

Abstract

1-Octadecyl-2-Octadecyl-2-methyl-glycerophosphocholine (ET18-OCH₃) is an ether lipid with selective antiproliferative properties whose mechanism of action is still unresolved. We hypothesized that since ET18-OCH₃ affects a wide variety of cells, its mechanism of action was likely to involve the inhibition of a common pathway used for transducing growth signals such as the mitogen-activated protein kinase (MAPK) cascade. To test this, we established conditions whereby quiescent MCF-7 cells took up ET18-OCH₃ in sufficient quantities that inhibited cell proliferation subsequent to the addition of growth medium and examined the activation of components of the MAPK cascade under these conditions. ET18-OCH₃ inhibited the sustained phosphorylation of MAPK resulting in a decrease in the magnitude and duration of activation of MAPK in cells stimulated with serum or EGF. ET18-OCH₃ had no effect on the binding of EGF to its receptors, their activation, or p21ras activation. However, an interference in the association of Raf-1 with membranes and a resultant decrease in Raf-1 kinase activity in membranes of ET18-OCH₃-treated cells was observed. ET18-OCH₃ had no direct effect on MAPK or Raf-1 kinase activity. A direct correlation between ET18-OCH₃ accumulation, inhibition of cell proliferation, Raf association with the membrane, and MAPK activation was also established. These results suggest that inhibition of the MAPK cascade by ET18-OCH₃ as a result of its effect on Raf-1 activation may be an important mechanism by which ET18-OCH₃ inhibits cell proliferation. (J. Clin. Invest. 1996. 98:934–944.) Key words: phospholipid ethers • antineoplastic agents • cell division • signal transduction • protein kinases

Introduction

1-Octadecyl-2-Octadecyl-2-methyl-glycerophosphocholine (ET18-OCH₃), also known as edelfosine, is the prototype of a class of antitumor ether lipids, the alkyllysophospholipids (ALPs), with a wide range of antitumor activity that are undergoing clinical trials (1–3). Two characteristics of the antiproliferative effect exhibited by these compounds have generated considerable interest. Firstly, inhibition of cell proliferation appears to be achieved without any interaction with cellular DNA and secondly, these antiproliferative effects are cell selective (1). The mechanism of inhibition of cell proliferation by ALPs is still unresolved and although they affect a large number of cellular events including nutrient transport, TGF-α secretion, estradiol uptake, transferrin binding, and intracellular-free Ca²⁺ levels (reviewed in 1–3), the role of these perturbations in inhibiting cell growth has yet to be established. ET18-OCH₃ also perturbs lipid metabolism but recent studies have suggested that this is unlikely to be the underlying cause for its antiproliferative effect (4–7).

There is a growing recognition that ALPs may inhibit cell growth by interfering in signal transduction processes. Inhibition of protein kinase C (PKC) has been proposed as the mechanism by which ET18-OCH₃ and related compounds inhibit cell proliferation (8), but this is contentious. ET18-OCH₃ may inhibit (9) or activate (10) PKC activity in vitro assays depending on the mode of presentation of the ALP in the assay, but a number of studies have revealed no correlation between inhibition of cell growth and inhibition of PKC activity (2, 11, 12). Decreased inositol-1,4,5-trisphosphate (InsP₃) production in response to incubation of cells with antitumor ether lipids has been reported (13) and could be due to inhibition of phosphatidylinositol-specific phospholipase C (PI-PLC) (14). Observations that ET18-OCH₃ inhibited PI-3-OH-kinase (PI-3K) activity in purified preparations and cell lysates and reduced the production of 3-phosphorylated inositol lipids after PDGF stimulation of NIH 3T3 cells (15) led to the suggestion that the mechanism by which ALPs inhibit cell proliferation may involve the inhibition of PI-3K. PI-3K activation may not be sufficient to initiate cell proliferation and its contribution to proliferation is yet to be fully established (16). The wide range of cancer cells whose growth is inhibited by ET18-OCH₃ suggests that a common mechanism of action, if one existed, was likely to involve perturbation of a widely used signal transduction mechanism that initiates proliferation. One such pathway is the Ras-dependent activation of the mitogen-activated protein kinase (MAPK) cascade (Ras/Raf-1/MEK/ERK) which transduces signals from receptor tyrosine kinases, oncogenic tyrosine kinases, and G-protein-coupled receptors (17, 18).

