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Differential Regulation of Phospholipase A_2 in Human Leukemia Cells by the Etherphospholipid Analogue Hexadecylphosphocholine

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ABSTRACT. Hexadecylphosphocholine (HePC) is the main representative of a new group of antineoplastic agents, the alkylphosphocholines, which were originally derived from cytotoxic etherlysophospholipids. HePC shows antiproliferative action against a whole variety of tumor cells and tumors *in vitro* and *in vivo*. Furthermore, it also induces differentiation in some hematologic cell lines and prevents invasive growth of neoplastic cells *in vitro*. To date, the precise molecular mechanisms mediating the biological effects of HePC have not been identified yet. As etherlysophospholipids seem to inhibit some pathways of lipid-dependent intracellular signalling, similar effects may be relevant for HePC. We therefore investigated the influence of HePC on phospholipase A_2 (PLA_2-EC 3.1.1) in the human leukemia cell line U 937. HePC seems to inhibit enzyme activity independently of protein kinase C (PKC) in differentiated U 937 cells stimulated by tumor necrosis factor α (TNFα). Inhibition of purified secretory PLA_2 from snake venom (EC 3.1.1.4) *in vitro* shows characteristics of a non-competitive mode. In contrast, HePC leads to an enhancement of PLA_2 activity in immature cells which cannot be explained by changes in membrane composition. Our data suggest that PLA_2 inhibition is most probably not the mechanism by which HePC mediates its antiproliferative effects. BIOCHEM PHARMACOL **53**;11:1725–1733, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. hexadecylphosphocholine; etherlysophospholipids; cytotoxicity; phospholipase A_2; differentiation; signal transduction

Hexadecylphosphocholine (HePC) is the first and best characterized representative of a new group of etherlysophospholipid-derived antineoplastic agents, the alkylphosphocholines. HePC shows remarkable antiproliferative activity on a broad spectrum of tumor cell lines and in some tumors *in vivo* [1–6]. HePC also induces differentiation in leukemic cell lines and in solid tumors [3, 4, 7, 8]. In an *in vitro* model, treatment of HePC inhibits the invasion of malignant cells into normal tissue [9]. So far, several potential mechanisms of action have been postulated by which HePC and probably other etherlipids may mediate their biological effects. The inhibition of protein kinase C (PKC) [10–14] and perturbations of membrane phospholipid metabolism [15–19] have been discussed as possible targets. However, it has recently been shown that these effects either do not correlate with growth inhibition [20–22, 23, 24] or have not yet been tested with regard to their significance for cell toxicity. To date, interactions of etherlysophospholipids with lipid-dependent cellular signalling are currently under investigation. The etherlipid 1-O-octadecyl-2-O-methyl-rac-3-glycero-phosphocholine ($ET\text{-}18\text{-}OCH_3$) and HePC seem to inhibit the phosphatidylinositol phospholipase C [25–27] and the phosphatidylinositiol-3-kinase [28]. In this study, we therefore were interested whether and how HePC affects another phospholipid-dependent signal transduction pathway via phospholipase A_2 (PLA_2). In addition, we investigated whether these effects change when cells become resistant towards HePC by differentiation.

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Abbreviations: AA, arachidonic acid; DPPC, dipalmitoylphosphatidylcholine; $ET\text{-}18\text{-}OCH_3$, 1-O-octadecyl-2-O-methyl-rac-3-glycero-phosphocholine; HePC, hexadecylphosphocholine; HPTLC, high performance thin layer chromatography; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PKC, protein kinase C; PLA_2, phospholipase A_2; SM, sphingomyeline; TNF-α, tumor necrosis factor alpha; TPA, phorbol 12-myristate 13 acetate.

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MATERIALS AND METHODS

Cell Culture and Biological Reagents

U 937 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Tumor necrosis factor alpha (TNF-α), H-7, bisindolylmaleimide II (B II), phospholipids and Naja mocambique PLA_2 were purchased from Sigma (Munich, Germany). Cell culture material was from Gibco (Glasgow, Scotland). Other reagents: Silicagel 60 high performance thin layer chromatography (HPTLC)

plates were obtained from Merck (Darmstadt, Germany) and organic solvents were from Baker (Deventer, Holland). Hexadecylphosphocholine was provided by Asta Medica (Frankfurt/M., Germany). Radioactively labelled substances were from Amersham (Braunschweig, Germany).

