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Hexadecylphosphocholine disrupts cholesterol homeostasis and induces the accumulation of free cholesterol in HepG2 tumour cells

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HePC, hexadecylphosphocholine
APC, alkylphosphocholine
PC, phosphatidylcholine
PE, phosphatidylethanolamine
PS, phosphatidylserine
SM, sphingomyelin
DAG, diacylglycerol
TAG, triacylglycerol
CE, cholesteryl ester
FC, free cholesterol
ACAT, acyl CoA:cholesterol acyltransferase
CEH, cholesteryl ester hydrolase
FBS, foetal bovine serum
MEM, minimum essential medium
DTT, dithiothreitol
TLC, thin-layer chromatography

ABSTRACT

Hexadecylphosphocholine (HePC) is a synthetic lipid belonging to the alkylphosphocholines (APC), a new group of antiproliferative agents that are proving to be promising candidates in anticancer therapy. We reported in a previous study that HePC interferes with phosphatidylcholine (PC) synthesis in HepG2 cells via both CDP-choline and phosphatidylethanolamine (PE) methylation. We have subsequently extended our studies to show that HePC interferes with sphingolipid metabolism by hindering the formation of sphingomyelin (SM), an effect accompanied by a substantial increase in the incorporation of the exogenous lipogenic precursors into ceramides. Interestingly, we demonstrate for the first time that HePC strongly inhibits the esterification of free cholesterol (FC) by acting at the level of acyl CoA:cholesterol acyltransferase (ACAT) (EC 2.3.1.26) activity. This effect is accompanied by a considerable increase in the synthesis of cholesterol, which leads to a rise in the levels of FC in cells. We are left in no doubt that the imbalance in the metabolism of membrane-lipid components vital to cell survival may well be responsible for the observed DNA fragmentation and activation of caspase-3, an enzyme involved in the cell apoptosis found in this study.

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1. Introduction

Most cytostatic agents impair cell division by acting either upon DNA or the cytoskeleton. In a new approach to cancer chemotherapy, however, it is the cell membrane, which is targeted with cytostatic agents. One such agent is hexadecylphosphocholine (HePC), a lipid analogue belonging to the alkylphosphocholine (APC) group, which exerts antitumoral activity against a broad spectrum of established tumour cell lines [1]. There is growing interest in the biological activity of these lipid analogues as they do not interact with DNA but selectively inhibit the growth of transformed cells, and thus could well complement existing DNA-directed anticancer chemotherapies. The inhibition of tumour cell proliferation caused by agents such as HePC may be the result not only of direct cell damage but also the induction of apoptosis [2]. Initial clinical studies have shown promising results and HePC is being used for the treatment of cutaneous metastases of mammary carcinomas [3].

Due to its amphiphylic properties HePC interacts with the cell membrane and rapidly reaches other subcellular membranes [4], thus being able to affect cell metabolism at different levels. The enzymes involved in lipid metabolism are mainly located in the membranes of the endoplasmic reticulum and thus would be a target for HePC activity. In fact, both our research group [5] and others [6,7] have already shown that HePC inhibits the biosynthesis of phosphatidylcholine (PC) by impairing the translocation of CTP:phosphocholine cytidylyltransferase from the cytosol, where it is inactive, to membranes, where it expresses activity. Furthermore, as a result of additional experiments we have been able to report that HePC also inhibits the biosynthesis of PC by phosphatidylethanolamine (PE) methylation in HepG2 cells [8], thus suggesting a link between the regulation of PC biosynthesis and cell proliferation.

This present study was undertaken to investigate the effects of the antitumoral drug HePC on the metabolism of neutral lipids and phospholipids and its possible induction of apoptosis. We used various exogenous radiolabeled precursors and found that HePC inhibited the incorporation of different fatty acids into PC whilst at the same time increasing their incorporation into diacylglycerol (DAG) and ceramide. Synthesis and esterification of cholesterol was also dramatically altered after exposure to HePC. In addition, we provide experimental evidence for the induction of apoptosis in HepG2 cells mediated by caspase-3.

2. Materials and methods

2.1. Materials

All radiolabeled compounds were supplied by American Radiolabeled Chemicals (St. Louis, MO, USA). Foetal bovine serum (FBS) was from Roche Diagnostics (Barcelona, Spain). The caspase-3 assay kit was from Molecular Probes (Poortgebouw, The Netherlands). HePC, minimum essential medium (MEM) and thin-layer chromatography (TLC) plates came from Sigma–Aldrich (Madrid, Spain). All other reagents were of analytical grade. The enzymatic colorimetric test to determine total and free cholesterol (FC) was from LabKit (Madrid, Spain).