1. Abbreviations used in this paper: AEBSF, aminoethylbenzenesulfonyl fluoride; ALP, alkyllysophospholipid; DME/FBS (or/BSA), DME supplemented with 10% FBS (or with 0.5 mg/ml BSA); EGF, EGF receptor; ERK, extracellular signal-regulated kinase; ET18-OCH₃, 1-Octadecyl-2-Octadecyl-2-methyl-glycerophosphocholine; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/extracellular signal-regulated kinase; PI-3K, phosphatidylinositol-3-OH kinase; PI-PLC, PI-specific phospholipase C; PKA, protein kinase A; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate.
We, therefore, investigated the effects of ET18-OCH₃ on signaling via the MAPK cascade in the human breast adenocarcinoma cell line, MCF-7, which is very sensitive to the anti-proliferative effects of ET18-OCH₃ (19). Here we show that ET18-OCH₃ inhibits the transduction of growth signals via the MAPK cascade in MCF-7 cells by perturbing the association of Raf-1 with the membrane. This truncates Raf-1 kinase activity and prevents the sustained activation of the MAPK cascade.

**Methods**

**Materials.** ET18-OCH₃ was obtained from Medmark (Gruenwald, Germany). EGF, myelin basic protein (MBP), all protease inhibitors, calmidazolium and Na₃VO₄, were purchased from Sigma Chemical Co. (St. Louis, MO). Coomassie protein assay reagent was a product of Pierce (Rockford, IL). FBS (Cellest Gold), [³²P]thymidine, 2 Ci/ mmol (γ³²P)ATP, and [³²P]orthophosphate were obtained from ICN Pharmaceuticals (Montreal, Quebec). Cell culture medium and sub-culturing reagents were purchased from Gibco BRL (Burlington, Ontario). PEI cellulose TLC plates were purchased from E. Merek (Darmstadt, Germany). Anti-EGFR1 (C-16), -ERK2 (C-14), -Ras (259), and -Raf-1 (C-12) antibodies and MEK-1 (FL) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sheep polyclonal anti-EGF receptor Ab and mouse antiphosphotyrosine mAb were procured from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-Raf-1 antisera (SP63) was generously provided by Dr. U. Rapp (National Cancer Institute, NIH). aCAMP[H] assay kit was from Amersham Canada (Oakville, Ontario). PKI and Kemptide were purchased from Bachem (Torrance, CA).

**Cell culture.** MCF-7 (human breast adenocarcinoma) cells from frozen stocks originally obtained from American Type Culture Collection (Rockville, MD) were cultured in DME/FBS (19). Quiescent cells were obtained by seeding cells in tissue culture dishes overnight with buffer C (buffer A + 1% [wt/vol] Triton X-100, 0.5% [wt/vol] NP-40), vortexed immediately, and centrifuged at 200,000 g for 30 min. Cell cytosol was precleared followed by immunoprecipitation of p42map and p44map with agarose-conjugated polyclonal Abs to ERK1 (C-16) and ERK2 (C-14). Pellets were washed (eight times) with buffer C followed by boiling in SDS sample buffer. The proteins were separated on 10% SDS gels and phosphorylated bands were visualized by autoradiography.

EGF receptor phosphorylation. After the appropriate treatment and stimulation of cells with EGF, the cells were washed twice and lysed in 50 mM Hepes (pH 7.5), 150 mM NaCl, 1% (wt/vol) Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, protease inhibitors (aprotinin, leupeptin, benzamidine, AEBFS), 0.5 mM Na₃VO₄ and 100 mM β-glycerophosphate. The extracts were sonicated and centrifuged at 200,000 g for 30 min. The supernatant was used for immunoprecipitation with sheep polyclonal anti-EGF Ab followed by Western blot analysis with the same Ab or mouse antiphosphotyrosine mAb.

p21⁺ras activation. Ras was immunoprecipitated with a rat anti-v-H-Ras mAb (259) from [³²P]orthophosphate (300 μCi/ml, 4 h) labeled quiescent cells stimulated for selected periods with EGF. The GTP/(GTP + GDP) content was determined as previously described (23).

Translocation of Raf-1. This was performed as described by Wartmann and Davis (24). Quiescent cells treated with or without ET18-OCH₃ were stimulated with EGF or serum. The cytosolic fraction and solubilized membrane fraction were prepared (24) and subjected to Western blot analysis with anti–Raf-1 (C-12) Ab after electrophoresis on 10% SDS-PAGE.