Cell Culture

Cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 10 mM glutamine, 10000 u/mL penicillin and 120 U/mL streptomycin in a 5% CO_2 atmosphere at 37°C. For experiments, cells were harvested in their exponential growth phase, washed twice in fresh medium and counted in a Neubauer chamber (Hecht-Assistent, Germany). Cell viability was checked by trypan blue dye exclusion tests. For induction of differentiation, cells were treated with dimethylsulfoxide (DMSO) 1.25% for 3 days. The effects of HePC on cell proliferation were tested in a standardized 48 hr incubation with escalating HePC concentrations followed by cell count and determination of cell viability, 50% inhibitory and lethal concentrations (IC_{50} and LC_{50}) values were extrapolated from the growth curves obtained by these tests and represent the HePC concentrations at which cell number and cell viability were reduced by 50%.

Radioactive Labelling

Cell membrane lipids were labelled metabolically by incubation of cells with 0.2 μCi/mL [^{14}C]arachidonic acid (55 mCi/mmol) and [^{14}C]choline (55 mCi/mmol) over 48 hr. Specific labelling of phosphatidylcholine was performed by a 4-hr incubation with 1 μCi/mL L-lyso-3-phophatidylcholine, 1-[^{14}C]-palmitoyl (56 mCi/mmol).

Cell Stimulation and Extraction

Cells were washed free of radioactive label in medium and then serum starved in medium supplemented with 5% bovine serum albumin for 4 hr. HePC treatment was performed for the last 2 hr of incubation at a concentration of 25 nmol/mL. Protein kinase C inhibitors H 7 (30 μM) and bisindolylmaleimide II (65 nM) were added together with HePC 2 hr prior to cell stimulation. Cell aliquots of 5×10^6 were suspended in 500 μL PBS with 2% BSA at 37°C and stimulated for indicated time periods with 100 ng/mL TNF-α. Incubations were stopped by addition of 1 mL ice-cold phosphate buffered saline (PBS) and immersion of reaction tubes in methanol/dry ice for 10 sec. Cells were pelleted at 4°C in an Eppendorff microfuge (Eppendorff, Hamburg, Germany) and then extracted according to the method of Bligh and Dyer [29].

Thin Layer Chromatography

Cell lipid extracts were concentrated under N_2 and spotted on thin layer plates by a semi-automated applicator (Camag Linomat IV, Berlin, Germany). Phospholipids were separated on silicagel HPTLC plates in a solvent system containing either chloroform/methanol/acetic acid/H_2O 100/60/20/5 (by vol.) or chloroform/methanol/triethylamine/H_2O 30/34/35/8 (by vol.). Plates were exposed to Kodak XAR films and the autoradiographs were analyzed by two-dimensional scanning in a Personal Densitometer (Molecular Dynamics, Krefeld, Germany). Silicagel plates were stained in coomassie brillant blue in order to visualize lipid standards. Analysis of not radioactively labelled lipids was also performed by densitometry after dipping silicagel plates in 10% coppersulfate in 8% phosphoric acid and charring at 180°C.

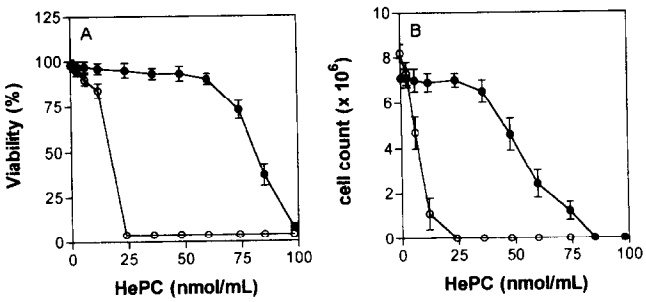

FIG. 1. Inhibition of cell proliferation: 2×10^6 normal (○) and differentiated (●) U 937 cells were incubated for 48 hr at 37°C with growing concentrations of HePC as indicated. Cell viability (A) and number (B) were determined by trypan blue dye exclusion test in a Neubauer chamber. Data represent mean values of five independent experiments ± SD.

In vitro *Enzyme Assay with Purified PLA_2*

A mixture of [^{14}C] labelled dipalmitoylphosphatidylcholine (40 nCi/μmol) (DPPC) and HePC in different concentrations of each substance was prepared in 1 mL TRIS-HCl, 20 mM $CaCl_2$, pH 8.0 by intensive sonication. Reactions were initiated by addition of 0.1 U snake venom PLA_2 (EC 3.1.1.4) at 37°C. After 10 min, the reactions were stopped by ice-cold chloroform/methanol 1:2 (by vol.). Lipids were extracted twice and spotted on a silicagel G 60 plate which was developed in chloroform/methanol/acetic acid/H_2O 100/60/20/5 (by vol.). Lipid spots corresponding to lysophosphatidylcholine (LPC) standard were scraped off the plate and radioactivity was quantified by liquid scintillation counting.

