Original Article

The antitumor ether lipid edelfosine (ET-18-O-CH_3) induces apoptosis in H-*ras* transformed human breast epithelial cells: by blocking ERK1/2 and p38 mitogen-activated protein kinases as potential targets

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We previously reported that a novel alkylphospholipid type antitumor agent edelfosine (ET-18-O-CH_3 ; 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine) induced apoptosis in human breast epithelial cells transfected with the H-*ras* oncogene (MCF10A-*ras*) which was causally linked to cyclooxygenase-2 (COX-2) up-regulation and production of 15-deoxy-$\Delta^{12,14}$-prostaglandins J_2 (15d-PGJ2). ET-18-O-CH_3 treatment also enhanced the production of prostaglandin E_2 (PGE_2), a major COX-2 product. In this study, we found that ET-18-O-CH_3 treatment resulted in elevated mRNA expression of the PGE_2 receptor subunit, EP2 receptor. Exogenously added PGE_2 inhibited the growth of MCF10A-*ras* cells and induced proteolytic cleavage of caspase 3. ET-18-O-CH_3 also inhibited constitutive activation of ERK1/2, p38 MAPK, and Akt/protein kinase B, which was blunted by a selective COX-2 inhibitor SC58635. In addition, ET-18-O-CH_3 inhibited DNA binding activity of NF-κB in MCF10A-*ras* cells, and this was again attenuated by SC58635. Based on these findings, it is likely that ET-18-O-CH_3 inactivates ERK1/2, Akt, and NF-κB signaling via COX-2 induction in MCF10A-*ras* cells, thereby inducing apoptosis of these cells.

Key Words: ET-18-O-CH_3, edelfosine, apoptosis, COX-2, MCF10A-*ras* cells

INTRODUCTION

A synthetic ether lipid edelfosine (ET-18-O-CH_3; 1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine' structure shown in Fig. 1), has been found to exert potent anti-tumorigenic effects.[1-3] The compound has been known to be a potent inducer of apoptosis in several tumor cell lines and primary tumor cells from cancer patients.[4] Unlike most conventional chemotherapeutic drugs, ET-18-O-CH_3 does not target DNA but rather acts on the tumor cell membranes, thereby inducing apoptosis.[5] The molecular mechanism underlying ET-18-O-CH_3-induced apoptosis is associated with inhibition of *de novo* synthesis of phosphatidylcholine at the endoplasmic reticulum.[6] Inhibition of protein kinase C, phophatidylinositol 3-kinase, and coenzyme A-independent transacylase, as well as the blockcade of arachidonate-phospholipid remodelling, also contributed to ET-18-O-CH_3-induced apoptosis.[7-10] In addition, ET-18-O-CH_3-induced apoptosis was accompanied by intracellular activation of the death receptor Fas/CD95 and its recruitment together with downstream signal molecules into lipid rafts, independently of FasL ligand.[11]

There is a substantial body of data supporting the idea that cyclooxygenase-2 (COX-2) overexpression provides tumor cells with a survival advantage, by conferring resistance to apoptosis and increasing invasiveness or angiogenesis.[12,13] Selective COX-2 inhibitors have been shown to exert anti-carcinogenic activity *in vivo* and *in vitro* experiments.[14,15] However, recent reports have suggested that the induction of COX-2 does not necessarily contribute to cell survival or tolerance in response to proapoptotic stimuli. Certain anticancer agents with pro-apoptotic activity were found to upregulate COX-2 expression in human hepatic myofibroblasts[16] cells. and neuroglioma cells.[17] Thus, COX-2-derived prostaglandins