Raf-1 kinase assays. Membranes were obtained from control and ET18-OCH₃-treated cells, solubilized (24), and were precleared. Immunoprecipitation was achieved with anti–Raf-1 antisera (SP63) (25). Raf-1 kinase activity was assayed (25) by using 10 μg of histone H1 or 2.5 μg of MEK (FL) as substrates. Radiolabeled histone H1 or MEK was separated on 12% SDS gels and visualized by autoradiography.

³²PThymidine incorporation in cells. Quiescent cells in 6-well plates were preincubated with or without ET18-OCH₃ (10 μg/ml) for selected periods. After washing, the cells were incubated with DME/BSA or DME/FBS for 20 h followed by the addition of [³²P]thymidine 0.5 μCi/well for an additional 4 h. Radioactivity in TCA precipitable material was determined (26).

**Phosphorylation of MAPK.** Quiescent cells were washed with Krebs buffer and incubated with phosphate-free DME/BSA containing 300 μCi/ml [³²P]orthophosphate for 3 h followed by washing and incubation with or without ET18-OCH₃ (10 μg/ml) for 3 h. At the end of the incubation the cells were washed and incubated with DME/BSA and stimulated for selected periods with EGF. The cells were then quickly washed three times with ice-cold Krebs buffer and scraped into buffer C (buffer A + 1% [wt/vol] Triton X-100, 0.5% [wt/vol] NP-40), vortexed immediately, and centrifuged at 200,000 g for 30 min. Cell cytosol was precleared followed by immunoprecipitation of p42map and p44map with agarose-conjugated polyclonal Abs to ERK1 (C-16) and ERK2 (C-14). Pellets were washed (eight times) with buffer C followed by boiling in SDS sample buffer. The proteins were separated on 10% SDS gels and phosphorylated bands were visualized by autoradiography. EGF receptor phosphorylation. After the appropriate treatment and stimulation of cells with EGF, the cells were washed twice and lysed in 50 mM Hepes (pH 7.5), 150 mM NaCl, 1% (wt/vol) Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, protease inhibitors (aprotinin, leupeptin, benzamidine, AEBFS), 0.5 mM Na₃VO₄ and 100 mM β-glycerophosphate. The extracts were sonicated and centrifuged at 200,000 g for 30 min. The supernatant was used for immunoprecipitation with sheep polyclonal anti-EGF Ab followed by Western blot analysis with the same Ab or mouse antiphosphotyrosine mAb.

p21⁺ras activation. Ras was immunoprecipitated with a rat anti-v-H-Ras mAb (259) from [³²P]orthophosphate (300 μCi/ml, 4 h) labeled quiescent cells stimulated for selected periods with EGF. The GTP/(GTP + GDP) content was determined as previously described (23).

Translocation of Raf-1. This was performed as described by Wartmann and Davis (24). Quiescent cells treated with or without ET18-OCH₃ were stimulated with EGF or serum. The cytosolic fraction and solubilized membrane fraction were prepared (24) and subjected to Western blot analysis with anti–Raf-1 (C-12) Ab after electrophoresis on 10% SDS-PAGE.

Raf-1 kinase assays. Membranes were obtained from control and ET18-OCH₃-treated cells, solubilized (24), and were precleared. Immunoprecipitation was achieved with anti–Raf-1 antisera (SP63) (25). Raf-1 kinase activity was assayed (25) by using 10 μg of histone H1 or 2.5 μg of MEK (FL) as substrates. Radiolabeled histone H1 or MEK was separated on 12% SDS gels and visualized by autoradiography.

³²PThymidine incorporation in cells. Quiescent cells in 6-well plates were preincubated with or without ET18-OCH₃ (10 μg/ml) for selected periods. After washing, the cells were incubated with DME/BSA or DME/FBS for 20 h followed by the addition of [³²P]thymidine 0.5 μCi/well for an additional 4 h. Radioactivity in TCA precipitable material was determined (26).

**Flow cytometry.** Cells were processed for flow cytometry analysis as previously described (27). Data were collected and analyzed with a cell sorter EPICS 753; Coulter Electronics Inc., Hialeah, FL and the PARA 1 analysis software (Coulter Electronics Inc.).