Statistical Calculations

Statistical analysis of experimental data was performed by the GraphPad Instat program (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Cell Proliferation

U 937 cells and differentiated U 937 cells were incubated with escalating concentrations of HePC at 37°C. After 48

TABLE 1. Basic activity of acylation/deacylation in U 937 cells

	pmol [^{14}C]arachidonic acid incorporated			
Time (hr)	SM	PC	PA/PI	PE
U 937				
0	630 ± 71	1108 ± 103	2936 ± 310	3399 ± 368
2	694 ± 54	1284 ± 144	3060 ± 356	3544 ± 560
4	766 ± 110	1159 ± 98	3310 ± 378	3205 ± 491
8	738 ± 126	1193 ± 116	3271 ± 319*	3627 ± 544
U 937 + HePC				
0	641 ± 66	1061 ± 123	2723 ± 276	3472 ± 331
2	761 ± 94	1138 ± 112	2439 ± 233	3520 ± 371
4	712 ± 99	1279 ± 101	2414 ± 243	3617 ± 355
8	679 ± 84	1138 ± 98	2287 ± 183*	3396 ± 321

2×10^6 cells were labelled with 0.2 μCi/mL [^{14}C]AA for 12 hr at 37°C. Cells were washed and exposed to 25 nmol/mL HePC for indicated time periods. Total cellular phospholipids were extracted and separated by thin layer chromatography as described in the Methods section. The amount of incorporated AA was calculated by measurement of radioactivity in single phospholipid bands by a linear analyzer. Data are mean values of four independent experiments ± SD. * Significant by Student's *t*-test ($P < 0.05$).

hr cell count and viability was determined. Fig. 1 shows dose-response curves obtained by this method. Normal U 937 cells are susceptible towards HePC with intrapolated IC_{50}/LC_{50} values of 5.8 and 17.3 nmol/mL respectively, representing the half-maximal cytostatic and cytotoxic effects of HePC. Differentiated U 937 cells appear to be much more resistant to HePC, with an IC_{50} concentration of 45.7 and a LC_{50} concentration of 81.6 nmol/mL.

Basal Activity of Acylation/Deacylation

U937 cells were preincubated with radiolabelled arachidonic acid (AA) for 12 hr and then exposed to 25 nmol/mL HePC for indicated time periods or left untreated. The changes in AA distribution were measured in the major cellular phospholipids sphingomyeline (SM), phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylethanolamine (PE). Table 1 shows experiments performed in U 937 cells while Table 2 summarizes data obtained from differentiated U 937 cells. In U 937 cells, HePC induced a decrease of AA incorporated into PA/PI of approximately 17%. The other phospholipids remained unchanged. In differentiated cells, HePC treatment led to a significant accumulation of AA in PC of 52% over control whereas in PA/PI AA levels were reduced by 22%. These data indicate that the turnover of AA is increased by HePC in PA/PI either by inhibition of reacylation or stimulation of PLA_2 activity. In HePC-treated differentiated U 937 cells, higher level of AA in PC rather suggest reduced PLA_2 activity or intensified reacylation processes.

Cellular Acylation Activity

To further discriminate between what effects were induced by changes in acylation or deacylation, cells were treated

TABLE 2. Basic activity of acylation/deacylation in differentiated U 937 cells

	pmol [^{14}C]arachidonic acid incorporated			
Time (hr)	SM	PC	PA/PI	PE
U 937 diff.				
0	1069 ± 156	1065 ± 131	2810 ± 211	3355 ± 314
2	922 ± 113	1197 ± 107	2949 ± 274	2952 ± 302
4	837 ± 93	1098 ± 117	2771 ± 271	3131 ± 324
8	1016 ± 123	1157 ± 120*	2813 ± 234*	2848 ± 304
U 937 diff. + HePC				
0	912 ± 117	1198 ± 124	2904 ± 214	3402 ± 364
2	848 ± 93	1141 ± 111	2506 ± 261	3385 ± 344
4	770 ± 81	1488 ± 127	2144 ± 244	2880 ± 329
8	1001 ± 98	1762 ± 156*	2214 ± 213*	2890 ± 310

2×10^6 cells were labelled with 0.2 μCi/mL [^{14}C]AA for 12 hr at 37°C. Cells were washed and exposed to 25 nmol/mL HePC for indicated time periods. Total cellular phospholipids were extracted and separated by thin layer chromatography as described in the Methods section. The amount of incorporated AA was calculated by measurement of radioactivity in single phospholipid bands by a linear analyzer. Data are mean values of four independent experiments ± SD. * Significant by Student's *t*-test ($P < 0.05$).

