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MODULATION OF ATPase ACTIVITY BY CHOLESTEROL AND SYNTHETIC ETHER LIPIDS IN LEUKEMIC CELLS

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Abstract—Synthetic ether lipids (EL) exert their antiproliferative action on leukemic cells through localization in the plasma membrane with subsequent biochemical effects which are still being elucidated. In the present study, the modulation of membrane-linked ATPase activity was investigated in relation to changes in membrane fluidity of HL60 and K562 human leukemic cells. Incubation of HL60 and K562 cells with EL under non-cytotoxic conditions caused significant membrane fluidization which was related to the membrane cholesterol (CHOL) levels. HL60 cells, which are sensitive to the cytotoxic action of EL, had a lower basal CHOL content. When HL60 cells were loaded with CHOL, Na^+,K^+-ATPase activity was reduced significantly compared to that of untreated cells. In contrast, CHOL-deprived K562 cells had twice the Na^+,K^+-ATPase activity of unmodified K562 cells. Na^+K^+- and Mg^{2+}-ATPase activities were stimulated significantly in both cell lines by EL at concentrations lower than 20 μM. This stimulation was greater in cells richer in CHOL, such as K562 cells and CHOL-enriched HL60 cells. In contrast, Na^+,K^+-ATPase in both cell lines was inhibited by EL above 20 μM regardless of the CHOL content. Mg^{2+}-ATPase activity was not related to cell CHOL content and was not inhibited by EL above 20 μM.

Synthetic ether lipids (EL‡) express their antitumor properties by direct cytostatic and cytotoxic action, by activation of macrophages and by differentiation of malignant cells [1, 2]. Their toxic and antiproliferative effects have been reported to be relatively selective for leukemic cells compared with normal bone marrow progenitors [3–6].

The cellular effects of EL are apparently accompanied by no direct alterations in nuclear DNA [7] and EL are believed to be specifically membrane-interactive drugs since they accumulate in the plasma membrane [8]. EL have significant biochemical effects [9] and alter the biophysical properties of cancer cells such as membrane fluidity [10, 11]. Among the biochemical effects, EL are known to inhibit major membrane enzymes playing a pivotal role in cell function, like Na^+,K^+-ATPase and protein kinase C (PKC) [12–14]. For this reason it has been suggested that the antineoplastic properties of these drugs may be related to this interaction. In particular, the sodium pump appears to be either stimulated or inhibited by changes in lipid membrane composition or fluidity [15]. Membrane cholesterol (CHOL) content plays a crucial role in the modification of ATPase activity [15]. Indeed, we found recently that membrane lipid composition, particularly CHOL content, has important repercussions on the membrane permeability and toxicity of EL in leukemic cells [16, 17]. HL60 cells, which are more sensitive to the cytotoxic action of EL deficient than K562 cells, are in CHOL [16]. Enrichment in CHOL of HL60 cells to levels similar to those of K562 cells gave a cell line (HL60-CHOL) more resistant to EL inhibition than the native HL60 cells. On the contrary, when K562 cells are deprived of membrane CHOL (K562-AL cells) they become more sensitive to EL. It thus appeared that EL cytotoxicity could be modified by raising or lowering the membrane CHOL level [16, 17].

Zheng *et al.* [14] suggested that the antineoplastic effect of EL on intact cells was the combined result of their synergistic action on individual targets, such as Na^+,K^+-ATPase, PKC and phospholipid-metabolizing enzymes. In order to investigate the influence of changes in leukemic cell membrane composition and membrane environment on the inhibitory effects of EL, we investigated: (1) whether different CHOL levels might lead to different ATPase activities in these cells and (2) whether inhibition of the sodium pump by EL could be related to the different CHOL levels in these leukemic cells.

MATERIALS AND METHODS

Chemicals. CHOL was from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Erythrosin B (tetraiodofluorescein) was purchased from BDH (Poole, U.K.). DPH was purchased from Janssen (Beerse, Belgium). The racemic ether lipid, ET-16S-OEt, was synthesized by Dr C. Piantadosi and colleagues at the School of Pharmacy, University of North Carolina, Chapel Hill, NC, U.S.A. [18]. Active lecithin (AL), a 7:2:1 mixture of egg

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‡ Abbreviations: EL, synthetic ether lipids; PKC, protein kinase C; CHOL, cholesterol; DPH, 1,6-diphenyl-1,3,5-hexatriene; ET-16S-OEt, 1-hexadecylthio-2-ethyl-*rac*-glycero-3-phosphocholine; AL, AL721, active lecithin; FBS, fetal bovine serum.

yolk neutral glycerides, phosphatidylcholine and phosphatidylethanolamine [19], was kindly provided by Prof. Meir Shinitzky from the Weizmann Institute of Science (Rehovot, Israel).