**Quantitation.** Quantitation of immunoblots and autoradiographs were obtained by densitometric analysis with a high resolution color scanner model PDI 325oe (Protein + DNA Imageware Systems, Huntington Station, NY) using the ImageMaster scanning program (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

**Results**

**ET18-OCH₃ inhibits the serum-induced cell proliferation and ³²PThymidine incorporation.** The approach adopted for this study was to investigate the effect of ET18-OCH₃ on the proliferation of quiescent cells. To correlate any observed cellular perturbations with the inhibition of cell growth, incubation conditions were established whereby quiescent MCF-7 cells accumulated sufficient ET18-OCH₃ to inhibit cell proliferation after the readdition of DME/FBS without loss of cell viability. The addition of DME/FBS to serum-deprived MCF-7 cells stimulated proliferation, while continued deprivation of serum did not (Fig. 1A). Preincubation of the cells with ET18-OCH₃ (10 μg/ml) for 1 h and subsequent incubation with DME/FBS-de-
increased their rate of proliferation relative to controls while incubation for 2 h or more completely inhibited cell proliferation. In cells preincubated with ET18-OCH₃ for 3 h before incubation in DME/FBS for up to 4 d, the proportion of cells excluding trypan blue dye (>-90%) was similar to that of controls, indicating that the viability of the cells had not been compromised. Preincubation of quiescent MCF-7 cells with ET18-OCH₃ followed by stimulation with serum also inhibited the incorporation of [³H]thymidine into the cells (Fig. 1B) indicating that progression of the cells into S phase had been inhibited. The quantities of ET18-OCH₃ taken up by the cells after incubation for varying periods were determined with [³H]thymidine incorporation in cells pretreated with ET18-OCH₃ for 3 h, stimulated with EGF, and MAPK activity was measured in the cytosolic fractions. The results displayed in Fig. 2A show that in control cells, MAPK activity peaked at 642 pmol/min per mg protein after 10 min stimulation whereas in cells preincubated with ET18-OCH₃, a peak activity of 395 pmol/min per mg protein was observed after 5 min. At 10 min, MAPK activity was 2.5 times higher in the controls compared with the cells preincubated with ET18-OCH₃. The in-gel MAPK assay revealed a similar inhibitory effect of ET18-OCH₃ preincubation on MBP phosphorylation in response to EGF (Fig. 2B). There was little or no differences between the controls and experimental cells after 2 and 4 min but at 10 min, phosphorylation of MBP by p42mapk and p44mapk in the controls was still evident whereas that in the ET18-OCH₃-treated cells had declined considerably. ET18-OCH₃ had a similar effect on MAPK activation in cells stimulated with serum (Fig. 2A). Peak phosphorylation of MBP in control cell cytosol occurred 15 min after serum stimulation. In contrast, preincubation of the cells with ET18-OCH₃ before serum stimulation yielded a peak activity at 10 min which was twofold lower than the peak activity in controls. 15 min after serum stimulation, MAPK activity in the ET18-OCH₃-loaded cell cytosol was fourfold lower than the activity in controls. Thus, the effect of preincubating MCF-7 cells with ET18-OCH₃ under conditions that inhibit their proliferation was a decrease in both the intensity and duration of MAPK activation in response to serum or EGF. The observed inhibition of MAPK activation in cells preincubated with ET18-OCH₃ is unlikely to be due to a direct inhibition of the enzyme activity by the compound because the activity of purified recombinant ERK2 and MAPK activity in the cytosolic fractions from stimulated cells were unaffected by the addition of up to 20 μg/ml exogenous ET18-OCH₃ (data not shown).

ET18-OCH₃ decreases EGF-induced phosphorylation of p42mapk and p44mapk. As phosphorylation of MAPK is required for its activation (28) we investigated the effect of ET18-OCH₃ on this event. p42mapk and p44mapk were immunoprecipitated from [³P]orthophosphate-labeled cells incubated with or without ET18-OCH₃ and stimulated with EGF. Phosphorylation was assessed by autoradiography (Fig. 2C). 4 min after EGF stimulation there was little difference in the phosphorylation of the MAPK immunoprecipitates from the control and experimental cells. However, while phosphorylation had not decreased after 10 min in MAPK immunoprecipitated from the control, this had declined significantly in MAPK immunoprecipitates from cells incubated with the ALP (3.2- and 3.7-fold decrease in p42mapk and p44mapk by densitometric analysis, respectively). Western blot analysis of parallel gels revealed no differences in MAPK protein content (data not shown).

