phosphorylated GST-c-Jun-1–223 (GST-c-Jun) is indicated. Experiment shown is representative of three performed.

**Fig. 11.** PMA induces a rapid and transient JNK activation but inhibits the sustained JNK activation induced by ET-18-OCH₃ in HL-60 cells. Top, HL-60 cells were treated with 100 ng/ml PMA or 5 µg/ml ET-18-OCH₃ for the indicated times and assayed for JNK activation as described in Materials and Methods. Control untreated cells were run in parallel in the same gel. The position of phosphorylated GST-c-Jun-1–223 (GST-c-Jun) is indicated. Experiment shown is representative of three performed. Bottom, HL-60 cells were treated with 100 ng/ml PMA and 5 µg/ml ET-18-OCH₃ for the indicated times and assayed for JNK activation as described in Materials and Methods. HL-60 cells treated with 5 µg/ml ET-18-OCH₃ alone for 2 hr were analyzed for JNK activation and run in parallel in the same gel to compare the inhibitory effect of PMA on ET-18-OCH₃-induced JNK activation. Control untreated cells were run in parallel in the same gel. The position of phosphorylated GST-c-Jun-1–223 (GST-c-Jun) is indicated. Values are representative of three experiments.
JNK. These results are in agreement with data recently reported by Verheij et al. (1996), which showed that stress-induced apoptosis, but not JNK activation, was inhibited in cells expressing a dominant-negative c-Jun mutant.

**Discussion**

Because (1) ET-18-OCH₃ strongly induces c-jun expression (Mollinedo et al., 1994a; current report); (2) JNK activates the transcriptional activity of c-Jun, stimulating c-jun transcription (Karlin, 1995); and (3) JNK signaling has been reported to be involved in the induction of cell death by different agents in distinct systems (Sluss et al., 1994; Chen et al., 1996; Verheij et al., 1996; Zanke et al., 1996), we suspected that the JNK signaling route might be involved in the induction of apoptosis by ET-18-OCH₃. The results described here indicate the first time the association of persistent JNK activation with ET-18-OCH₃-induced apoptosis, as well as the requirement of c-Jun for this apoptotic response. Thus, these data unveil a signaling route involved in the induction of apoptosis by ET-18-OCH₃. We found that ET-18-OCH₃ induces a persistent JNK activation in HL-60 cells, which undergo rapid apoptosis on treatment with this ether phospholipid (Fig. 2) (Mollinedo et al., 1993a). This JNK activation may mediate the increased AP-1 activity observed in ET-18-OCH₃-treated cells (Fig. 1) (Mollinedo et al., 1994a). However, ET-18-OCH₃-induced JNK activation is not detected in ET-18-OCH₃-resistant K562 cells. Subtle modifications in the molecular structure of ET-18-OCH₃ that abolish its apoptotic properties also abrogate its capacity to induce JNK activation. In this regard, ET-18-OCH₃ analogues, in which the methoxy group is substituted for an OH or H group in the sn-2 position of the glycerol backbone, lack the capacities to activate JNK and induce apoptosis in HL-60 cells. Thus, the results reported here indicate that ET-18-OCH₃-induced JNK activation is associated with the capacity of this ether phospholipid to induce apoptosis.

We also found that this JNK activation seems to precede the onset of apoptosis induced by ET-18-OCH₃. JNK activation was detected before DNA fragmentation and phosphatidyserine externalization, two early and well-known processes involved in the apoptotic response. Furthermore, both dose-response and time course analyses indicated that JNK activation preceded DNA fragmentation in ET-18-OCH₃-treated HL-60 cells. On the other hand, our data suggest that in ET-18-OCH₃-treated HL-60 cells, DNA fragmentation, assessed through visualization of the typical internucleosomal DNA fragments in agarose gels and through TUNEL analysis, precedes phosphatidyserine exposure at the outer leaflet of the plasma membrane, as assessed through FITC-labeled annexin V binding.

PMA was found to block both ET-18-OCH₃-induced apoptosis and JNK activation. Although the molecular basis for these effects remains to be determined, the protective effect of PMA against apoptosis has been related to its ability to induce cellular alkalization (Pérez-Sala et al., 1995). In this regard, an association has been reported between intracellular acidification and DNA degradation (Barry et al., 1993;...
Pérez-Sala et al., 1995). Thus, it will be of interest to study a putative relationship between intracellular pH and JNK activation.

The requirement of c-jun in ET-18-OCH₃-induced apoptosis was determined by the blockade of apoptosis by antisense c-jun oligonucleotides. These results are in agreement with previous reports showing a crucial role for c-Jun in the induction of apoptosis in different systems, as demonstrated by the use of c-Jun dominant-negative mutants, neutralizing antibodies, or antisense oligonucleotides (Colotta et al., 1992; Ham et al., 1995; Verheij et al., 1996). However, antisense c-fos oligonucleotides did not prevent ET-18-OCH₃-induced apoptosis, indicating that c-fos is not needed for the onset of ET-18-OCH₃-induced apoptosis. Although some previous reports have implicated c-fos in programmed cell death (Colotta et al., 1992), the results herein described are in agreement with recent evidence from c-Fos-deficient mice demonstrating that c-fos is not essential for the induction of apoptosis (Gajate et al., 1996).