TABLE 3. Acylation of major phospholipids in U 937 cells

Time (hr)	pmol [^{14}C]arachidonic acid incorporated			
	SM	PC	PA/PI	PE
U 937				
0	8 ± 2	16 ± 5	70 ± 11	25 ± 8
2	312 ± 44	456 ± 37	1070 ± 93	568 ± 66
4	371 ± 50	585 ± 68	1192 ± 104	1116 ± 87
8	429 ± 56*	680 ± 70	1261 ± 114	1332 ± 126
U 937 + HePC				
0	7 ± 4	11 ± 6	69 ± 14	12 ± 4
2	83 ± 21	446 ± 47	1131 ± 104	484 ± 37
4	137 ± 31	583 ± 49	1355 ± 117	934 ± 101
8	220 ± 47*	747 ± 88	1475 ± 127	1178 ± 108

1×10^7 cells were simultaneously incubated with 25 nmol/mL HePC and 0.2 μCi/mL [^{14}C]AA at 37°C. At indicated time periods aliquots of 2×10^6 cells were taken and cellular phospholipids were extracted as described above. Extracts were subjected to separation by thin layer chromatography and the amount of incorporated AA was calculated from determinations of radioactivity in phospholipid bands by a linear analyzer. Data represent mean values of four independent experiments ± SD. * Significant by Student's *t*-test ($P < 0.05$).

with HePC and radiolabelled AA simultaneously. Table 3 shows data from U 937 cells and Table 4 data from differentiated U 937 cells. The acylation rate of SM in U937 was significantly reduced by HePC by 49%. In differentiated cells, HePC led to a slight but significant increase in SM acylation and an increase in PC acylation which, however, was not statistically significant. Taken together, these findings indicate that cellular acylation processes are not affected by HePC, with the sole exception being normal U 937 cells with regard to SM. The accumulation of AA in PC from differentiated cells, shown in Table 2, is therefore most probably due to an inhibitory effect on PLA_2 activity.

TNF-α Induced Activation of Phospholipase A_2

To study the effects of HePC on ligand-induced PLA_2 activation, we prelabelled cellular phospholipids with either [^{14}C]LPC, [^{14}C]choline or [^{14}C]AA. Cells were then treated with HePC for 2 hr at a concentration of 25 nmol/mL and stimulated with 100 ng/mL TNF-α for indicated time periods. Both reaction products of PLA_2 activation, LPC and AA, were measured in membrane lipid extracts. In addition, free AA was also determined in culture supernatants. Figure 2A shows the generation of LPC labelled either by [^{14}C]choline or [^{14}C]LPC in U 937 cells. In control cultures, no activation of PLA_2 apparently took place, as the LPC level did not increase. On the contrary, there was a reduction in LPC at 12 min of incubation. In HePC-treated cells, a transient peak activity of PLA_2 between 8 to 12 min of TNF-α exposure was observed, suggesting an enhancement of PLA_2 activity. Measurements of free AA seemed to confirm this observation (Figure 2B). There was once again a transient peak of free AA in the cell membrane followed by a continuous rise of free AA in culture supernatants in HePC treated cells.

TABLE 4. Acylation of major phospholipids in differentiated U 937 cells

Time (hr)	pmol [^{14}C]arachidonic acid incorporated			
	SM	PC	PA/PI	PE
U 937 diff.				
0	8 ± 2	6 ± 2	64 ± 8	12 ± 6
2	163 ± 16	474 ± 34	921 ± 96	522 ± 70
4	277 ± 18	596 ± 55	995 ± 88	892 ± 81
8	284 ± 24*	616 ± 73	1083 ± 98	992 ± 105
U 937 diff. + HePC				
0	10 ± 2	6 ± 2	67 ± 8	10 ± 3
2	130 ± 17	337 ± 48	894 ± 97	437 ± 57
4	201 ± 21	514 ± 66	1050 ± 96	606 ± 74
8	345 ± 27*	757 ± 80	923 ± 101	887 ± 84

1×10^7 U 937 cells differentiated with DMSO for 3 days were simultaneously incubated with 25 nmol/mL HePC and 0.2 μCi/mL [^{14}C]AA at 37°C. At indicated time periods aliquots of 2×10^6 cells were taken and cellular phospholipids were extracted as described above. Extracts were subjected to separation by thin layer chromatography and the amount of incorporated AA was calculated from determinations of radioactivity in phospholipid bands by a linear analyzer. Data represent mean values of four independent experiments ± SD. * Significant by Student's *t*-test ($P < 0.05$).