2.2. Cell culture

The human hepatoma cell line HepG2 and monkey kidney Vero cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). Cells were cultured in MEM supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 1% non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin at pH 7.4. Cells were seeded on 60-mm tissue-culture plates (Nunc^{TM}) at densities of 3×10^4 cells cm^{-2} and incubated in a humidified atmosphere of 5% CO_2 at 37 °C to be used in experimental radiolabeling assays after 6 or 7 days' culture.

2.3. Metabolic labeling assays

2.3.1. Fatty acid incorporation

For metabolic labeling assays tissue-culture HepG2 cells were incubated at 37 °C for 6 h in 3 ml MEM/10% FBS containing [1-^{14}C]palmitate (100 μM, 17 Ci/mol), [9,10-^{3}H]oleate (100 μM, 40 Ci/mol) or [1-^{14}C]arachidonate (100 μM, 58 Ci/mol) supplemented with 50 μM HePC (treated cells) or without HePC (control cells). The incubation medium was also supplemented with 25 μM glycerol, 60 μM choline and 50 μM ethanolamine.

Fatty-acid sodium salt was prepared by drying the radioactive fatty acid by evaporation under a stream of nitrogen. The lipid residue was dissolved in 0.2 M NaOH to a molar ratio of 1:56 and the mixture sonicated until an optically clear solution was obtained. Defatted BSA dissolved in PBS was added to the fatty acid/NaOH solution to obtain the desired fatty acid/albumin ratio of 1:0.5. The medium was then adjusted to pH 7.4 with 1 N HCl. The fatty acid/BSA solution was diluted to its final working concentration in MEM/10% FBS and filtered before use.

2.3.2. Cholesterol synthesis

Synthesis of cholesterol was determined by measureing the incorporation of radioactive acetate into cellular free and esterified cholesterol. HepG2 and Vero cells were incubated at 37 °C for different time periods in MEM/10% FBS in the presence or absence of HePC. [1-^{14}C]acetate (3, 4 μM, 60 Ci/mol) was added during the last 6-h incubation period.

Lipid biosynthetic activity was estimated according to the level of incorporation of the radiolabel of each exogenous precursor into the lipids.

2.3.3. Lipid extraction and analysis

After incubation the medium was collected and the cells washed twice with ice-cold PBS before being harvested by scraping into PBS. The lipids were extracted from the cells following Bligh and Dyer's procedure [9]. The main phospholipids were separated by TLC using a mixture of chloroform/methanol/acetic acid/water (60/50/1/4, v/v/v/v) as solvent. The different neutral lipids were separated using a solvent of *n*-hexane/ethyl ether/acetic acid (80/20/2, v/v/v). Ceramides were separated in chloroform methanol (9/1, v/v). The spots were rendered visible by exposure to iodine vapour. Radiometric measurements of scraped lipid spots were made by liquid scintillation using a Beckman 6000-TA counter (Madrid, Spain).

2.4. Determination of cholesterol content

Lipids were isolated from both control and HePC-treated cells as described above. Total and free cholesterol contents were measured by enzymatic colorimetric kits from LabKit (Madrid, Spain). The free and total cholesterol concentrations were determined enzymatically by solubilizing the lipids in a single aqueous reagent containing cholesterol oxidase or cholesterol esterase/cholesterol oxidase, respectively. These reactions produced Δ^4-cholestenone and hydrogen peroxide (H_2O_2). H_2O_2 reacts with phenol and 4-aminophenazone by peroxidase activity to yield a stable, rose-colored product absorbing at 500 nm. Esterified cholesterol was determined as the difference between total and free cholesterol.

2.5. Enzymatic assays

To measure neutral cholesteryl ester hydrolase (nCEH) and acyl CoA:cholesterol acyltransferase (ACAT) activity in HepG2 lysates, the HePC-treated cell monolayers and controls were washed with PBS, scraped into 1 ml of 100 mM phosphate buffer (pH 7.4) and sonicated for 5 s.

2.5.1. Neutral cholesteryl ester hydrolase assay

nCEH activity was measured directly in lysates as described by O'Rourke et al. [10]. Briefly, the substrate was prepared by drying of cholesterol [^{14}C]oleate (610 nmol, 4 Ci/mol) under nitrogen. The lipid was then resuspended in 15 μl of ethanol and added to 2.5 ml of 200 mM phosphate buffer (pH 7.0) containing 72 mg BSA. A total of 175 μl substrate was added to a 50 μl sample (~2.5 mg/ml) and incubated for 45 min at 37 °C. When the reaction was finished the lipids were extracted and separated by TLC as described above. The fatty-acid band was scrapped and radioactivity was determined by liquid scintillation.