Cells. Human leukemia HL60 and K562 cells were obtained from Istituto Zooprofilattico Sperimentale (Brescia, Italy), and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), and were free of mycoplasma contamination. In order to obtain HL60 cells preloaded with CHOL (HL60-CHOL), HL60 cells were cultured for 48 hr in 10% FBS RPMI medium containing an additional 25 μg/mL of CHOL [16]. To obtain CHOL-deprived K562 cells (K562-AL), K562 cells were incubated for 4 hr in 10% FBS RPMI medium containing 1 mg/mL of AL from a stock solution of 100 mg/mL in ethanol [17]. Ethanol concentration did not exceed 0.5% in the final volume and viability was 100% at the end of either CHOL or AL incubations.

Cholesterol determination. Lipids were extracted from the cell suspension (10×10^6 cells) according to Folch *et al.* [20] and CHOL content was measured according to Svanborg and Svennerholm [21].

Fluorescence polarization studies. Fluorescence polarization values (P values) were measured in cell suspensions according to Shinitzky and Barenholz [22], using DPH as the fluorescent probe. In a first series of experiments cells ($2–5 \times 10^5$) were incubated for 2 hr with 5 μM ET-16S-OEt, with cell viability 100% at the end of the incubation. Control cells were incubated with the same volume of medium alone. Cells were then washed twice with Dulbecco's PBS and resuspended in 2 mL of PBS-containing DPH (10^{-6} M). After incubation at 37° for 30 min, the P value was measured at 25°, using an MV-1 microviscosimeter (Elscint, Haifa, Israel). In another set of experiments, the cells ($2–5 \times 10^5$) suspended in 2 mL of DPH–PBS solution were incubated for 30 min at 37°. Then, increasing concentrations of the drug (1.5–200 μM) were added to the cells, and the P value was recorded immediately at 25°. The P value correlated directly with the apparent microviscosity, as already reported [16].

Determination of ATPase activities. Cells, washed twice with 0.9% NaCl, were resuspended at 5×10^5/mL and kept in ice, and composite Na^+,K^+ and Mg^{2+}-ATPase activities in intact cells were determined spectrophotometrically by the coupled enzyme assay of Scharschmidt *et al.* [23]. The ATPase activities were stable for at least 4 hr and linear with time/cell (between 0.1×10^5 and 1×10^5 cells) or protein concentrations. Na^+,K^+-ATPase was defined as oubain-inhibitable activity (3 mM final concentration). When required, 2, 20 and 200 μM ET-16S-OEt were added immediately before starting the reaction by ATP addition. The protein concentration was determined according to Lowry *et al.* [24].

Statistical analysis. Duncan's test for multiple comparisons was used.

RESULTS

Table 1 reports the composite, Na^+,K^+ and Mg^{2+}-ATPase activities for two leukemic cell lines, HL60 and K562, with different membrane CHOL contents [16]. The composite ATPase activity was about three times higher in the EL-resistant K562 cells than in the EL-sensitive HL60 line.

After enrichment of HL60 cells with CHOL (HL60-CHOL cells), with a subsequent CHOL content 1.7 times higher, the Na^+,K^+-ATPase activity was reduced by about 16% compared with the activity in HL60 cells ($P \leq 0.05$). The Na^+,K^+-ATPase activity of K562 cells deprived of CHOL (K562-AL cells), was double that of the parent cells ($P \leq 0.01$).

Under non-toxic conditions EL lowers the bulk fluidity of cell membranes [10, 11]. This effect may depend on the CHOL content of HL60 and K562 cells [16]. Figure 1 reports the effects of increasing doses of ET-16S-OEt on the P value measured at 25° in the different leukemic cell lines. HL60-CHOL cells, richer in CHOL (Table 1), were more rigid than normal HL60 cells and less sensitive to the fluidizing action of EL (Fig. 1, Panel A). A dose of 5 μM ET-16S-OEt increased significantly the bulk fluidity in HL60 cells, whereas in HL60-CHOL cells doses above 38 μM were needed to cause a significant change. As already reported, the basal P values for K562 and HL60-CHOL cells (Fig. 1, Panel B) were very similar; both cell lines were resistant to the toxic action of EL and had similar CHOL contents (Table 1). K562 cells deprived of CHOL (K562-AL) were more fluid than parent K562 cells; however the two cell lines were equally sensitive to the action of EL in increasing the bulk fluidity (Fig. 1, Panel B).