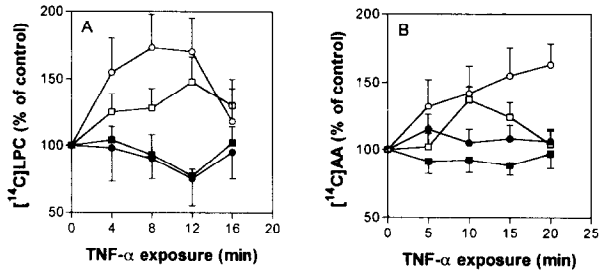

FIG. 2. TNF-α induced activation of PLA_2 in U 937 cells: (A) Cells were labeled with [^{14}C]LPC (■, □) or [^{14}C]choline (●, ○) as described in the Methods section and either treated with 25 nmol/mL HePC (○, □) for 2 hr or left untreated (●, ■). 5 × 10^6 cells were stimulated with 100 ng/mL TNF-α for indicated time periods. Cell lipid extracts were separated by thin layer chromatography and the amount of liberated radioactive LPC was measured from autoradiographs of thin layer plates densitometrically. (B) Cells were prelabelled with [^{14}C] AA as described before and treated with (○, □) or without (●, ■) 25 nmol/mL HePC for 2 hr. After stimulation with TNF-α the amount of free radioactive AA was measured in cellular supernatants (●, ○) by liquid scintillation counting. Membrane-bound AA (■, □) was determined in lipid extracts by thin layer chromatography separation, autoradiography and densitometry. Data are mean values of five independent experiments ± SD.

Surprisingly these effects were reversed in differentiated U 937 cells (Figure 3). Here, TNF-α treatment alone produced a high elevation of LPC (Figure 3A) and of free AA in membrane and supernatant (Figure 3B) which was totally inhibited by preincubation with HePC. These data indicate a different regulation of PLA_2 activity in U 937 cells by HePC depending on the state of maturation of the cells.

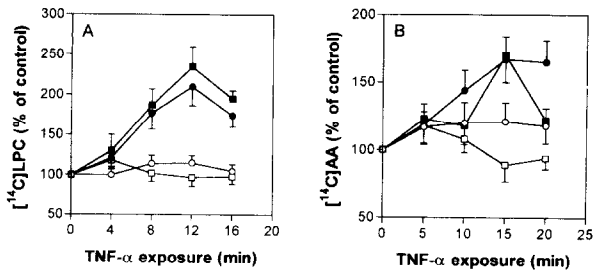

FIG. 3. TNF-α induced activation of PLA_2 in differentiated U 937 cells: (A) Cells were labelled with [^{14}C]LPC (■, □) or [^{14}C]choline (●, ○) as described in the methods section and either treated with 25 nmol/mL HePC (○, □) for 2 hr or left untreated (●, ■). 5 × 10^6 cells were stimulated with 100 ng/mL TNF-α for indicated time periods. Cell lipid extracts were separated by thin layer chromatography and the amount of liberated radioactive LPC was measured from autoradiographs of thin layer plates densitometrically. (B) Cells were prelabelled with [^{14}C] AA as described before and treated with (○, □) or without (●, ■) 25 nmol/mL HePC for 2 hr. After stimulation with TNF-α the amount of free radioactive AA was measured in cellular supernatants (●, ○) by liquid scintillation counting. Membrane bound AA (■, □) was determined in lipid extracts by thin layer chromatography separation, autoradiography and densitometry. Data are mean values of five independent experiments ± SD.

TABLE 5. Distribution of major cellular lipids

	Phospholipid distribution (%)	
	U 937	U 937 differentiated
LPC	1.5 ± 1.5	4.1 ± 1.5*
SM	11.0 ± 3.5	11.4 ± 2.1
PC	30.1 ± 5.1	24.8 ± 4.5
PS	1.3 ± 1.3	0.8 ± 0.8
PE	7.7 ± 3.2	6.0 ± 2.3
PI/PA	2.6 ± 1.2	4.2 ± 2.2
PG	9.6 ± 3.4	7.9 ± 3.2
CL	8.1 ± 3.0	16.4 ± 4.1*
NL	26.1 ± 4.8	24.4 ± 6.5

Normal and differentiated U 937 cells (5 × 10^6) were extracted by the method of Bligh and Dyer. Cellular phospholipids in organic phases were separated by thin layer chromatography. The amount of single phospholipids was determined by charring and densitometry and expressed as % of total plate extinction. Data are mean values of eight independent measurements ± SD. * Significant by Student's *t*-test ($P < 0.05$).