We found that antisense c-jun oligonucleotides were unable to prevent ET-18-OCH₃-mediated JNK activation. In this regard, Verheij et al. (1996) reported that expression in U937 and BAEC cells of a dominant-negative c-Jun mutant lacking the amino terminus, the portion that is phosphorylated by JNK, blocked stress-induced apoptosis but did not inhibit JNK activation. Taken together, these data indicate that inhibition of the apoptotic response to ET-18-OCH₃ or to stress, by antisense c-jun oligonucleotides or by a dominant-negative c-Jun mutant, occurs at a level downstream of JNK. These data also suggest a critical role for c-Jun in these apoptotic processes. The mechanism by which c-Jun could mediate apoptosis is unknown. It is tempting to speculate that activated c-Jun regulates apoptosis through modulation of certain proteins required in the apoptotic response or through sequestration of inhibitors of apoptosis. A putative hypothesis for the c-Jun action on apoptosis might stem from the capacity of c-Jun to complex with different proteins.

Immediate and transient kinetics of JNK activation in response to various stimuli that promote cell activation and proliferation have been reported (Karin et al., 1995; Chen et al., 1996). We also found that induction of monocyte/macrophage differentiation of HL-60 cells by TNF-α or PMA was accompanied by a rapid and transient JNK activation, reaching a maximum JNK activation after 15 and 30 min of treatment, respectively. However, the JNK activation reported here on ET-18-OCH₃ treatment was delayed and persistent. JNK activation was detected after 30–60 min of ET-18-OCH₃ treatment; increased strongly with the incubation time, being very potent by 2 hr of treatment; and was sustained for a long period of time. Chen et al. (1996) recently reported that activation of Jurkat cells with different stimuli rendered a rapid and transient induction of JNK activity, whereas JNK remained persistently activated during γ-radiation- and UV-C-induced apoptosis. Thus, JNK activation occurs in different physiological processes, including cell activation, cell differentiation, and apoptosis. This indicates that JNK can function as a common kinase shared by different physiological processes. The different timing or duration of JNK induction, or both, may lead to opposite outcomes, namely cell activation/proliferation/differentiation (rapid and transient JNK activation) versus apoptosis (sustained JNK activation), as postulated previously (Chen et al., 1996). The participation of JNK activation in different physiological outcomes suggests that additional coactivators or JNK-interacting proteins could play a role in promoting a final cell response. The prolonged JNK activation in ET-18-OCH₃ may cause the persistent activation of some cellular factors, such as c-Jun, and results in detrimental effects on the cells.

The JNK protein kinases comprise a family of ∼10 isoforms, which correspond to alternatively spliced isoforms derived from the JNK1, JNK2, and JNK3 genes (Gupta et al., 1996). These JNK isoforms are detected as proteins of 46 kDa (JNK1), 55 kDa (JNK2), and 48 and 57 kDa (JNK3) (Gupta et al., 1996). With in-gel assays, we found that ET-18-OCH₃ induces activation of two JNK protein kinase forms with apparent molecular masses of 46 and 55 kDa in HL-60 cells. JNK protein kinases are members of the wide family of the MAPK-related family (Karin et al., 1995; Gupta et al., 1996), which includes two great subfamilies: MAPK/ERK, mainly involved in mitogenesis and differentiation; and JNK, supposedly involved in growth arrest and cell death. Mitogens are potent ERK activators but activate JNK poorly, and stress stimuli are powerful inducers of JNK but induce ERK relatively weakly (Karin et al., 1995; Gupta et al., 1996). Our current results demonstrate that ET-18-OCH₃ strongly activates the JNK route in a sustained way. Zhou et al. (1996) recently reported that this ether phospholipid inhibited the MAPK/ERK cascade. Thus, ET-18-OCH₃ arrests mitogenic signals (MAPK/ERK) and strongly induces apoptotic signaling pathways (JNK). These actions could explain the rapid and effective induction of apoptosis exerted by this ether phospholipid (Diomede et al., 1993, 1994; Mollinedo et al., 1993a, 1997).

In conclusion, the current results indicate an association between persistent JNK activation and induction of apoptosis in response to ET-18-OCH₃, suggesting that JNK activation is involved in the initiation of programmed cell death by ET-18-OCH₃. Furthermore, the present data indicate that c-Jun is required for the rapid induction of apoptosis by ET-18-OCH₃, suggesting that activation of c-Jun plays a critical role in this apoptotic response.

References

Jun Kinase in Ether Phospholipid-Induced Apoptosis 611


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