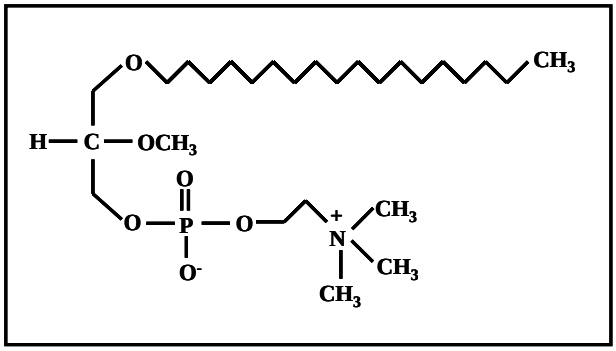

Figure 1. The chemical structure of ET-18-O-CH_3

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(PGs) are likely to be implicated in sensitizing these cells to apoptotic death. In this context, it is noticeable that some COX-2 products induced apoptosis in serveral types of cancer cells.[17,18] We previously reported that up-regulation of COX-2 expression and subsequent production of 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), a ligand of peroxisome proliferator-activated receptor gamma (PPARγ), induced apoptotic death of the *ras*-transformed human mammary epithelial (MCF10A-*ras*) cells treated with ET-18-O-CH_3.[19]

In the present study, we found investigated the possibility that another COX-2 product prostaglandin E_2 (PGE_2) can could also induce mediate apoptosis in the MCF10A-*ras* cells treated with ET-18-O-CH_3. In addition, ET-18-O-CH_3-induced apoptosis as well as COX-2 upregulation was associated with the suppression of extracellular-signal-regulated kinase1/2 (ERK1/2) and Akt.

MATERIALS AND METHODS

Cell culture

The MCF10A cell line transfected with a virus carrying the H-*ras* oncogene (MCF10A-*ras*) was cultured as described previously.[19]

Cell growth assay

MCF10A-*ras* cells at 50-60 % confluence were exposed to the medium containing chemicals. Cell viability was determined by the conventional MTT reduction assay. All samples were prepared in triplicates.

Western blot analysis

Protein isolation, electrophoresis and immunoblot analysis were conducted as described previously.[19] Antibodies against COX-2, extracellular signal regulated kinase1/2 (ERK1/2), pERK1/2, p38 mitogen-activated protein kinase (MAPK), pp38 MAPK, c-Jun N-terminal kinase (JNK), pJNK, Akt and pAkt were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cleaved caspase 3 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA).

Reverse-transcription polymerase chain reaction (RT-PCR)

Isolation of total RNA and reverse transcription were performed as previously reported.[19] The primer pairs were as follows (forward and reverse, respectively): EP2, 5'-GCCACGATGCTCATCCTCTTCGCC-3' and 5'-CTTGTGTTCTTAATGAAATCCGAC-3', EP4, 5'-GCCACGATGCTCATCCTCTTCGCC-3' and 5'-CTTGTGTTCTTAATCAAATCCGAC-3'.

Electrophoretic mobility shifty assay

The oligonucleotide harboring the NF-κB consensus sequence (Promega, Medicine, USA) was end-labeled with [γ-P^{32}]ATP using T4 polynucleotide kinase (Takara, Japan). Electrophoretic mobility shifty assay (EMSA) was conducted according to the previous report.[19]

RESULTS

ET-18-O-CH_3 induced COX-2 and EP2 expression

When MCF-10A-*ras* cells were treated with a concentration of 2.5 μM ET-18-O-CH_3, it induced COX-2 expression in a time dependent manner with concomitant proteolytic cleavage of caspase 3 (Figure 2A). As a selective COX-2 inhibitor, SC58635 suppressed the ET-18-O-CH_3-induced COX-2 expression (Fig. 2B). ET-18-O-CH_3 was reported to induce the production of PGE_2 which was attenuated by a selective COX-2 inhibitor, SC58635.[19] We observed that ET-18-O-CH_3 upregulated the expression of the PGE_2 receptor subunit EP2, but not EP4 (Fig. 2C). As an initial approach to elucidate a link between COX-2 upregulation and induction of apoptosis by ET-18-O-CH_3 in MCF10A-*ras* cells, we attempted to examine the effect of the major COX-2 product PGE_2 on the viability of these cells. Exogenously added PGE_2 inhibited cell growth (Fig. 3A) and induced apoptosis as revealed by proteolytic cleavage of caspase 3 (Fig. 3B).