Comparison of Membrane Phospholipids

To investigate whether the differential effects on PLA_2 activity may be explained by a change in membrane phospholipid composition during cellular maturation, we analyzed the distribution pattern of major membrane phospholipids (Table 5). As can be seen, differentiation induced a shift in phospholipid distribution towards a higher content of LPC and cardiolipin (CL). There also seemed to be less PC in the membranes of mature U 937 cells, although the difference with normal U 937 was statistically not significant. SM, PS, PE, PI/PA, phosphatidylglycerol (PG) and neutral lipid (NL) amounts showed no relevant changes.

Effects of Inhibition of Protein Kinase C

As regulation of PLA_2 activity is often mediated by phosphorylation through PKC, we tested PLA_2 activation by LPC formation induced by TNF-α or phorbolester (TPA) in untreated (Figure 4A) or HePC exposed (Figure 4B) differentiated U 937 cells. After stimulation of cells for 10 min, TNF-α and TPA led to a strong activation of PLA_2, as was expected. When cells were incubated with the PKC inhibitor B II prior to stimulation, the TPA-induced stimulation of PLA_2 was reduced from 259% to 178% of control, whereas TNF-α-induced enzyme activation remained unchanged. Almost identical results were obtained with the second PKC inhibitor H7 (data not shown). In HePC pretreated cells, neither TNF-α nor TPA could induce PLA_2 activation whether PKC inhibitors were present or not. This observation suggests that HePC does not influence PLA_2 activity via PKC but rather by direct inhibition.

Effects of HePC on Purified PLA_2

To further characterize the inhibitory action of HePC on PLA_2, *in vitro* assays with purified enzyme from Naja

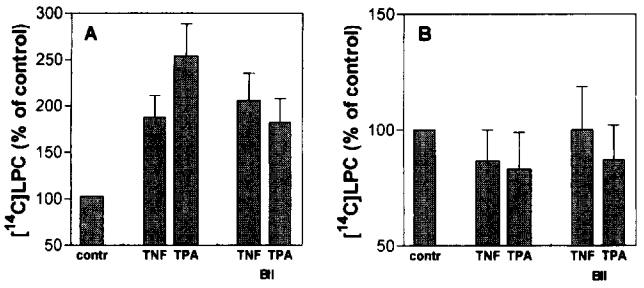

FIG. 4. Protein kinase C regulated activation of PLA_2: Differentiated U 937 cells were labelled with [^{14}C]LPC. A) shows control cells and B) cells treated with HePC 25 nmol/mL for two hr. PKC inhibitor B II was added two hr prior to stimulation of cells with either TNF-α 100 ng/mL or TPA 50 ng/mL for 10 min. Cellular lipids were extracted and resolved by HPTLC as described above. The amount of radiolabeled LPC was determined densitometrically form autoradiographs. Data show mean values of three independent experiments ± SD. Significant differences according to Student's t test ($P < 0.05$) were only observed between TPA- and TPA + B II-treated cells in Figure 4A.

mocambique were performed with a mixture of DPPC as substrate and HePC (Figure 5). HePC produced a dose-dependent inhibition of the enzyme (A). A half-maximal inhibition was achieved at approximately 50 nmol/mL HePC, making up 5% of the total DPPC in the assay. In a second experiment 25 nmol/mL HePC were mixed with growing amounts of DPPC (B). Lineweaver–Burke-plot analysis of this data revealed a non-competitive mode of inhibition, with a K_m value of 38.3×10^{-3}M (C).

DISCUSSION

In the past, many biological phenomena induced by HePC and other etherlipid analogues have been described. However, little is known about the biochemical mechanisms and target structures by which these substances elicit their effects. So far, the most promising approach to investigate the mode of action of HePC and etherlipids have been studies on their involvement in lipid-dependent intracellular signalling. It has been demonstrated that ET-18-OCH_3 inhibits ligand-induced inositoltrisphosphate formation and Ca^{2+} release in Swiss 3T3 cells [25]. HePC seems to prevent inositoltrisphosphate formation in bombesin-stimulated NIH 3T3 fibroblasts in a similar manner [26]. Both effects indicate an inhibition of the phosphatidylinositol-specific phospholipase C. Another enzyme inhibited by both compounds is the phosphatidylinositol-3-kinase in Swiss 3T3 cells [28]. *In vitro*, HePC shows inhibitory activity on purified phosphatidylinositol-specific phospholipase Cδ [30], and ET-18-OCH_3 decreases the activity of this enzyme in cell membrane preparations while it hardly affects other phospholipases such as the phosphatidylcholine-specific phospholipase C or phospholipase D [26].