2.5.2. Acyl CoA:cholesterol acyltransferase assay

Enzyme activity was measured both in control and HePC-treated cells using [1-^{14}C]oleoyl CoA as radioactive substrate, as described elsewhere [11]. Briefly, 200 μg of protein from HepG2 lysate were preincubated for 5 min at 37 °C in 100 mM phosphate buffer (pH 7.4) containing 2 mM dithiothreitol (DTT) and 6 mg/ml BSA. Reactions were initiated by the addition of [1-^{14}C]oleoyl CoA (100 μM, 13.5 Ci/mol) and then further incubated for 10 min in a shaken water bath at 37 °C. The lipids were extracted and separated as described above. The CE band was removed by scraping and radioactivity was determined by liquid scintillation.

2.5.3. Caspase-3 assay

The activity of caspase-3-like proteases was measured to provide evidence that the cells were apoptotic. The cells were treated with 100 μM HePC in MEM. They were then washed twice with PBS and harvested in ice-cold PBS. Cell pellets were suspended in ice-cold 50 μl lysis buffer (10 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.01% Triton X-100). After 3 cycles of freeze/thawing the samples were centrifuged for 10 min at 5000 × *g*. The supernatants were incubated with 100 μM Z-DEVD-AMC in a 96-well plate at room temperature for 30 min. The fluorescence of the cleaved substrate was measured every 5 min for 45 min at 37 °C in a microplate reader (Perkin-Elmer) at 360 nm excitation and 446 nm emission.

2.6. DNA fragmentation

To determine apoptosis by DNA fragmentation, cells were grown as above on a 150-mm dish. After treatment the cells were lysed with 20 mM Tris–HCl pH 7.5, 2 mM EDTA, 0.4% Triton X-100. Fragmented DNA was prepared from the 13,000 × *g* supernatant after incubation overnight in a lysing buffer containing 10 mM Tris–HCl pH 8, 40 mM EDTA, 150 mM NaCl, 1% SDS and 200 μg/ml proteinase K. DNA was extracted with phenol/chloroform/isoamylalcohol (25/24/1, v/v/v). The aqueous phase was made up to 150 mM with NaCl and precipitated with 2 vol. of ethanol at −20 °C overnight. The pellets were air-dried, resuspended in 15 μl of distilled water and incubated for 1 h at 37 °C with 50 μg/ml ribonuclease A.

Horizontal electrophoresis of DNA was done in 1.5% agarose gel with 40 mM Tris–HCl pH 8, 40 mM sodium acetate, 1 mM EDTA (TAE buffer) as running buffer. DNA was visualized after electrophoresis by ethidium-bromide staining.

2.7. Other analysis

Cell protein content was determined in the cell homogenates by Bradford's method [12] using BSA as standard.

Results are expressed as the mean ± S.E.M. for three different experiments. Statistical comparisons were made by Student's *t*-test using the SPSS 9.0 program. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

We have investigated the effects of HePC on the synthesis of neutral lipids and phospholipids in HepG2 cells by using three radiolabeled fatty acids, palmitate, oleate and arachidonate, each with a different chain-length and degree of unsaturation. These fatty acids can be used for the de novo biosynthesis of glycerolipids and sphingolipids as well as for the retailoring of membrane phospholipids.

The results shown in Table 1 indicate that the profile of incorporation into phospholipids in the control cells is similar for all three fatty acids assayed. Thus, as might be expected by their higher intracellular levels, PC and PE incorporate higher radioactivity whilst the other phospholipids are labeled to a lesser extent. It can be seen in the same table that the effect of 6 h HePC-treatment upon the incorporation of fatty acids into phospholipids depends very much upon the fatty acid in question. Thus, when oleate was assayed as radioactive precursor there was a significant increase in the radioactive label of PE in HepG2-treated cells, whilst the other phospholipids remained unchanged. PE also incorporated more of the polyunsaturated fatty acid after exposure to HePC, this effect being accompanied by a significant decrease in the label of PC and phosphatidylserine (PS). Interestingly, the incorporation of palmitate into PS, PC and sphingomyelin (SM) was significantly hindered by HePC.