ET18-OCH₃ inhibits the activation of MAPK in MCF-7 cells. The activation of MAPK (ERK) is crucial for cell proliferation after stimulation by extracellular ligands, such as EGF, acting on receptor tyrosine kinases (17). To determine if the inhibitory locus of ET18-OCH₃ was upstream or downstream of MAPK, cells were preincubated with or without ET18-OCH₃ (10 μg/ml) for 3 h, stimulated with EGF, and MAPK activity was measured in the cytosolic fractions. The results displayed in Fig. 2A show that in control cells, MAPK activity peaked at 642 pmol/min per mg protein after 10 min stimulation whereas in cells preincubated with ET18-OCH₃, a peak activity of 395 pmol/min per mg protein was observed after 5 min. At 10 min, MAPK activity was 2.5 times higher in the controls compared with the cells preincubated with ET18-OCH₃. The in-gel MAPK assay revealed a similar inhibitory effect of ET18-OCH₃ preincubation on MBP phosphorylation in response to EGF (Fig. 2B). There was little or no differences between the controls and experimental cells after 2 and 4 min but at 10 min, phosphorylation of MBP by p42mapk and p44mapk in the controls was still evident whereas that in the ET18-OCH₃-treated cells had declined considerably. ET18-OCH₃ had a similar effect on MAPK activation in cells stimulated with serum (Fig. 2A). Peak phosphorylation of MBP in control cell cytosol occurred 15 min after serum stimulation. In contrast, preincubation of the cells with ET18-OCH₃ before serum stimulation yielded a peak activity at 10 min which was twofold lower than the peak activity in controls. 15 min after serum stimulation, MAPK activity in the ET18-OCH₃-loaded cell cytosol was fourfold lower than the activity in controls. Thus, the effect of preincubating MCF-7 cells with ET18-OCH₃ under conditions that inhibit their proliferation was a decrease in both the intensity and duration of MAPK activation in response to serum or EGF. The observed inhibition of MAPK activation in cells preincubated with ET18-OCH₃ is unlikely to be due to a direct inhibition of the enzyme activity by the compound because the activity of purified recombinant ERK2 and MAPK activity in the cytosolic fractions from stimulated cells were unaffected by the addition of up to 20 μg/ml exogenous ET18-OCH₃ (data not shown).
shown). These results suggest that the truncated activation observed in cytosol from cells preincubated with ET18-OCH₃ was due to the decreased phosphorylation of MAPK, an indication that ET18-OCH₃ probably affected an event upstream of MAPK that was critical to its sustained phosphorylation and activation. Experiments were initiated to investigate the effect of preincubating cells with ET18-OCH₃ on the signaling events from EGF receptor to MAPK activation (17, 28–31).

ET18-OCH₃ does not affect activation of the EGF receptor. Studies on the binding and affinity of [¹²⁵I]EGF to its receptors in cells incubated with or without ET18-OCH₃ at 4 and 37°C revealed no significant differences in the quantity of EGF bound or affinity of EGF to the receptors (data not shown). Decreased internalization of EGF in cells preincubated with ET18-OCH₃ relative to controls was observed at 37°C, but this was only apparent after 20 min incubation with the ligand (data not shown). The effect of ET18-OCH₃ on EGF receptor (EGFr) activation was assessed by monitoring the tyrosine phosphorylation of EGFr (32). Immunoblotting of the immunoprecipitated EGFr with anti-EGF (αEGFr) Abs showed

Figure 2. Preincubation of MCF-7 cells with ET18-OCH₃ attenuates activation of MAPK in response to stimulation by EGF or serum. (A) Quiescent cells were incubated without (open symbols) or with (closed symbols) ET18-OCH₃ (10 μg/ml) for 3 h, washed and stimulated with EGF (circles) or DME/FBS (squares). MAPK activity was measured in cell cytosol as the phosphorylation of MBP. These results are from a single experiment that is representative of four separate experiments and the values are the mean of triplicate incubations. (B) Assessment of MAPK activity by the in-gel assay. Results from control and ET18-OCH₃–treated cells stimulated with EGF are displayed. The results are from a single experiment that is representative of two identical experiments. (C) Assessment of p42mapk and p44mapk phosphorylation. Quiescent cells were prelabeled with [³²P]orthophosphate, washed, and subsequently incubated with or without ET18-OCH₃ (10 μg/ml) for 3 h and stimulated with EGF. MAPK was immunoprecipitated from 750 μg of cell cytosol protein with 3 μg each of anti-ERK1 and -ERK2 Abs and resolved on 10% SDS-PAGE. MAPK phosphorylation was visualized by autoradiography. The results are from a single experiment that was repeated with three different cell preparations with similar results.