In order to investigate whether differences in membrane fluidity influenced the action of EL on the ATPase activities, we studied the effect of ET-16S-OEt on Na^+,K^+ and Mg^{2+}-ATPase activities in cells with different membrane CHOL contents. As reported in Fig. 2, Panel A, 2 μM EL raised the Na^+,K^+-ATPase activity by 228% in HL60 and 333% in HL60-CHOL cells, with respect to control values. The stimulatory effect was reduced, at 20 μM EL, to 144% and 181% of control values in HL60 and HL60-CHOL cells, respectively. At 200 μM EL the Na^+,K^+-ATPase activity was 64% of the control for HL60 cells and 83% for HL60-CHOL cells. Figure 2, Panel B, illustrates the effect of ET-16S-OEt on Na^+,K^+-ATPase activity in K562 and K562-AL cells. Activity increased to 173 and 134% of control values in K562 cells at 2 and 20 μM EL, respectively, followed by inhibition (66% of control) at 200 μM EL. No significant variation in Na^+,K^+-ATPase activity was measured in K562-AL cells exposed to 2 or 20 μM EL (Fig. 2, Panel B). In these cells, inhibition was seen at 200 μM only (63% of control). Figure 3 reports the effect of ET-16S-OEt on Mg^{2+}-ATPase activity in these cell lines. Stimulation of the Mg^{2+}-ATPase activity was observed at 2 μM EL in both HL60 and HL60-CHOL cells, the activity increasing to 212% and 163% of the control, respectively (Fig. 3, Panel A). At the higher EL concentrations no stimulation was seen and enzyme activities were at control levels. In K562 and K562-AL cells treated with 2 μM EL (Fig. 3, Panel B), the activity increased to 120% and 128%, respectively, but returned to the control value at higher concentrations. No inhibition of Mg^{2+}-ATPase activity was observed at 200 μM.

Table 1. ATPase activity in leukemic cell lines with different cholesterol contents

Cell type	CHOL ($\mu g/10^6$ cells)	ATPase activity*		
		Composite	Na^+,K^+	Mg^{2+}
HL60	4.34 ± 0.2	1.572 ± 0.26	0.175 ± 0.01	1.389 ± 0.37
HL60-CHOL	7.35 ± 0.5‡	2.008 ± 0.45	0.147 ± 0.01†	1.860 ± 0.44
K562	8.75 ± 0.5	5.127 ± 0.66	0.442 ± 0.05	4.710 ± 0.60
K562-AL	3.50 ± 0.2‡	6.900 ± 0.56	0.884 ± 0.16‡	6.017 ± 0.49†

* ATPase activity is expressed as mmol $P_i/hr/10 \times 10^6$ cells.
Each value is the mean ± SD of at least five different determinations.
† $P \leq 0.05$ and ‡ $P \leq 0.01$ compared with the parent cell line, by Duncan's test for multiple comparisons.