We also studied the synthesis of neutral lipids from these exogenous fatty acids (Table 2). It can be seen that in the

Table 1 – Influence of HePC upon the incorporation of [1-^{14}C]palmitate, [9,10-^{3}H]oleate and [1-^{14}C]arachidonate into phospholipids in HepG2 cells

	Palmitate		Oleate		Arachidonate	
	Control	HePC	Control	HePC	Control	HePC
Phosphatidylcholine	40.13 ± 0.73	36.15 ± 0.24**	15.69 ± 1.27	15.13 ± 0.39	12.29 ± 0.20	10.93 ± 0.14**
Phosphatidylethanolamine	18.70 ± 0.71	18.85 ± 0.18	12.66 ± 0.53	15.44 ± 0.06**	11.66 ± 0.35	14.05 ± 0.31**
Phosphatidylinositol	3.18 ± 0.16	3.19 ± 0.08	2.12 ± 0.03	2.37 ± 0.04	4.11 ± 0.20	4.01 ± 0.09
Phosphatidylserine	2.01 ± 0.08	1.45 ± 0.08**	1.03 ± 0.06	0.96 ± 0.05	2.34 ± 0.07	2.03 ± 0.04*
Sphingomyelin	1.09 ± 0.06	0.82 ± 0.06*	0.17 ± 0.01	0.18 ± 0.02	0.21 ± 0.01	0.19 ± 0.01

Cells growing in log-phase were incubated for 6 h in MEM/10% FBS containing [1-^{14}C]palmitate (100 μM, 17 Ci/mol), [9,10-^{3}H]oleate (100 μM, 40 Ci/mol) and [1-^{14}C]arachidonate (100 μM, 58 Ci/mol). Fifty micromolar HePC was added at the beginning of the 6-h incubation period. Untreated cells were used as control. The incorporation of fatty acids into phospholipids was determined as described in Section 2. Results are expressed as nmol/mg protein and are the mean ± S.E.M. of four determinations.

* $P < 0.05$.

** $P < 0.01$ when compared with control values.

Table 2 – Influence of HePC upon the incorporation of [1-^{14}C]palmitate, [9,10-^{3}H]oleate and [1-^{14}C]arachidonate into neutral lipids in HepG2 cells

	Palmitate		Oleate		Arachidonate	
	Control	HePC	Control	HePC	Control	HePC
Triacylglycerol	39.86 ± 1.91	49.23 ± 2.01*	126.37 ± 5.01	136.94 ± 1.58	10.45 ± 0.40	11.35 ± 0.32
Diacylglycerol	2.45 ± 0.25	5.57 ± 0.55**	1.91 ± 0.10	2.21 ± 0.02*	0.26 ± 0.01	0.39 ± 0.01**
Ceramide	0.74 ± 0.03	1.03 ± 0.06*	0.16 ± 0.01	0.22 ± 0.01**	0.08 ± 0.01	0.13 ± 0.01*
Cholesteryl esters	0.92 ± 0.05	0.48 ± 0.06**	2.86 ± 0.12	0.66 ± 0.02**	0.47 ± 0.04	0.07 ± 0.01**

Cells growing in log-phase were incubated for 6 h in MEM/10% FBS containing [1-^{14}C]palmitate (100 μM, 17 Ci/mol), [9,10-^{3}H]oleate (100 μM, 40 Ci/mol) and [1-^{14}C]arachidonate (100 μM, 58 Ci/mol). Fifty micromolar HePC was added at the beginning of the 6-h incubation period. Untreated cells were used as control. The incorporation of fatty acids into neutral lipids was determined as described in Section 2. Results are expressed as nmol/mg protein and are the mean ± S.E.M. of four determinations.

* $P < 0.05$.

** $P < 0.01$ when compared with control values.

control cells all three fatty acids were incorporated mainly into triacylglycerol (TAG), which contained around 90% of the total radioactivity found in the neutral lipid fraction. The levels of oleate incorporated into TAG were clearly higher than those found with the other fatty acids, indicating the high specificity of diacylglycerol acyltransferase for oleate. As far as CE is concerned, the results of incorporating fatty acids into this neutral lipid agree with earlier studies with rat liver microsomes, where it was shown that ACAT exhibits variable specificity against different fatty acids, e.g., oleoyl CoA > palmitoyl CoA > linoleoyl CoA [13]; the lowest specificity was for arachidonoyl CoA in the current study.

With regard to the effects produced in HepG2 cells after 6 h HePC-treatment, it can also be seen in Table 2 that there were similar modifications to the incorporation of all three fatty acids: the radiolabels in DAG and ceramide increased significantly whilst radioactivity in TAG only increased to any significant extent when the exogenous substrate was palmitate. A good way of analyzing the effect of HePC upon the biosynthesis of SM is to look at the ratio of radioactivity between SM and its immediate precursor, ceramide. The data in Fig. 1 clearly demonstrate that the exposure of HepG2 cells to this APC produced a marked decrease in the SM/ceramide ratio for all three fatty acids assayed.