Figure 3. Preincubation with ET18-OCH₃ does not affect the phosphorylation of EGF receptors in MCF-7 cells. Western blot analysis of immunoprecipitates of EGF receptor from cells incubated with or without ET18-OCH₃ and subsequent stimulation by EGF. Antibodies used for immunoblotting are indicated in the figures. The results are from a single experiment. Two independent experiments showed similar results.

Figure 4. Pretreatment with ET18-OCH₃ does not affect p21rass activation. p21rass was immunoprecipitated from cell lysates (500 μg protein) obtained from [³²P]orthophosphate-labeled cells preincubated with or without ET18-OCH₃ (10 μg/ml) and subsequent stimulation with EGF. Guanine nucleotides were extracted from the immunoprecipitates and separated by TLC and the amount of associated radioactivity was visualized by autoradiography. Spots were scraped from the TLC plates and the radioactivity was quantitated. The results are from a single experiment that is representative of two identical experiments.
that similar amounts of the receptor were present in cells incubated with or without ET18-OCH₃ (Fig. 3). Immunoblotting with antiphosphotyrosine (α-Tyr) Abs revealed that the extent and kinetics of EGFr phosphorylation were similar in both groups (Fig. 3). This was confirmed by densitometric analysis (data not shown).

ET18-OCH₃ does not affect the activation of p21ras. The activation of EGFr leads to the formation of a complex with Grb2/mSos that leads to the activation of p21ras (29) which in turn leads to Raf-1 activation to initiate the kinase cascade. Inhibition of p21ras activity could lead to decreased activation of MAPK. Fig. 4 shows that preincubation of the cells with ET18-OCH₃ had no effect on the activation of p21ras which peaked between 2 and 4 min and declined to resting values by 8 min.

ET18-OCH₃ perturbs Raf-1 association with cell membranes. Activated p21ras mediates the translocation of Raf-1 from the cytosol to the membrane where it is activated by undefined events (17, 30, 31, 33). To investigate the effect of ET18-OCH₃ on Raf-1 association with the cytosol to the membrane in control cells and those preincubated with ET18-OCH₃, in cells stimulated with EGFr (Fig. 5 A), densitometric analysis of the blots revealed that the membrane-associated Raf-1 in controls and cells preincubated with ET18-OCH₃ increased similarly at 2 min while at 4 and 7 min, 1.8- and 3.4-fold more Raf-1, respectively, was associated with control membranes relative to membranes from ET18-OCH₃-loaded cells. Results from serum-stimulated cells (Fig. 5 B) also revealed that Raf-1 association with membranes from cells preincubated with ET18-OCH₃ never attained the levels of the controls and decreased more rapidly. After 5 and 10 min the levels in membranes from ET18-OCH₃-treated cells were 1.6- and 2.6-fold less than the levels in control membranes, respectively. Western blot analy-
sis was performed on cell lysates to investigate whether the above differential association of Raf-1 with membranes was due to differences in the Raf-1 content in cells treated with or without ET18-OCH₃. The results revealed that there were no differences in the Raf-1 content between ET18-OCH₃–treated and control cells (Fig. 5 C).

**Preincubation of cells with ET18-OCH₃ inhibits Raf-1 kinase activity.** To investigate if the decreased Raf-1 association caused by ET18-OCH₃ preincubation translated into decreased Raf-1 kinase activity in the membrane, Raf-1 kinase activity was measured in Raf-1 immunoprecipitates from membranes of control and ET18-OCH₃–loaded cells. The results clearly showed that there was greater phosphorylation of MEK-1 and histone H1 in assays with immunoprecipitates from control cells compared to those from cells preincubated with ET18-OCH₃ (Fig. 5, D and E), and the time course of the activity paralleled that of Raf-1 membrane association. The ratio of the increase in phosphorylation of the substrates over blanks in controls relative to ET18-OCH₃–loaded cells as assessed densitometrically ranged from 1.5 at 2 min to 12 at 10 min for MEK-1 and 1.2 to 4.3 for histone H1 from 2 to 10 min, respectively. Addition of ET18-OCH₃ to the assays did not have any effect on the kinase activity of Raf-1 immunoprecipitates from EGF-stimulated and unstimulated cells (data not shown). Thus, ET18-OCH₃ has no direct effect on Raf-1 kinase activity.

