Induction of Ceramide-mediated Apoptosis by the Anticancer Phospholipid Analog, Hexadecylphosphocholine*

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The prototype of a new class of antiproliferative phospholipid analogs, hexadecylphosphocholine (HePC), has been shown to inhibit tumor growth and is currently used for the treatment of cutaneous metastases of mammary carcinomas. Although several cellular targets of HePC, e.g. protein kinase C and CTP:phosphocholine cytidylyltransferase, have been proposed, the mechanisms of HePC-induced anticancer activity are still unclear. Considering that the antiproliferative effect of HePC correlates with inhibition of phosphatidylcholine biosynthesis, which is tightly coupled to sphingomyelin biosynthesis, we tested the hypothesis that treatment of cells with the anticancer drug leads to increased cellular ceramide and subsequently to apoptotic cell death. In the present study, we showed that 25 μmol/liter HePC induced apoptosis. In further experiments, we demonstrated that HePC inhibited the incorporation of radiolabeled choline into phosphatidylcholine and at a later time point into sphingomyelin. This was confirmed by metabolic labeling of the lipid backbone using radiolabeled serine, and it was shown that HePC decreased the incorporation of serine into sphingomyelin by 35% and simultaneously increased the incorporation of serine into ceramide by 70%. Determination of the amount of ceramide revealed an increase of 53% in HePC-treated cells compared with controls. In accordance with the hypothesis that elevated ceramide levels may be the missing link between the metabolic effects of HePC and its proapoptotic properties, HePC-induced apoptosis was blocked by fumonisin B1, an inhibitor of ceramide synthesis. Furthermore, we found that membrane-permeable ceramides additively increased the apoptotic effect of HePC.

In current anticancer therapies, most cytostatic agents impair cell division by cross-linking DNA (e.g. cisplatin or alkylating agents), disrupting the cytoskeleton (e.g. vinblastine), or rectifying the cytoskeleton (e.g. Taxol). In a new approach to cancer chemotherapy, the cell membrane was described as a target for cytostatic agents. It is known that alkyllysosphospholipids possess antineoplastic properties in vitro and in vivo (1), leading to the development of another class of antiproliferative phospholipid analogs, the alkylphosphocholines. The prototype of these phospholipid analogs hexadecylphosphocholine (HePC) (for chemical structure see Fig. 1), has been shown to inhibit cell proliferation and tumor growth (2–4). Due to its amphiphilic behavior and favorable penetration characteristics, HePC was expected to be a good candidate for topical treatment of skin metastases in breast cancer patients. Indeed, the first clinical studies showed promising results (5), and HePC is currently used for the treatment of cutaneous metastases of mammary carcinomas (6).

In search for the cellular targets of HePC it was demonstrated that HePC inhibits the biosynthesis of phosphatidylcholine (PC) in different cell lines (7–10). Subsequently, we and others systematically investigated the effects of different phospholipid analogs. Using alkylphosphocholines (11, 12), 1-O-octadecyl-2-O-methylglycerol-3-phosphocholine (13, 14) and N-acetylsphingosine-1-phosphocholeline (15), it has been demonstrated that inhibition of PC biosynthesis was paralleled by inhibition of cell growth. Interestingly, all biologically active phospholipid analogs contained a phosphocholine head group. Investigating hexadecylphosphoethanolamine and hexadecylphosphoserine (HePS), neither cell proliferation nor PC biosynthesis was inhibited (11).

In further experiments, inhibition of the rate-limiting enzyme of PC biosynthesis, CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15), was shown to be the mechanism underlying the inhibition of PC biosynthesis for all different classes of phospholipid analogs tested so far. Incubation of cells with the different analogs reduced the active, membrane-bound form of cytidylyltransferase (8, 13, 15), suggesting a link between the regulation of PC biosynthesis by cytidylyltransferase and the proliferative properties of cells. Experimental data supporting this hypothesis were provided by two recent studies which demonstrated that (i) inhibition of PC biosynthesis resulted in an arrest of the cell cycle (16) and (ii) Chinese hamster ovary mutant 58 cells carrying a temperature-sensitive mutation 58 cells carrying a temperature-sensitive mutation in cytidylyltransferase were driven into apoptosis when shifted to the nonpermissive growth temperature (17).

In the present study, we investigated the influence of the anticancer drug HePC on apoptosis and sphingomyelin (SM) biosynthesis, a metabolic pathway that is coupled to PC biosynthesis (18, 19). It was shown that HePC inhibited incorporation of [3H]choline and [3H]serine into SM, thereby increasing cellular ceramide. Furthermore, using cell permeable ceramides and fumonisin B1, a potent, competitive inhibitor of ceramide synthase (20), experimental evidence was provided...
that HePC-induced apoptosis is mediated by an elevation of cellular ceramide.