Of particular interest in our present study was the effect of HePC upon intracellular CE synthesis; the incorporation of fatty acids into CE was dramatically reduced in cells exposed to HePC (Table 2), this effect being observed with all the fatty acids assayed whatever their chain-length or degree of unsaturation, suggesting that HePC might affect the metabolism of CE. In general, the concentration of CE is a result of the balance

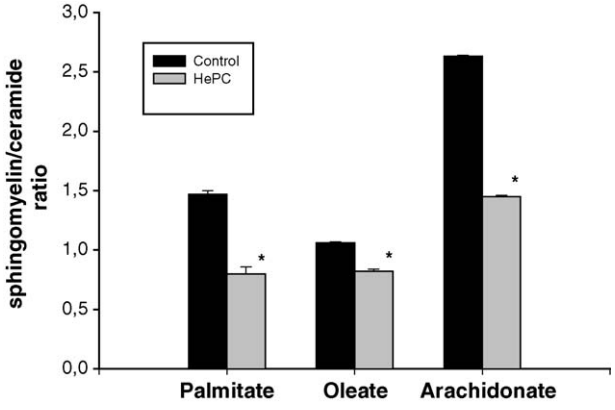

Fig. 1 – Values of the sphingomyelin/ceramide ratio after the incorporation of [1-^{14}C]palmitate, [9,10-^{3}H]oleate or [1-^{14}C]arachidonate into HepG2 cells incubated in the absence or presence of 50 μM HePC. Data were obtained as described in Tables 1 and 2. Results are expressed as the mean ± S.E.M. of four determinations. *$P < 0.001$ when compared with control values.

Table 3 – Influence of HePC upon acyl CoA:cholesterol acyltransferase activity and neutral cholesteryl ester hydrolase

	pmol/min mg protein	
	Control	HePC
ACAT activity	38.20 ± 1.41	23.9 ± 2.31*
nCEH activity	0.55 ± 0.02	0.62 ± 0.08

Cells growing in log-phase were incubated in MEM/10% FBS for 6 h in the presence or absence of 50 μM HePC. Cells lysates were obtained as described in Section 2. ACAT and nCEH activity was measured in both control and HePC-treated cells by using [1-^{14}C]oleoyl CoA (100 μM, 13.5 Ci/mol) and [1-^{14}C]cholesteryl oleate (190 μM, 4 Ci/mol) as substrates, respectively. Results are expressed as pmol/min mg protein and are the mean ± S.E.M. of three determinations.

* $P < 0.05$ when compared with control values.

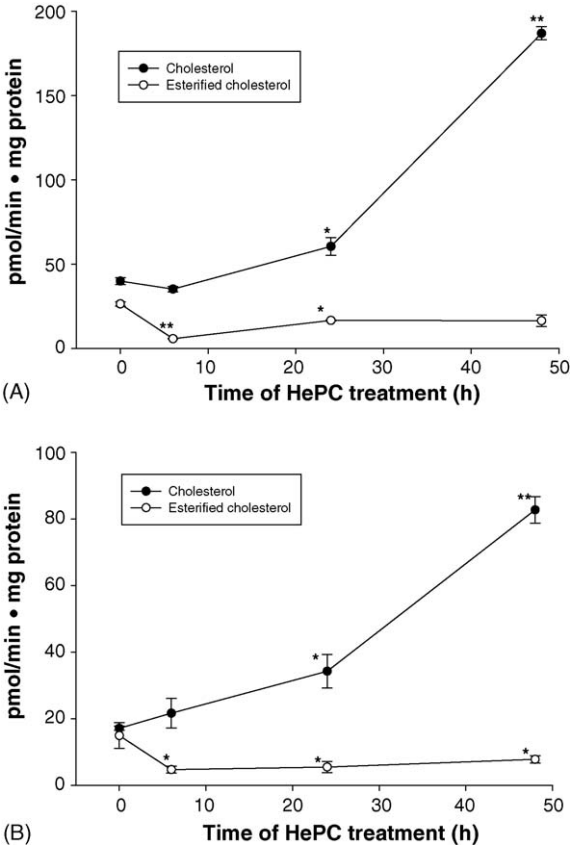

Fig. 2 – Effect of HePC on the biosynthesis of cholesterol and cholesteryl esters in HepG2 and Vero cells. HepG2 (A) and Vero (B) cells growing in log-phase were treated for 6, 24 and 48 h with 50 μM HePC in MEM/10% FBS. [1-^{14}C]acetate (3, 4 μM, 60 Ci/mol) was added during the last 6-h incubation period. Cells incubated in the absence of HePC (time 0) were used as control. The incorporation of acetate into cholesterol and CE was determined as described in Section 2. Results are expressed as the mean ± S.E.M. of four determinations. $^{*}P < 0.05$, $^{**}P < 0.001$ when compared with control values.