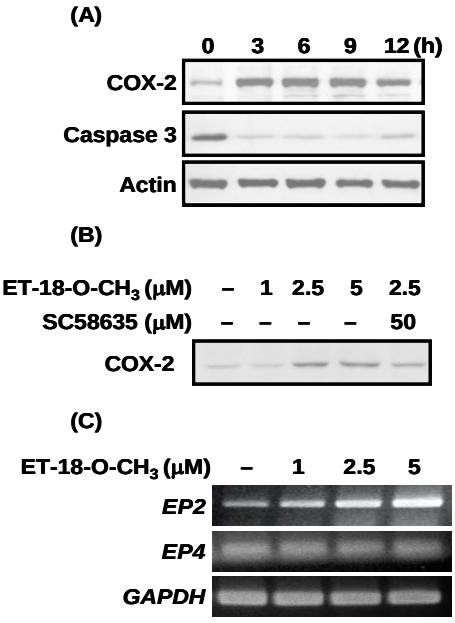

Figure 2. ET-18-O-CH_3 induced expression of COX-2 and the proteolytic cleavage of caspase 3 as determined by immunoblot analysis in MCF10A-*ras* cells (A, B). MCF10A-*ras* cells were treated with 2.5 μM ET-18-O-CH_3 for an indicated time, and mRNA expression of EP2 receptor was as determined by RT-PCR (C).

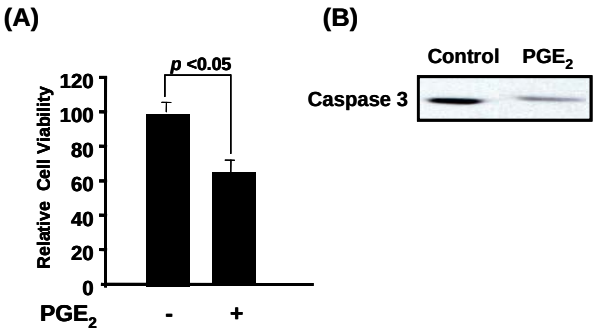

Figure 3. PGE_2 exerted anti-proliferative effects in MCF10A-*ras* cells. Cell viability was measured by conventional MTT reduction assay after the treatment of PGE_2 (200 μM) for 24 h (A). The Bars represent the mean ±S.E.M. of triplicate experiments. PGE_2 induced apoptosis in MCF10A-*ras* cells as evidenced by caspase-3 cleavage (B).

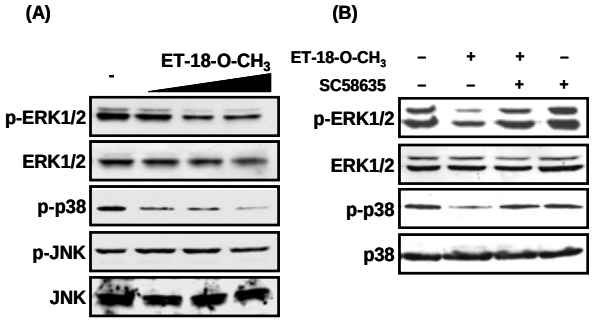

Figure 4. Effects of ET-18-O-CH_3 on the activation of representative MAPKs via phosphorylation (A) and inhibitory effects of SC58635 on phosphorylative activation of ERK1/2 and p38 MAPK (B). MCF10A-*ras* cells were treated with ET-18-O-CH_3 in the presence or absence of SC58635 (50 μM) for 3 days. ET-18-O-CH_3concentrations of were 1, 2.5, 5 μM for (A) and 2.5 μM for (B).

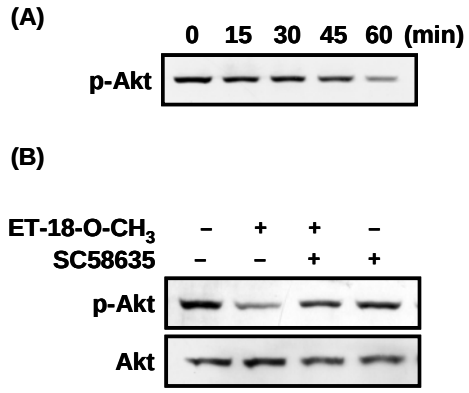

Figure 5. Effects of ET-18-O-CH_3 on the activation of Akt at the indicated time (A) and protective effect of SC58635 on the activation of Akt (B). MCF10A-*ras* cells were treated with 2.5 μM ET-18-O-CH_3 in the presence or absence of SC58635 (50 μM) for 1 h.