**Correlation between ET18-OCH₃ accumulation, Raf-1 membrane association and MAPK activation.** As we observed a time-dependent accumulation of ET18-OCH₃ (see above), which could account for the differential effect on proliferation observed in Fig. 1, we investigated whether this could be correlated with differences in the extent of Raf-1/membrane association and MAPK activity. Quiescent MCF-7 cells were incubated with ET18-OCH₃ for 0, 1, and 3 h before stimulation with EGF for 7 min and cell fractions processed for examination of Raf-1/membrane association, and MAPK activity. The results (Fig. 6) show that Raf-1 association with the membrane decreased as a function of increasing incubation with ET18-OCH₃ and that the order of decreasing MAPK activity correlated with decreased membrane-bound Raf-1 and increasing ET18-OCH₃ accumulation.

**Effect of ET18-OCH₃ on cellular cAMP levels.** Because PKA activation may lead to inhibition of Raf-1 activity (34), we determined the cAMP levels and PKA activity in ET18-OCH₃–pretreated and untreated MCF-7 cells to investigate whether the inhibitory effect of ET18-OCH₃ was mediated via activation of PKA. ET18-OCH₃ had no effect on unstimulated or EGF-stimulated cellular levels of cAMP (Fig. 7) or on PKA activity which remained unchanged from the basal level of 3.6±1.7 pmol/min per mg protein.

**Effect of transient MCF-7 cell stimulation on the cytostatic effect of ET18-OCH₃.** If the inhibition of the MAPK cascade by ET18-OCH₃ contributes significantly to the antiproliferative effect of the compound as the above studies suggest, these effects should be minimized if the ALP is added subsequent to the transient activation of the cells. Quiescent MCF-7 cells were therefore incubated with DME/FBS for 1 h to transiently activate MAPK (Fig. 2). The cells were washed, and incubated with ET18-OCH₃ for 3 h. At the end of this incubation the cells were washed and incubated with DME/FBS and subsequently processed for flow cytometry analysis. Table I shows that whereas preincubation with ET18-OCH₃ before the addition of FBS completely inhibited cell cycle progression into S phase, this inhibition was significantly blunted when the cells were transiently activated with FBS before addition of ET18-OCH₃. The above results demonstrate that inhibition of the early signaling events by ET18-OCH₃ contributes significantly to the antiproliferative action of the compound. However, the observation that there was a difference of 7% in the number of cells in S phase between control cells incubated without ET18-OCH₃ (FBS/BSA/FBS) and those incubated with ET18-OCH₃ after transient activation (FBS/ET18-OCH₃/ FBS) suggests that the inhibition of cellular events by the ALP after the transient activation may also contribute to the inhibition of MCF-7 cell proliferation by ET18-OCH₃.
Discussion

The results of the current study demonstrate that in an ET18-OCH₃-sensitive cell line, uptake of the drug inhibits the sustained phosphorylation and activation of MAPK as a consequence of its effect on Raf-1. This is based on the following: [a] ET18-OCH₃ had no effect on the binding of EGF to its receptors. Although EGFr internalization was inhibited as previously reported (8), this occurred after the decrease of MAPK activity to near-resting levels; [b] ET18-OCH₃ did not affect the extent and kinetics of tyrosine phosphorylation of the EGFr, suggesting that ET18-OCH₃ had no effect on EGFr kinase activity; [c] ET18-OCH₃ did not affect the activation of p21ras in the cells; [d] Preincubation of cells with ET18-OCH₃ did not reduce the Raf-1 content of the cells but significantly decreased the duration of the association of Raf-1 with the membrane within 4 min of stimulation. This reduced association correlated with a decrease in the membrane-associated Raf-1 kinase activity; [e] Since ET18-OCH₃ had no effect on the cellular cAMP levels and PKA activity, the inhibition of Raf-1 activity was not caused by a direct or indirect decrease of PKA activity by ET18-OCH₃; and [f] The decrease in MAPK activity paralleled that of Raf-1 activity and resulted from decreased phosphorylation rather than a direct effect of ET18-OCH₃ on MAPK activity. The reduction in magnitude and duration of MAPK and the decreased association of Raf-1 with membranes in MCF-7 cells preincubated with ET18-OCH₃ after EGF or serum stimulation have also been observed in insulin-stimulated cells (Richard, C. and G. Arthur, personal observations).