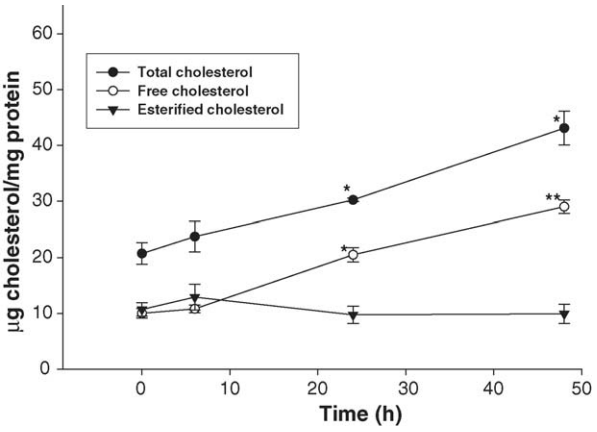

Fig. 3 – Effect of HePC on the cholesterol and cholesteryl ester content of HepG2 cells. HepG2 cells growing in log-phase were treated for 6, 24 and 48 h with 50 μM HePC in MEM/10% FBS. Cells incubated in the absence of HePC (time 0) were used as control. After incubation the medium was collected and the cells washed twice with ice-cold PBS before being harvested by scraping into PBS. The lipids were extracted from the cells following Bligh and Dyer's procedure, as described in Section 2. The lipid residue was resuspended in chloroform. An aliquot of this residue was used for enzymatic colorimetric determination of both total and free cholesterol. Results are expressed as mean ± S.E.M. of four determinations. $^{*}P < 0.05$, $^{**}P < 0.001$ when compared with control values.

between its esterification and de-esterification. De-esterification mainly results from the activity of the enzyme nCEH whilst the enzyme ACAT is responsible for the esterification of excess FC, and hence plays a key role in cellular cholesterol homeostasis. Thus, to analyse the above effects more closely we investigated the action of HePC on the synthesis and hydrolysis of CE in homogenates from cells treated with HePC for 6 h and controls. It can be seen in Table 3 that ACAT activity decreased by 37% concomitantly with the reduction of fatty-acid incorporation into CE. nCEH activity, on the other hand, was not affected when the cells were exposed to HePC.

Although we and other researchers have reported previously that the exposure of cells to HePC brings about a significant decrease in PC biosynthesis [6,8], to our knowledge there is no information about the effects it might exert on cholesterol metabolism in HepG2 or other cell lines. So, to obtain more detailed information about this alteration we went on to analyse the possible effect of HePC on cholesterol metabolism during a 48-h period by using [1-^{14}C]acetate as lipogenic precursor. Surprisingly, as can be seen in Fig. 2A, it had different effects on the synthesis and esterification of cholesterol. It markedly increased the synthesis of cholesterol over time, the incorporation of acetate into FC being about 370% higher in cells treated for 48 h compared to controls. With CE, on the other hand, the profile of acetate incorporation was very different: in spite of the enormous increase in the radioactivity of FC found after prolonged exposure to HePC, the radioactivity incorporated into CE was clearly lower in

treated cells when compared to controls. These results are consistent with the inhibition in ACAT activity induced by HePC found in our study.

To confirm that the results were not a unique or unusual effect specific to HepG2 cells, we also made experiments with Vero cells. Our results (Fig. 2B) demonstrate a similar profile of changes induced by HePC upon the incorporation of [1-^{14}C]acetate into cholesterol and CE. The synthesis of cholesterol increased throughout the time of exposure to HePC and once again 6 h HePC-treatment brought about a marked decrease in the incorporation of acetate into CE, which was sustained throughout the period assayed.

Interestingly, the increase in the cholesterogenic activity in HepG2 cells was accompanied by an increase in free-cholesterol levels of up to three fold in cells exposed to the APC for 48 h (Fig. 3). Since HepG2 cells contain small stores of esterified cholesterol it was quite difficult to observe any change in their content. In fact, in spite of the inhibition of ACAT activity CE levels were not significantly altered by HePC-treatment. As a consequence of these changes the cells treated with HePC contained higher quantities of total cholesterol mainly because FC had increased by 190% above basal levels.

