RESEARCH COMMUNICATION

The ether lipid 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine induces expression of *fos* and *jun* proto-oncogenes and activates AP-1 transcription factor in human leukaemic cells

Faustino MOLLINEDO,*† Consuelo GAJATE* and Manuel MODOLELL†

*Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Velázquez, 144, E-28006 Madrid, Spain and †Max-Planck-Institut für Immunobiologie, D-7800 Freiburg, Stübeweg 51, Germany

The ether lipid analogue 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH₃) has been recently shown to induce apoptosis in the human leukaemic HL-60 and U937 myeloid cell lines [Mollinedo, Martinez-Dalmau and Modolell (1993) *Biochem. Biophys. Res. Commun.* **192**, 603–609]. We have found that ET-18-OCH₃ is also able to promote apoptosis in the human leukaemic Jurkat T lymphoid cell line. This lymphoid cell line as well as the two myeloid HL-60 and U937 cell lines incorporated significant amounts of exogenously added radiolabelled ET-18-OCH₃. Addition of ET-18-OCH₃ to these human leukaemic cells induced an increase in the steady-state

mRNA levels of *fos* and *jun* proto-oncogenes, components of the transcription factor AP-1. These increases in *fos* and *jun* mRNA levels were associated with the activation of the AP-1 transcription factor after addition of ET-18-OCH₃ to human leukaemic cells, as assessed by an enhanced binding activity of transcription factor AP-1 to its cognate DNA sequence as well as by stimulation of transcription from an AP-1 enhancer element. These data demonstrate that the ether lipid ET-18-OCH₃ can affect gene expression by inducing expression of *fos* and *jun* proto-oncogenes and by modulating the activity of transcription factor AP-1.

INTRODUCTION

Synthetic ether phospholipids seem to constitute a novel class of anti-tumour agents [1]. The ether lipid 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH₃) is a synthetic analogue of 2-lysophosphatidylcholine and acts as a strong anti-tumour agent [2–4]. It has been recently reported that the ether lipid ET-18-OCH₃ promotes apoptosis in human myeloid leukaemic cells [5,6]. The precise mechanism involved in the ET-18-OCH₃ effect on apoptosis remains to be established, even though a number of biochemical processes have been described as being induced by alkyl phospholipids analogues, such as disturbance of phospholipid metabolism of the plasma membrane [7,8], inhibition of cellular transport systems [9], and modification of PtdIns phospholipase C and protein kinase C activities [10–12].

It has been suggested that expression of the proto-oncogenes *c-fos* and *c-jun* plays an active role in the onset of programmed cell death in lymphoid cells [13]. The nuclear proto-oncogenes *c-fos* and *c-jun* encode two components of the transcription factor AP-1 [14], which binds to the palindromic consensus sequence 5'-TGA(G/C)TCA-3', known as the AP-1 site, and affects the transcriptional control of AP-1 responsive genes [14]. Two other *c-jun*-related genes, *jun B* and *jun D*, encode proteins that show extensive amino acid identity with c-Jun [15,16]. Dimer formation between Fos/Jun or Jun/Jun proto-oncogenes is a prerequisite to constitute a functional active AP-1 complex [14]. In this study, we have examined the possibility that the ether lipid ET-18-OCH₃ may regulate gene expression by inducing the expression of *fos* and *jun* proto-oncogenes and enhancing the activity of

transcription factor AP-1 in human myeloid and lymphoid leukaemic cells.

MATERIALS AND METHODS

Materials

ET-18-OCH₃ was obtained from R. Berchtold (Biochemisches Labor, Bern, Switzerland). [³H]ET-18-OCH₃ (specific activity 42 Ci/mmol) was synthesized by tritiation of the 9-octadecenyl derivative (Amersham Buchler, Braunschweig, Germany). ET-18-OCH₃ was dissolved at 500 µg/ml as a stock solution in culture medium containing 20% (v/v) heat-inactivated fetal calf serum by heating at 50 °C for 30 min and sonication. The clear solution was sterilized by filtration through a sterile filter (pore size 0.22 µm) and stored at 4 °C. *D-threo*-[dichloroacetyl-1-¹⁴C]Chloramphenicol ([¹⁴C]chloramphenicol) (specific activity 57 mCi/mmol) was purchased from Amersham International. RPMI-1640 culture medium, fetal calf serum and L-glutamine were purchased from Flow Laboratories (Irvine, Ayrshire, U.K.). Antibiotics were from Laboratorios Llorente (Madrid, Spain). Guanidine thiocyanate and formamide were from Fluka (Buchs, Switzerland). Formaldehyde was from J. T. Baker Chemicals B. V. (Deventer, Holland). Acrylamide, bisacrylamide, ammonium persulphate and NNN'-tetramethylethylenediamine were from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were from Sigma (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany).

