The antitumor ether lipid edelfosine (ET-18-O-CH₃) 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-CH₃; 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-Δ12,14-prostaglandins J₂ (15d-PGJ₂). ET-18-O-CH₃ treatment also enhanced the production of prostaglandin E₂ (PGE₂), a major COX-2 product. In this study, we found that ET-18-O-CH₃ treatment resulted in elevated mRNA expression of the PGE₂ receptor subunit, EP2 receptor. Exogenously added PGE₂ inhibited the growth of MCF10A-ras cells and induced proteolytic cleavage of caspase 3. ET-18-O-CH₃ 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-CH₃ 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-CH₃ 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-CH₃, edelfosine, apoptosis, COX-2, MCF10A-ras cells

INTRODUCTION

A synthetic ether lipid edelfosine (ET-18-O-CH₃; 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine* structure shown in Fig. 1), has been found to exert potent anti-tumorigenic effects. The compound has been known to be a potent inducer of apoptosis in several tumor cell lines and primary tumor cells from cancer patients. Unlike most conventional chemotherapeutic drugs, ET-18-O-CH₃ does not target DNA but rather acts on the tumor cell membranes, thereby inducing apoptosis. The molecular mechanism underlying ET-18-O-CH₃-induced apoptosis is associated with inhibition of de novo synthesis of phosphatidylycerol at the endoplasmic reticulum. Inhibition of protein kinase C, phosphatidylinositol 3-kinase, and coenzyme A-independent transacylase, as well as the blockade of arachidonate-phospholipid remodelling, also contributed to ET-18-O-CH₃-induced apoptosis. In addition, ET-18-O-CH₃-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.

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. Selective COX-2 inhibitors have been shown to exert anti-carcinogenic activity in vivo and in vitro experiments. 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 and neuroglioma cells. Thus, COX-2-derived prostaglandins were shown to inhibit 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-CH₃ 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-CH₃, edelfosine, apoptosis, COX-2, MCF10A-ras cells

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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 several types of cancer cells. We previously reported that up-regulation of COX-2 expression and subsequent production of 15-deoxy-D<sub>12,14</sub>-PGJ<sub>2</sub> (15d-PGJ<sub>2</sub>), 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<sub>3</sub>. 

In the present study, we found that another COX-2 product prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) could also induce apoptosis in the MCF10A-ras cells treated with ET-18-O-CH<sub>3</sub>. In addition, ET-18-O-CH<sub>3</sub>-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. 

**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. 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. The primer pairs were as follows (forward and reverse, respectively): EP2, 5’-GCCACGATGCTCATCTCTTCGCC-3’ and 5’-CTTGTGTTCTTAATGAAATCCGAC-3’; EP4, 5’-GCCACGATGCTCATCTCTTCGCC-3’ and 5’-CTTGTGTTCTTAATCAAATCCGAC-3’.

**Electrophoretic mobility shifting assay**
The oligonucleotide harboring the NF-κB consensus sequence (Promega, Medicine, USA) was end-labeled with [γ-<sup>32</sup>P]ATP using T4 polynucleotide kinase (Takara, Japan). Electrophoretic mobility shifting assay (EMSA) was conducted according to the previous report. 

RESULTS

**ET-18-O-CH<sub>3</sub> induced COX-2 and EP2 expression**
When MCF-10A-ras cells were treated with a concentration of 2.5 μM ET-18-O-CH<sub>3</sub>, it induced COX-2 expression in a time dependent manner with concomitant 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<sub>3</sub> for an indicated time, and mRNA expression of EP2 receptor was as determined by RT-PCR (C).

**Figure 2.** ET-18-O-CH<sub>3</sub> 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<sub>3</sub> for an indicated time, and mRNA expression of EP2 receptor was as determined by RT-PCR (C).

**Figure 3.** PGE<sub>2</sub> exerted anti-proliferative effects in MCF10A-ras cells. Cell viability was measured by conventional MTT reduction assay after the treatment of PGE<sub>2</sub> (200 μM) for 24 h (A). The Bars represent the mean ± S.E.M. of triplicate experiments. PGE<sub>2</sub> induced apoptosis in MCF10A-ras cells as evidenced by caspase-3 cleavage (B).
regulation of cell proliferation and survival. To delineate such as ERK1/2, JNK and p38, play an important role in

It has been known that Akt/protein kinase B and MAPKs, MAPK and Akt

ET-18-O-CH₃ 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-CH₃-induced suppression of ERK1/2 and p38 MAPK phosphorylation (Fig. 4B). In addition, ET-18-O-CH₃ inhibited Akt activation at 1 h as assessed by the kinetic study (Fig. 5A). ET-18-O-CH₃-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. NF-κB activation appears to be facilitated through cooperation with CREB (cyclic AMP-responsive element binding protein)-binding protein (CBP). NF-κB is a major downstream molecular target of both ERK1/2 and Akt. Therefore, we conducted EMSA to determine whether the ET-18-O-CH₃-induced apoptosis is associated with down-regulation of NF-κB. ET-18-O-CH₃ 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-CH₃ induced apoptosis in MCF10A-ras cells, which was associated with the induction of COX-2 expression and subsequent production of PGE₂. In our previous study, we observed that ET-18-O-CH₃-induced COX-2 expression and production of 15d-PGJ₂ may be involved in ET-18-O-CH₃-induced apoptosis in MCF10A-ras cells. Therefore, some products of COX-2 play an important role in the induction of apoptosis by ET-18-O-CH₃. In addition, we have found that ET-18-O-CH₃ 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. Therefore, down regulation of NF-κB by ET-18-O-CH₃ 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-CH₃ 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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