The mechanism by which ET18-OCH₃ interferes in the association of Raf-1 with the membrane is not known. Since Ras activation, which localizes Raf-1 to the membrane (30, 31), is unaffected by ET18-OCH₃, it is not surprising that even though ET18-OCH₃ was already present in the membrane before stimulation of the cells with growth factors, it was unable to prevent the initial increase in Raf-1 association with membranes. We believe the initial activation of MAPK after cell stimulation is due to the Ras-induced translocation and subsequent activation of Raf-1, but the presence of ET18-OCH₃ in the membranes appears to activate or enhance a process that results in the dissociation of membrane-associated Raf-1 much earlier than in the control cells. However, we cannot yet categorically rule out an effect of ET18-OCH₃ on the translocation process. The enhanced dissociation of Raf-1 leads to the termination of the MAPK activity. Since growth inhibitory conditions with ET18-OCH₃ used in the study did not completely block MAPK activation, we can surmise that the magnitude and duration of activation of the enzyme were insufficient for the phosphorylation and activation of downstream molecules to levels required to initiate proliferation. This interpretation is consistent with the postulation that the magnitude and duration of activation of MAPK is crucial in determining cellular responses to stimulation (17).

We do not know whether ET18-OCH₃-induced inhibition of Raf-1 activation is due to a direct interaction of the compound with Raf-1, or whether this is achieved indirectly via its effects on unknown intermediary molecules required for the activation and/or attachment of Raf-1 to the membrane. It has been suggested that lipids may play a role in Raf-1 activation (35–37). Irrespective of the exact mode of action of ET18-OCH₃ on Raf-1, its ability to interfere in the activation of this key signaling molecule in a dose-dependent fashion should make it useful in assessing the effects of differential activation of the MAPK cascade on cellular responses.

The correlation between the accumulation of ET18-OCH₃, the extent of growth inhibition, Raf-1/membrane association and MAPK activation suggests that the inhibition of the MAPK pathway by ET18-OCH₃ is likely to contribute significantly to its antiproliferative effects. This view is supported by our recent studies demonstrating that R and S enantiomers of a double bond phosphonocholine ALP analog had differential effects on MCF-7 cell growth which correlated with their ability or inability to inhibit MAPK activation (Samadder, P., R. Bittman, H.-S. Byun, and G. Arthur, personal observations). The differential effects of the phosphonocholine enantiomers on cell proliferation also provides support for the widely held view that the inhibitory effects of ALPs are not due to nonspecific perturbation of membranes (reviewed in references 2, 3). The demonstration that inhibition of the early events in cell activation by ET18-OCH₃ significantly contributes to its antiproliferative effects clearly implicates the inhibition of the MAPK cascade in the mechanism of action of the compound. However, as other signaling molecules such as PI-3K, PLC, and PKC that may be activated within this period could be inhibited by ALPs, this cautions against attributing the inhibition of growth by ET18-OCH₃ solely to its ability to inhibit the MAPK cascade. An assessment of whether these molecules are inhibited under ET18-OCH₃ preincubation conditions that inhibit Raf-1 activation and whether their activation is essential for growth will be required before such a conclusion can be made. There is evidence that inhibition of PKC by ALPs does not correlate with inhibition of cell growth (2, 11, 12).

The localization of an inhibitory effect of ET18-OCH₃ downstream of Ras, implies that ET18-OCH₃ and related compounds should be effective against activating Ras mutations which have been observed in almost one-third of human cancers (38). Another implication of our study is that the apparent cell selective effects of ET18-OCH₃ may be due to fundamental differences in the regulation of Raf–membrane associations between the ALP-sensitive and -insensitive cells or differences in the signal transduction pathways (Raf-dependent or -independent pathways) that mediate cell proliferation in different cell types. Future studies to resolve the basis of the selective effects should lead to the rational use of these compounds in cancer therapy.

Acknowledgments

We are very grateful to Drs. N. Ahn for purified recombinant ERK2, and Dr U. Rapp for anti Raf-1 antisera (SP63).

This work was supported by a research grant from the National Cancer Institute of Canada (NCIC) with funds from the Canadian Cancer Society (to G. Arthur), a Research Scientist Award of NCIC (to D.W. Litchfield), and a Studentship Award from the Manitoba Health Research Council (to X. Zhou).

References

by the synthetic alkyllyso phospholipid 1-O-octadecyl-2-O-methylglycerol-3-phosphocholine do not correlate with inhibition of proliferation of MCF-7 and TH4 cell lines. *Cancer Res.* 52:2806–2812.