The anti-Fos antibody against the M peptide [17] was gen-

Abbreviations used: CAT, chloramphenicol acetyltransferase; ET-18-OCH₃, 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine; PBL, peripheral blood lymphocyte; PMA, 4β-phorbol 12-myristate 13-acetate.

† To whom correspondence should be addressed. Present address: Instituto de Biología y Genética Molecular, Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Consejo Superior de Investigaciones Científicas, Universidad de Valladolid, c/Ramón y Cajal s/n, E-47005 Valladolid, Spain.

erously given by Dr. J. R. Naranjo (Instituto Cajal, Madrid, Spain).

Cells

Human promyelocytic HL-60 cells, human promonocytic U937 cells and human T lymphoid Jurkat cells were grown in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 i.u./ml penicillin and 24 µg/ml gentamicin. Cells were incubated at 37 °C in a humidified CO₂/air (1:19) atmosphere. ET-18-OCH₃ was added to the cell cultures at 3 µg/ml for the times indicated in the Figures.

Incorporation of ET-18-OCH₃

Cells (10⁶) were incubated in 1 ml of culture medium containing 3 µg of ET-18-OCH₃ and 0.03 µCi of [³H]ET-18-OCH₃. At the times indicated in the corresponding Figure, the cells were washed once with 1 ml of PBS and mixed with 5 ml of water-miscible liquid scintillator (Ultrafluor, National Diagnostics, Manville, NJ, U.S.A.) to measure the incorporated radioactivity.

Northern blot analysis

Total RNA was isolated by the guanidine thiocyanate/CsCl method [18]. Samples of 20 µg of RNA were electrophoresed on 0.9% (w/v) agarose-formaldehyde gels, and then transferred to nitrocellulose membranes (Schleicher und Schuell, Dassel, Germany) as previously described [19]. ³²P-labelled cDNA probes were prepared using the random hexanucleotide priming method [20] (Oligo-Labeling Kit, Pharmacia, Uppsala, Sweden) to a specific radioactivity $\geq 7 \times 10^8$ c.p.m./µg of cDNA. cDNA probes for *c-fos* [21], *c-jun* [22], *jun B* [15] and *jun D* [16] were kindly provided by Dr. P. Sassone-Corsi (Laboratoire de Genetique Moleculaire des Eucaryotes, Centre National de la Recherche Scientifique, Strasbourg, France) and Dr. R. Bravo (Squibb Institute, Princeton, NJ, U.S.A.). Under the experimental conditions used in the Northern blot analysis, there was no cross-reactivity among the above probes [23]. Conditions for blot hybridization and washing have been described elsewhere [19].

Gel retardation analysis

Mini-nuclear extracts were obtained from $\sim 3 \times 10^6$ cells as previously described [19], and aliquots (4 µg) from each nuclear extract were used in each binding assay. The assays were performed as described [19], using a synthetic 22 bp oligonucleotide containing the AP-1 consensus sequence 5'-CTAGTGATGAGTCAGCCGGATC-3'. Inhibition of complex formation with anti-(Fos M) peptide antiserum was performed as described [19].

Electroporation and CAT assays

Jurkat cells (20×10^6) in the exponential phase of growth were washed twice with RPMI-1640 medium and resuspended in 100 µl of RPMI-1640 medium containing 0.5 × HeBS (1 × HeBS: 25 mM Hepes, 140 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM D-glucose, pH 7.05). Cells were electroporated with 25 µg of the expression vector AP-1-TK-CAT plasmid [24], kindly provided by M. Karin (University of California, San Diego, CA, U.S.A.), that contains the chloramphenicol acetyltransferase (CAT) gene, and with 125 µg of salmon sperm DNA, used as