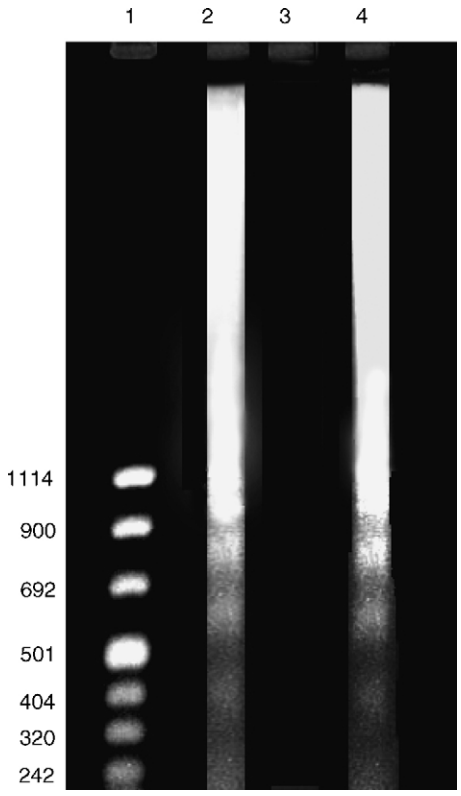

Fig. 4 – HePC induces genomic DNA fragmentation in HepG2 cells. Cells growing in log-phase were incubated for 24 h with TNF-α (30 ng/ml) + cycloheximide 40 μM (lane 2) as positive control of apoptosis or HePC 50 μM (lane 4). Untreated cells were used as controls (lane 3). Lane 1 was loaded with molecular-weight markers. DNA fragmentation was analyzed by agarose gel electrophoresis as described in Section 2.

All these results demonstrate that HePC drastically alters lipid metabolism. Having in mind that these alterations could be involved in programmed cell-death processes we investigated whether HePC might induce apoptosis. After 24 h exposure to HePC the tumoral cells showed a clearly rounded morphology and became increasingly detached from the plate the longer the exposure. Apoptotic cell death was confirmed by the presence of DNA ladders, a hallmark of apoptosis, which can be seen in the HepG2 cells after exposure to HePC (Fig. 4). In addition, we determined that HePC stimulates caspase-3 activity (29.11 ± 2.90 pmol/min mg protein in control cells versus 90.91 ± 3.41 pmol/min mg protein in HePC-treated HepG2 cells, $n = 3$; $^{*}P < 0.01$), which is entirely consistent with the induction of apoptosis.

4. Discussion

In recent papers we have reported that HePC exerts an antiproliferative effect on the HepG2 cell line, accompanied by considerable alterations to lipid metabolism. With regard to this, HePC inhibits PC biosynthesis via both CDP-choline [5] and the methylation of PE [8]. The experimental results we describe here indicate that HePC clearly alters the pattern of incorporation of different fatty acids into both phospholipids and neutral lipids. Although some authors have suggested that HePC might modulate phospholipid acylation by altering the activity of the reacylating acyltransferase enzymes [14], in vitro assays carried out in our laboratory using oleoyl-CoA as exogenous substrate indicate that it does not have any adverse effect on the retailoring of the different phospholipids in membranes isolated from control and HePC-treated cells (results not shown). Thus the effect of HePC on the incorporation of radiolabeled fatty acids found in our study may well be put down to its interfering with the de novo biosynthesis of membrane lipids.

The lower levels of palmitate and arachidonate incorporated into PC in cells exposed to HePC agree with our previous observations [5] and those of other authors [6,7] in different cell lines, where it was demonstrated that HePC inhibits PC biosynthesis by depressing the translocation of the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase to the microsomal membrane. Interestingly, we did not observe any decrease in PC labeling when we used oleate, probably due to the fact that this fatty acid stimulates PC synthesis by promoting the translocation of the enzyme to the membrane, where it becomes active [15], thus partially overcoming the inhibitory effect of HePC on PC biosynthesis. On the other hand, as we have previously shown, the decrease in radiolabeling in PS is a side-effect of the fall in radioactivity in PC, the substrate for the base-exchange reaction that produces PS [8].

In the cells treated with HePC we also observed a marked increase in the radiolabel of DAG from the three lipogenic precursors. This increase could not be attributed to an enhancement of PC degradation, since in cells in which PC was labeled to equilibrium with [methyl-^{14}C]choline the rate of PC turnover was unaltered by exposure to HePC, as we have demonstrated elsewhere [5]. Of particular interest in the present study is the fact that in HePC-treated cells the DAG synthesized from palmitate is shifted to the formation of TAG

whilst the DAG produced from oleate or arachidonate tends to be used to synthesise PE.

With regard to SM metabolism, this lipid is synthesized by the transfer of the phosphocholine head group from PC to ceramide, a reaction catalyzed by SM synthase, thereby liberating DAG. Due to this precursor-product relationship, the biosynthesis of SM might be influenced by the inhibition of the PC-synthesis pathway. Ceramide can also be produced in the cell by the breakdown of SM via sphingomyelinase activity, thus generating phosphocholine and ceramide (reviewed by [16]). In the presence of HePC the incorporation of palmitate into SM decreased by 20% whilst at the same time there was an increase of 30% in the labeling of ceramide. When oleate or arachidonate were used as lipogenic substrates radiolabeled ceramide also increased in HePC-treated cells with no concomitant change in the radioactivity of SM, thus indicating that HePC does not modify the degradation of this phospholipid. In accordance with this observation, Berkovic et al. [17] have demonstrated that HePC does not seem to alter sphingomyelinase activity in human leukaemia cells. The accumulation of radiolabeled ceramide found in our study may well be a result of diminished SM synthesis due to the lower availability of the choline-phosphate donor PC as a consequence of CTP:phosphocholine cytidylyltransferase inhibition produced by HePC. Our results agree with Wieder's findings in HaCaT cells that the incorporation of [^{3}H]choline into SM is inhibited concomitantly with an increase in intracellular ceramide levels [18].