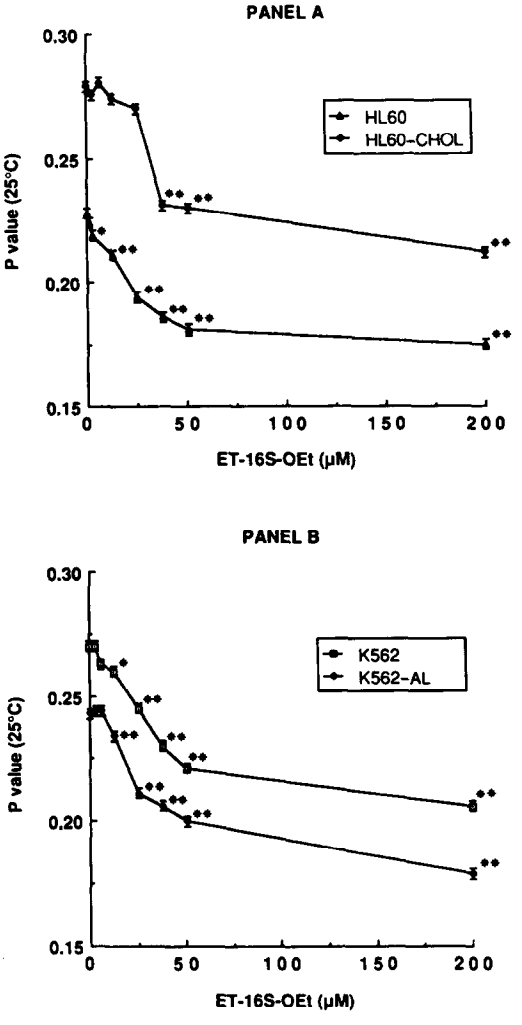

Fig. 1. Effect of increasing doses of ET-16S-OEt on P value, measured at 25° on HL60 and HL60-CHOL cells (Panel A) and K562 and K562-AL cells (Panel B). Each value is the mean ± SD of three measurements. * $P \leq 0.05$ and ** $P \leq 0.01$ according to Duncan's test for multiple comparisons vs each basal value.

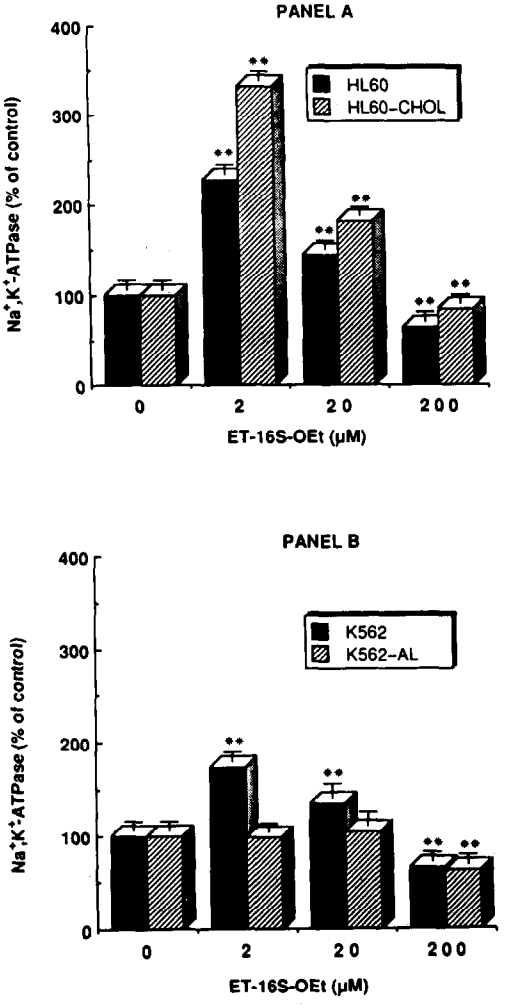

Fig. 2. Effect of ET-16S-OEt on Na^+,K^+-ATPase activity, expressed as a percentage of the control value, in HL60 and HL60-CHOL cells (Panel A) and in K562 and K562-AL cells (Panel B). Each value is the mean ±SD of five measurements. ** $P \leq 0.01$ according to Duncan's test for multiple comparisons.

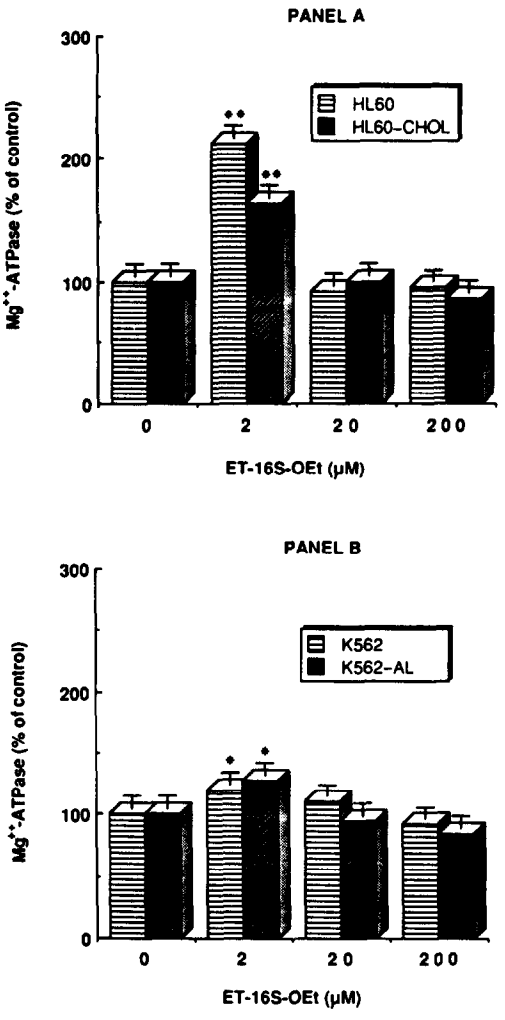

Fig. 3. Effect of ET-16S-OEt on Mg^{2+}-ATPase activity, expressed as a percentage of the control value, in HL60 and HL60-CHOL cells (Panel A) and in K562 and K562-AL cells (Panel B). Each value is the mean ± SD of five measurements. * $P \leq 0.05$ and ** $P \leq 0.01$ according to Duncan's test for multiple comparisons.