carrier, in a BTX electroporator (Biotechnologies & Experimental Research, San Diego, CA, U.S.A.) at 200 V, 950 µF and 48 Ω. Treatments were initiated after cell resuspension in 7 ml of culture medium. At 24 h after treatment, cells were pelleted, washed twice with PBS, resuspended in 80 µl of 250 mM Tris/HCl, pH 8.0, and stored at -20 °C for CAT assay. Cells were freeze-thawed four times, heated at 65 °C for 10 min to inactivate deacetylases, and the homogenate was spun for 10 min in a microcentrifuge. The supernatant was collected and protein content was determined using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, U.S.A.). An aliquot (25 µg) of protein from each experimental condition was added to a reaction mixture (150 µl) containing 470 mM Tris/HCl, pH 7.4, 0.53 mM acetyl-CoA and 0.3 µCi of [¹⁴C]chloramphenicol. After 2 h of incubation at 37 °C, 4 µl of 40 mM acetyl-CoA was added and the reaction mixture was incubated overnight at 37 °C. Then, the acetylated forms of chloramphenicol were extracted with ethyl acetate and spotted on to a silica gel 60 F₂₅₄ t.l.c. plastic plate. After chromatography in chloroform/methanol (95:5, v/v), the plate was dried, and an autoradiogram was developed after 24 h exposure. The percentage of acetylation of each treatment was determined by scraping off both the acetylated and non-acetylated chloramphenicol and counting radioactivity with 2 ml of Ready Safe liquid scintillation cocktail (Beckman Instruments, Fullerton, CA, U.S.A.).

RESULTS AND DISCUSSION

We have previously found that low doses of ET-18-OCH₃ (3 µg/ml) induce apoptosis in the human myeloid leukaemic HL-60 cells (promyelocytic) and U937 cells (promonocytic) [5]. We have also found that this ether lipid promotes programmed cell death in the human T lymphoid leukaemic Jurkat cell line, as evidenced by DNA degradation into oligonucleosome-size fragments (F. Mollinedo, R. Martinez-Dalmau and M. Modolell, unpublished work). Thus, ET-18-OCH₃ is able to induce apoptosis in human myeloid and T lymphoid cells, supporting the possible use of ET-18-OCH₃ in selectively purging myeloid and lymphoid leukaemic cells from remission bone marrows for bone marrow transplantation [25].

Figure 1 Incorporation of ET-18-OCH₃ in different human leukaemic cells and human PBL

The human myeloid leukaemic U937 (◆) and HL-60 (●) cells, the human lymphoid leukaemic Jurkat cells (◇), and human PBLs (△) were incubated with 3 µg/ml ET-18-OCH₃ and 0.03 µCi of [³H]ET-18-OCH₃ for the times indicated to measure incorporation of the ether lipid into the cell as described in the Materials and methods section. Data are shown as means ± S.D. ($n \geq 3$).

Figure 2 Expression of (a) *c-fos* and *c-jun* and (b) *jun B* and *jun D* proto-oncogenes in ET-18-OCH₃-treated HL-60 cells

Northern blot analysis of mRNA levels after cell treatment with 3 μ g/ml ET-18-OCH₃ for 0.5, 1, 2 and 4 h. Basal control levels in untreated HL-60 cells are also shown (lane 1). Ethidium bromide staining of the same gel reveals equivalent amounts of rRNA (c). Autoradiograms were developed after 24–48 h of exposure. The positions of molecular size markers (S) are given. For further details, see the Materials and methods section.

The three human leukaemic cell lines HL-60, U937 and Jurkat were able to accumulate a significant amount of ET-18-OCH₃ (Figure 1). After 6 h treatment with 3 μ g/ml ET-18-OCH₃, when the ether-lipid-induced apoptosis in the three human leukaemic cell lines was clearly observed by DNA degradation into oligonucleosome-size fragments (results not shown) [5], HL-60, U937 and Jurkat cells incorporated 732 ± 138 , 663 ± 146 and 329 ± 14 ng of ET-18-OCH₃/10⁶ cells respectively (Figure 1). However, human peripheral blood lymphocytes (PBLs) did not incorporate this ether lipid (Figure 1), and these cells were not susceptible to undergoing apoptosis upon ET-18-OCH₃ treatment (F. Mollinedo, R. Martinez-Dalmau, C. Gajate and M. Modolell, unpublished work). Thus, uptake of this ether lipid into the cell seems to be required for ET-18-OCH₃-induced programmed cell death.