Of special interest in our study are the effects caused by HePC upon cholesterol metabolism. To our knowledge there are no extant data concerning the effects of HePC and other APC on the homeostasis of cholesterol and thus our results demonstrate for the first time that whilst HePC reduces the synthesis of CE in HepG2 cells as a consequence of the inhibition of ACAT activity, it also stimulates the synthesis of cholesterol. We have also demonstrated this effect in other cell lines such as Vero. Furthermore, we have found in other experiments with HepG2 cells that other APC such as erucylphosphocholine produce the same alterations to cholesterol synthesis and esterification, although the effect is somewhat higher than that produced by HePC (results not shown).

All these observations point to the fact that HePC in general alters cholesterol metabolism, which in turn affects cholesterol homeostasis in the cell. In fact, the observed increase in cholesterol synthesis brought about by HePC-treatment and the decrease in cholesterol esterification lead to increased FC levels in HepG2 cells.

Considerable evidence has been gathered in recent years to suggest that cholesterol produced via the cholesterol biosynthetic pathway plays a key role in normal and neoplastic eukaryotic cell proliferation [19]. Further to this, recent in vitro studies have also shown a positive correlation between cholesterol esterification and cell-proliferation rate, indicating the possible involvement of the cholesterol esterification pathway in the regulation of cell growth and division in several cell lines [20–22]. Hepatic cells, which internalize exogenous cholesterol, have evolved mechanisms to prevent the accumulation of excess unesterified cholesterol, amongst which is the esterification of excess FC mediated by ACAT. The maintenance of intracellular cholesterol homeostasis is vital for the cell since, as has been demonstrated recently, an increase in FC levels may trigger the apoptotic process [23,24]. Rafts are distinct plasma membrane microdomains, comprised of cholesterol tightly packed with sphingomyelin. FC may modulate attractive forces between reversible palmitolated signaling proteins and lipids in rafts [25]. Some rafts proteins may contain specific FC binding sites [26]. Thus, the increase in the synthesis of cholesterol associated with an inhibition in cholesterol esterification found by us might be potentially important in terms of cell functionality and could be related to the antiproliferative action of HePC on HepG2 cells that we have described in a previous paper [5].

As we have tried to show in this work, the metabolic lipid alteration produced by exposing cells to HePC is quite complicated because not only are alterations caused to interrelated phospholipid metabolic pathways but also to cholesterol metabolism, which is coordinately regulated with the metabolism of choline-bearing phospholipids [27]. It is generally accepted nowadays that the maintenance of a strict FC/PC ratio is crucial to optimum cell behaviour and that alterations to this ratio may lead to necrosis and/or apoptosis [28]. Our results have considerable bearing on this idea because an increase in cholesterol biosynthesis associated with a decrease in PC synthesis and cholesterol esterification led to a modification in the FC/PC ratio.

All these alterations to lipid metabolism can be related to the induction of an apoptotic cell-death program. As far as this is concerned, we have observed that cells exposed to HePC show typical features of apoptosis, such as DNA laddering and caspase-3 activation. Caspases, a family of the interleukin 1β-converting, enzyme-like cysteine proteases, are involved in apoptotic signaling [29] and caspase activity is likely to be the most specific indicator of apoptosis. It is interesting to note that these apoptotic signs were not found in cells exposed to APC for periods of less than 24 h. Since substantial changes in PC and cholesterol metabolism are observed after 6 h of treatment, the changes in lipid balance precede and probably elicit apoptosis induced by HePC.

In conclusion, the results of our most recent study show that the effects of HePC on lipid metabolism within the cell are much more pronounced than previously imagined since it affects not only the biosynthesis of PC but also that of sphingolipids, acylglycerols and cholesterol. The complex network of effects that HePC seems to exert upon the metabolism of important molecules involved in cell-signaling makes it difficult, however, to pinpoint the precise mechanism(s) by which these events might participate in apoptosis. So far we have not been able to ascertain, which lipid metabolic intermediates might be involved but our observations are consistent with the hypothesis that HePC induces this process by interfering with the metabolism of cholesterol and/or PC and their related secondary messengers, and that the final effect is probably the result of a consequent imbalance in the metabolism of membrane-lipid components vital to cell survival.

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