In this study, we were primarily interested in whether HePC influences the activity of another very important phospholipase involved in cellular signalling, the phospholipase A_2 (PLA_2), and whether this possibly correlates with the antiproliferative effects of HePC. In the U 937 cells which we used for our investigations, HePC triggered early biological effects after an incubation time of 2 hr and concentrations of 25 nmol/mL. Due to its uptake mechanism via endocytosis, these effects can also be achieved at lower concentrations after longer exposition [8]. Under these conditions, approximately 3% of cellular phospholipids are comprised of HePC and mere physicochemical detrimental effects on the cells by its "lyso"-structure should not exist [31]. As in HL 60 cells, differentiation of U 937 induces a higher resistance of this cell line towards the cytostatic and cytotoxic effects of etherlipid analogues, thereby offering a useful model to study possible mechanisms responsible for growth inhibition [32].

When analyzing basal activity of acylation and deacylation in [^{14}C] AA labelled U 937 cells, we found changes in

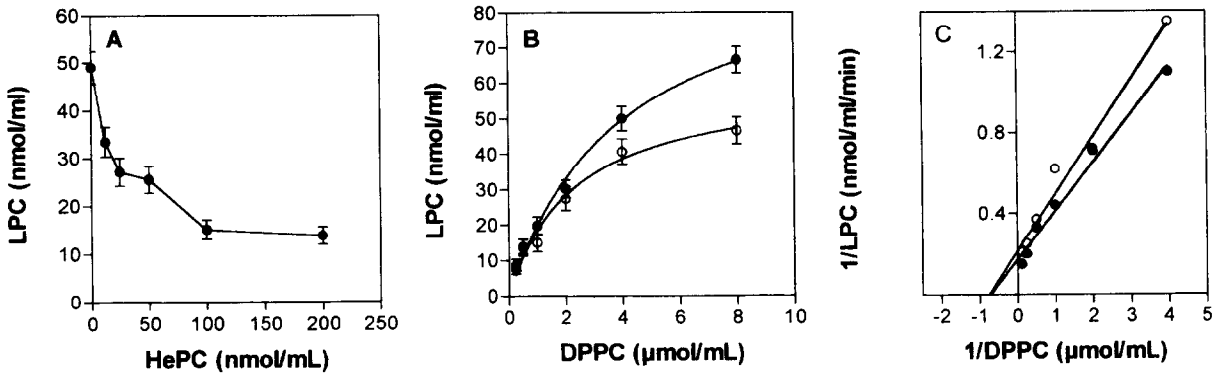

FIG. 5. Effects of HePC on purified PLA_2: A mixture of [^{14}C]DPPC and HePC was dissolved in 1 mL TRIS-HCl buffer by sonication. The reaction was started by addition of 0.1 U of purified snake venom PLA_2 from Naja mocambique and stopped after 10 min by addition of chloroform/methanol. Lipids were extracted and resolved by HPTLC. Lipid spots comigrating with LPC standards were scraped off the plate and radioactivity was determined by liquid scintillation counting. Figure 5A shows the effects of escalating concentrations of HePC at 1 µmol/mL DPPC. Figure 5B demonstrates the effect of increasing DPPC amounts at 25 nmol/mL HePC (○) or control (●). Figure 5C shows Lineweaver-Burke plot analysis of the data from Figure 5B. Data are mean values of three experiments ± SD.

AA turnover depending on the state of cellular maturation. In normal cells, there was only a slight decrease of AA content in the PI/PA fraction, whereas in differentiated U 937 a reduction of AA level in PI/PA and a concomitant accumulation in PC suggested an HePC-induced increased turnover of AA. As it was not possible to discriminate whether these effects were the result of intensified, PLA_2-mediated cleavage of AA from phospholipids or of reduced reacylation, we examined the HePC effects on incorporation of AA into cellular phospholipids. Here we found a significantly lower acylation rate in the SM fraction in U 937, indicating that the major changes in AA redistribution were probably due to inhibition of deacylation, i.e., an inhibition of PLA_2 activity. Lu and Gilbert noticed various changes in ET-18-OCH_3-treated cell lines when they investigated the incorporation of several fatty acids. However, they primarily observed changes in acylation with decreased incorporation of fatty acids into PC and an increased incorporation into other phospholipids, which rather suggests inhibitory activity of this etherlipid on reacylation processes [23, 24].