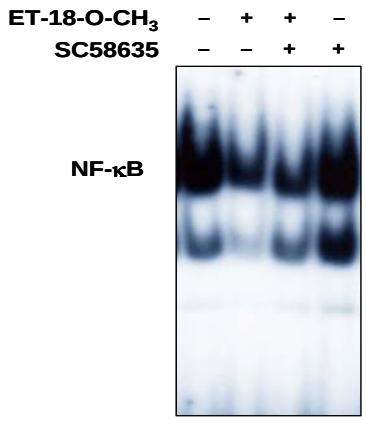

Figure 6. Inhibitory effects of ET-18-O-CH_3 on the DNA binding activity of NF-κB in MCF10A-*ras* cells. Nuclear extracts were isolated from the cells treated with ET-18-O-CH_3 (2.5 μM) for 3 days and incubated with the [γ-P^{32}] labelled oligonucleotide harbouring the -κB consensus sequence.

ET-18-O-CH_3 inhibited the activation of ERK1/2, p38 MAPK and Akt

It has been known that Akt/protein kinase B and MAPKs, such as ERK1/2, JNK and p38, play an important role in regulation of cell proliferation and survival. To delineate the intracellular signaling pathways responsible for the proapoptotic activity of ET-18-O-CH_3, we examined its effect on the activation of afore-mentioned kinases. ET-18-O-CH_3 inhibited activation of ERK1/2 and p38 MAPK through phosphorylation, but not JNK (Fig. 4A). A selective COX-2 inhibitor, SC58635, attenuated the ET-18-O-CH_3-induced suppression of ERK1/2 and p38 MAPK phosphorylation (Fig. 4B). In addition, ET-18-O-CH_3 inhibited Akt activation at 1 h as assessed by the kinetic study (Fig. 5A). ET-18-O-CH_3-induced Akt inactivation was also blunted by SC58635 (Fig. 5B).

ET-18-O-CH3 inhibited the DNA binding activity of NF-κB

The ubiquitous transcription factor, nuclear factor-kappa B (NF-κB), is involved in: inflammation, cell proliferation, and apoptosis.[20] NF-κB activation appears to be facilitated through cooperation with CREB (cyclic AMP-responsive element binding protein)-binding protein (CBP).[21,22] NF-κB is a major down stream molecular target of both ERK1/2 and Akt. Therefore, we conducted EMSA to determine whether the ET-18-O-CH_3-induced apoptosis is associated with down- regulation of NF-κB. ET-18-O-CH_3 inhibited DNA binding activity of NF-κB, which was again attenuated by SC58635 (Fig. 6).

DISCUSSION

In this study we have found that the antitumor alkyl-lysophospholipid ET-18-O-CH_3 induced apoptosis in MCF10A-*ras* cells, which was associated with the induction of COX-2 expression and subsequent production of PGE_2. In our previous study, we observed that ET-18-O-CH_3-induced COX-2 expression and production of 15d-PGJ_2 may be involved in ET-18-O-CH_3-induced apoptosis in MCF10A-*ras* cells.[19] Therefore, some products of COX-2 play an important role in the induction of apoptosis by ET-18-O-CH_3. In addition, we have found that ET-18-O-CH_3 inhibited the activation of ERK1/2 and Akt, which are central upstream kinases in the proliferation and survival pathways. NF-κB is a major transcription factor regulating the expression of the antiapoptotic protein Bcl-2.[23] Therefore, down regulation of NF-κB by ET-18-O-CH_3 is likely to reduce Bcl-2 levels, leading to the induction of apoptosis in MCF10A-*ras* cells. Additional studies are necessary to unravel the molecular link between the COX-2 inducing effects of ET-18-O-CH_3 and its anti-proliferative activity in MCF10A-ras cells and other transformed or cancerous cell lines.

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AUTHOR DISCLOSURES

Hye-Kyung Na and Young-Joon Surh, no conflicts of interest.

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