A role for *c-fos* and *c-jun*, components of the AP-1 transcription factor, in the induction of apoptosis has been recently suggested [13]. Thus, we have examined the effect of ET-18-OCH₃ on the expression of *c-fos* and of the three members of the *jun* family proto-oncogenes in human myeloid and lymphoid leukaemic cells. Addition of ET-18-OCH₃ to promyelocytic HL-60 cells induced an increase in the 2.2 kb *c-fos*, 2.7 kb *c-jun*, 2.1 kb *jun B* and 2.0 kb *jun D* steady-state mRNA levels (Figure 2). After overexposure of the autoradiograms corresponding to *c-jun*, an additional minor band of 3.4 kb, suggested to be a precursor molecule [26], was also evident. Similar results were obtained with U937 cells (Figure 3). A remarkable *c-jun* mRNA induction was achieved within 2 and 4 h of ET-18-OCH₃ treatment in HL-60 and U937 cells respectively (Figures 2 and 3), and a sustained and high accumulation of *jun D* transcripts was observed in both HL-60 and U937 cells upon ET-18-OCH₃ treatment (Figures 2 and 3). On the other hand, the addition of ET-18-OCH₃ to the human T lymphoid Jurkat cells induced a remarkable increase in the steady-state mRNA levels of *c-jun* and *jun B* proto-oncogenes (Figure 3). An appreciable, but weak, induction of the *c-fos* mRNA level was also detected when Jurkat cells were incubated with ET-18-OCH₃ (Figure 3). This ET-18-OCH₃-induced increase in the *c-fos* mRNA level in Jurkat cells was lower than that detected in both HL-60 and U937 cells. Unlike HL-60 and U937 cells, exposure of Jurkat cells to ET-18-OCH₃ did not modify the

Figure 3 Expression of *c-fos*, *c-jun*, *jun B* and *jun D* proto-oncogenes in ET-18-OCH₃-treated U937 (a) and Jurkat (b) cells

Northern blot analysis of mRNA levels after cell treatment with 3 μ g/ml ET-18-OCH₃ for 0.5, 1, 2, 4 and 24 h. Basal control levels in untreated U937 and Jurkat cells are also shown (lane 1). Ethidium bromide staining of the same gel reveals equivalent amounts of rRNA (bottom of the Figure). Autoradiograms were developed after 24–48 h of exposure. The positions of molecular size markers (S) are given. For further details, see the Materials and methods section.

Figure 4 Binding to an AP-1 sequence of nuclear extracts from ET-18-OCH₃-treated HL-60 and U937 cells

Nuclear extracts from (a) HL-60 and (b) U937 cells treated with 3 μ g/ml ET-18-OCH₃ were prepared at several time points (1–24 h). Gel-retardation analysis of nuclear extracts from untreated HL-60 and U937 cells (lanes 1 and 6) are also shown. (c) Binding inhibition using the specific anti-(Fos M) antibody (FM). Nuclear extracts from HL-60 or U937 cells treated with 3 μ g/ml ET-18-OCH₃ for 3 h were incubated with pre-immune serum (PI; lanes 11 and 13) or with anti-(Fos M) antibody (lanes 12 and 14), and then tested for AP-1 binding capacity. The DNA-binding complex (arrowheads) and the free oligoprobe containing the consensus AP-1 site (arrows) are indicated. For further details, see the Materials and methods section.

Table 1 Induction of AP-1 enhancer activity by ET-18-OCH₃ in Jurkat T cells

Plasmids containing AP-1-TK-CAT and TK-CAT were transfected into Jurkat cells by electroporation. Then, the cells were incubated with 10 ng/ml PMA or with the indicated concentrations of ET-18-OCH₃. Untreated control cells were run in parallel. After 24 h, cells were disrupted and CAT activity was determined as described in the Materials and methods section. The percentage of acetylation of each treatment was divided by the control value from unstimulated cells to obtain the fold induction. Data are shown as means $\pm$ S.D. ($n = 3$).