DISCUSSION

The mechanism of action and consequently the reason for the selectivity of EL toward leukemic versus normal cells remains to be clarified. Recent data from our group show that cells relatively resistant to the cytotoxic action of EL, such as K562 cells, are also richer in CHOL than sensitive HL60 cells [16, 17]. By modifying the CHOL content in these two cell lines, their sensitivity to EL toxicity can be modulated [17].

We have reported that fluidization of the cell membrane by EL is an important biophysical effect preceding the cytotoxic action of these drugs [11], and that cellular CHOL content may influence the EL action on membrane permeabilization of leukemic cells [16]. As shown in Fig. 1, in agreement with previous reports, cells with different lipid compositions showed different sensitivities to the fluidizing action of EL [17].

It has been suggested that changes in membrane lipid composition may affect the cell membrane properties and functions governing the interaction of the acidic boundary phospholipids with Na^+,K^+-ATPase [14, 15]. The data here reported show that CHOL-rich cell membranes exhibit lower Na^+,K^+-ATPase activity (HL60-CHOL vs HL60 cells), whereas CHOL-deprived cells exhibit a higher enzymatic activity (K562-AL vs K562 cells). We confirmed that Mg^{2+}-ATPase activity was only slightly affected by changes in membrane CHOL content, as already suggested [15].

Inhibition of the sodium pump in HL60 cells by EL has been reported [12–14]. We observed that EL at concentrations above 20 μM reduced Na^+,K^+-ATPase activity in HL60 and K562-AL cells, which are sensitive to EL cytotoxicity. Moreover, at concentrations up to 20 μM EL, inhibition of the sodium pump was also observed in K562 and HL60-CHOL cells, which are considerably less susceptible to EL toxic action [16, 17].

In contrast, the present data demonstrate that EL levels below 20 μM stimulate the sodium pump and that this stimulation is greater in cells less sensitive to the cytotoxic action of EL (HL60-CHOL and K562 cells). Stimulation was also observed for Mg^{2+}-ATPase activity but only at 2 μM EL. In HL60 cells, a short exposure to EL below 20 μM significantly fluidizes the cell membrane whereas treatment of HL60 or K562 cells with EL at concentrations exceeding 200 μM for 2 hr damages the cell membrane integrity and consequently the cell viability. We can speculate that the Na^+ pump is inhibited by EL at cytotoxic concentrations (up to 20 μM). Na^+,K^+-ATPase activity is stimulated by EL concentrations that significantly permeabilize the cell membranes. The stimulation was lower in cells less sensitive to the fluidizing action of EL (HL60-CHOL and K562 cells). We conclude that the Na^+,K^+-ATPase activity of rigid cells, which are less sensitive to EL cytotoxic action, is lower than in cells with more fluid membranes. Some authors [12–14, 25] have suggested that the antineoplastic and differentiating properties of EL might be due, at least in part, to the inhibition of both PKC and Na^+,K^+-ATPase, and that the combination of these two effects might be synergistic. The present findings indicate the existence of a relationship between cell membrane CHOL content and inhibition of Na^+,K^+-ATPase activity by EL, and may help explain the resistance of some leukemic cell lines to EL cytotoxic action.

On the basis of our recent studies, we conclude that CHOL content is certainly an important factor in the biophysical and biochemical effects of EL, including stimulation or inhibition of the sodium pump, and that it may attenuate EL effects modulating the toxic action of the drug. Thus, in order to reduce the residual leukemic cells the membrane CHOL content should be taken into consideration in bone marrow purging. In clinical studies *ex vivo* [3], bone marrow cells were treated for 4 hr with EL at 50–100 μM, concentrations that

inhibit the sodium pump, and returned to the host. As discussed already, the inhibition was greater in cells sensitive to the cytotoxic action of EL and with a significantly lower cholesterol content.

The metabolic fate of EL *in vivo* and the concentrations of the drugs or possible metabolites in target cells require further study. Further investigations are necessary to define a possible relationship among the *in vitro, ex vivo* and *in vivo* effects of EL.

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