We tested the hypothesis that HePC inhibits PLA_2 activity in TNF-α-stimulated cells. In U 937, TNF-α induces an activation of PLA_2 with slower kinetics than other phospholipases such as phospholipase C [33]. This effect is usually more pronounced in differentiated cells, most probably because of higher amounts of cytosolic enzyme [34, 35]. In contrast to our expectations, in normal U 937 cells HePC seemed to induce TNF-α-stimulated PLA_2 activity. In untreated cells, TNF-α alone did not induce PLA_2 activation at all. However, when cells were first differentiated by DMSO, HePC very effectively inhibited PLA_2 activation. The mechanisms of these effects are not clear. Analysis of membrane lipid composition did not offer an acceptable explanation of this inverse effect because total phospholipid and cholesterol content, the phospholipid to cholesterol ratio (data not shown) and the distribution of most of the single phospholipids in the membrane did not change through differentiation. Whether the increased content of LPC and cardiolipin in mature U 937 cells leads to this effect is highly questionable. One would rather expect that the reduced PC level in differentiated cells may play a more important role, but the observed decrease of PC was statistically not significant. Perhaps this HePC effect is based on conformational lipid membrane alterations or on changes in phospholipid-protein interactions which we could not detect by measurement of lipid distribution.

As HePC and other etherlipids are also inhibitors of PKC and PKC activates PLA_2, we checked whether the effects of HePC in differentiated U 937 cells may be mediated indirectly by inhibition of PKC. Combination treatment of cells with TNF-α, TPA and specific PKC inhibitors revealed a significant decrease in TPA-induced PLA_2 activation by B II and H7, while TNF-α-induced enzyme activation remained unchanged. In HePC-treated cells, regardless of the stimulus employed, there was no PLA_2 activation detectable. These results indicate that HePC inhibits PLA_2 directly, and further that TNF-α-induced PLA_2 activation is probably also independent of PKC. The regulation of signal transduction involving PKC and PLA_2 is complex. In recent years, there have been several reports of TNF-α and other agonist signalling pathways bypassing PKC [36]. PKC inhibition prevents PLA_2 activation in vascular smooth muscle cells stimulated with H_2O_2 or angiotensin II, whereas PKC inhibition augments the fMet-Leu-Phe-induced PLA_2 activation in neutrophils and the PLA_2 activation by epidermal growth factor in Chinese hamster ovary cells [37–40]. It has been suggested that phorbolester may also stimulate other AA-releasing enzymes such as phospholipase A_1 or diacylglycerol/monoacylglycerol lipases [41]. Another report demonstrated that the PKC inhibitor B II suppressed PLA_2 activation by phorbolester and zymosan, whereas okadaic acid-induced PLA_2 activation was independent of PKC inhibition [42]. It has been shown that PKC regulates a mitogen-activated protein kinase in macrophages, which in turn activates PLA_2. However, TNF-α and interleukin-1 apparently seem to use an alternative mechanism of mitogen-activated kinase activation via neutral sphingomyelinase and generation of ceramide, independent of PKC [43].

To further characterize the action of HePC on PLA_2, we investigated the HePC effects on a purified secretory form of enzyme from snake venom. HePC itself is not a substrate for PLA_2 [44], and the structural similarity to physiologic phospholipids may suggest an interaction with PLA_2 as a false substrate. However, in our test system HePC clearly showed a non-competitive mode of inhibition, probably explained by modification of the substrate interface as reported for other PLA_2 inhibitors [45, 46]. It must be stressed that this inhibition occurred at HePC to substrate lipid ratios of 1–5%, which are also achieved in cell membranes *in vivo* and elicit early biological effects [8, 31]. These results should of course always be interpreted with care, because secretory and cytosolic PLA_2 do cleave phospholipids in a similar manner but are in fact different enzymes with no sequence homologies [47, 48].

In summary, these results show that HePC differentially regulates the activity of PLA_2 in a human tumor cell line depending on its state of maturation by a so far unidentified mechanism. In differentiated U 937 cells, PLA_2 seems to be inhibited directly, probably by a non-competitive mechanism. However, the influence of HePC on PLA_2 and fatty acid turnover does not seem to correlate with the antiproliferative effects of HePC. First, HePC only shows inhibitory activity on PLA_2 in differentiated cells, which are rather resistant towards HePC-induced growth inhibition. Second, alterations of membrane phospholipid basal acylation and deacylation processes induced by HePC also do not show any correlation with the cytotoxic effects. This is supported by similar conclusions with regard to ET-18-OCH_3-induced effects on cellular acylation and cytotoxicity in other cell lines [23, 24]. Furthermore, there is no apparent synergism or antagonism between HePC- and

TNF-α-mediated cell death in combination treatment of U937 cells (Berkovic D., unpublished observation).

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