Plasmid	Treatment	Fold induction
AP-1-TK-CAT	None	1.0
AP-1-TK-CAT	PMA (10 ng/ml)	59.2 $\pm$ 7.8
AP-1-TK-CAT	ET-18-OCH ₃ (0.5 μ g/ml)	0.9 $\pm$ 0.1
AP-1-TK-CAT	ET-18-OCH ₃ (1 μ g/ml)	1.2 $\pm$ 0.1
AP-1-TK-CAT	ET-18-OCH ₃ (2 μ g/ml)	4.1 $\pm$ 0.6
AP-1-TK-CAT	ET-18-OCH ₃ (3 μ g/ml)	16.3 $\pm$ 2.2
TK-CAT	None	1.0
TK-CAT	PMA (10 ng/ml)	1.2 $\pm$ 0.2
TK-CAT	ET-18-OCH ₃ (3 μ g/ml)	0.9 $\pm$ 0.3

jun D mRNA level (Figure 3). These data could suggest that distinct AP-1 dimers are formed in myeloid and lymphoid leukaemic cells upon ET-18-OCH₃ treatment.

Furthermore, addition of ET-18-OCH₃ to U937 or HL-60 cells induced an enhancement of DNA-binding activity of the transcription factor AP-1 to its cognate sequence in both cell types (Figure 4). We confirmed with specific anti-(Fos M) antibody that the DNA-binding complex required the presence of Fos protein (Figure 4c).

As treatment of Jurkat T cells with ET-18-OCH₃ produced an increase in the steady-state mRNA levels of components of the transcription factor AP-1, we examined the effect of ET-18-

OCH₃ on the functional activation of the AP-1 transcription factor. Thus, we electroporated into Jurkat cells the AP-1-TK-CAT plasmid [24], which contains a single copy of the AP-1 site inserted upstream of position -109 of the herpes simplex virus thymidine kinase promoter and fused to the structural gene coding for CAT gene, used as reporter gene. The addition of 4 β -phorbol 12-myristate 13-acetate (PMA) to these cells stimulated CAT activity from the construct containing an AP-1 site (Table 1), but not the activity of the plasmid TK-CAT lacking the AP-1 enhancer element (Table 1). Interestingly, addition of ET-18-OCH₃ (3 μ g/ml) to Jurkat cells electroporated with the AP-1-TK-CAT construct induced a 16.3-fold increase in CAT activity (Table 1). This effect was dose-dependent, with 2 μ g/ml ET-18-OCH₃ being the threshold concentration to yield a measurable effect on AP-1 enhancer activity. This ET-18-OCH₃ dose is within the concentration range of the ether lipid required to induce apoptosis of human leukaemic cells [5]. ET-18-OCH₃ had no effect on the CAT activity of the construct lacking the AP-1 enhancer element (Table 1).

The data reported herein demonstrate for the first time that the ether lipid ET-18-OCH₃ is able to induce expression of *fos* and *jun* proto-oncogenes and to activate the transcription factor AP-1 in human leukaemic cells. In this context, it has been very recently reported that ET-18-OCH₃ stimulates the expression of *c-fos* in rat primary astroglial cell cultures [27]. Previous reports suggested that ether lipids, including ET-18-OCH₃, exert their destructive actions on tumour cells by interfering with the cell membrane phospholipid metabolism or with other processes occurring in the cell membrane, but not by affecting nuclear processes [1,2]. However, the results reported herein demonstrate conclusively that the ether lipid ET-18-OCH₃ affects nuclear events, increasing the steady-state mRNA levels of *fos* and *jun* proto-oncogenes and stimulating the AP-1 transcription factor activity in human leukaemic cells. Thus, the results of the present paper indicate that addition of ET-18-OCH₃ to human leukaemic

cells can affect gene expression by modulating the activity of the transcription factor AP-1. The possible relationship between the observed increase in AP-1 transcription induced by ET-18-OCH₃ and the promotion of apoptosis is currently underway. The fact that ET-18-OCH₃ can affect gene expression may give new explanations for the previously observed effects of this ether lipid [1]. In this regard, it has been reported that ET-18-OCH₃ activates macrophages which acquire cytotoxicity against tumour cells [1,2]. As the transcription factor AP-1 regulates the expression of several cytokines and proteins that play a key role in macrophage function, it could be suggested that the enhancement of the tumouricidal activity of macrophages after incubation with ET-18-OCH₃ might be, at least in part, mediated by the action of this ether lipid on the activity of transcription factor AP-1.

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