**EXPERIMENTAL PROCEDURES**

**Materials**—[methyl-3H](Choline chloride (2.8–3.1 TBq/mmol), t-[3-3H]serine (1.11 TBq/mmol), and [1-3H] thymidine (185 TBq/mmol) were from Amersham (Braunschweig, Germany). Silica Gel 60 high performance thin layer chromatography plates and reagents were purchased from Merck (Darmstadt, Germany). Streptomyces sp. sphingomelainase, phospholipids, and ceramide standards were from Sigma (München, Germany). The cytotoxicity detection kit (lactate dehydrogenase, LDH), the in situ cell death detection kit, fluorescein, and the cell death detection enzyme-linked Immunosorbsent assay (ELISA) from Boehringer (Mannheim, Germany). sn-1,2-Diacylglycerol kinase from Escherichia coli was purchased from Lipidex, Inc. (Westfield, NJ). HePC was synthesized as described previously (21), and HePS was a gift from Asta Pharma (Frankfurt/Main, Germany). Confluent cells were maintained in keratinocyte growth medium (KGM) for the duration of the experiments. KGM was prepared elsewhere stated, HaCaT cells were maintained in keratinocyte growth factor, 5 mg/liter insulin, 0.5 mmol/liter hydrocortisone, 50 mg/liter penicillin, and streptomycin from Roche (Mannheim, Germany) the terminal deoxynucleotidyltransferase is used to catalyze the labeling of the free ends of the DNA-strand breaks with modified nucleotides in an enzymatic reaction. In the in situ cell death detection kit, fluorescein (Boehringer Mannheim, Germany) the terminal deoxynucleotidyltransfease is used to catalyze the labeling of the free ends of the DNA-strand breaks with fluorescein-containing nucleotides. The reaction was carried out according to the manufacturer’s instructions. Confluent HaCaT cells that have been equilibrated in KGM for 24 h were incubated in KGM supplemented with 25 mmol/liter HePC or 25 mmol/liter HePS. After 20 h, cells were washed with PBS and trypanblue, 100 μl of cell suspension were applied to a slide and centrifuged for 5 min at 500 rpm. The cytosins were dried for 30 min at room temperature, and the slides were put in a 4% paraformaldehyde solution for 30 min. For permeabilization, 200 μl of 0.1% Triton X-100 in 0.1% sodium citrate were added to the cells and incubated at 4 °C for 2 min. Subsequently, cells were washed with PBS and 50 μl of TUNEL solution were added to the cells. Then, the cells were covered with a cover glas and the slides were put in a humid chamber and incubated at 37 °C for 1 h. After washing with PBS, Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) was added, and the slides were again covered with a cover glass. Finally, the cytosins were analyzed under a fluorescence microscope.

**Ceramide Mediates Hexadecylphosphocholine-induced Apoptosis**

**Measurement of Apoptosis**—Apoptosis was measured using the cell death detection ELISA from Roche (Mannheim) according to the manufacturer’s instructions. Confluent HeCaT cells that had been equilibrated in KGM for 24 h were incubated in KGM supplemented with different concentrations of HePC or HePS. After 20 h of incubation, the media were removed and the cells were trypsinized using 250 μl of 0.1% trypsin, 0.02% ethylenediamine tetraacetic acid in PBS. The reaction was stopped by addition of 500 μl of RPMI medium containing 10% fetal calf serum, and the cell suspensions were combined with 50 μl of lysis buffer (component of the assay kit) for 30 min at 4 °C, and the lysate was centrifuged for 10 min at 13,000 rpm in an Eppendorf centrifuge. Then, the cytosolic nucleosomes in the supernatant were detected using the cell death detection ELISA from Roche (Mannheim). For this, 20 μl of the probe were transferred to a streptavidin-coated microtiter plate, and 80 μl of immunoreagent containing 1 part biotinylated anti-histone antibody, 1 part peroxidase-conjugated anti-DNA-antibody, and 18 parts incubation buffer were added. After 2 h of incubation at room temperature, the microtiter plate was washed three times with incubation buffer, and the amount of peroxidase retained in the immunocomplex was determined photometrically at 405 nm with 2,2’-azo-ni-di-(3-ethylbenzhiazonium sulfonate) as a substrate. The specificity of the method was checked by the use of known inducers of apoptosis, such as cell permeable ceramides, and the results of the ELISA were confirmed by typical morphological changes of apoptotic cells seen at the ultrastructural level, e.g. membrane blebbing and condensation of the chromatin.

**Cell Culture—HaCaT cells (22) were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat-inactivated fetal calf serum, 0.35 g/liter glutamine, 100 000 IU/liter penicillin, and 0.1 g/liter streptomycin in plastic culture dishes (Nunc, Wiesbaden, Germany). Media and culture reagents were obtained from Life Technologies, Inc. (Karlsruhe, Germany). Penicillin and streptomycin were from Boehringer (Mannheim, Germany). Confluent cells were cultured after detaching the cells with 0.1% trypsin, 0.02% ethylenediamine tetraacetic acid in PBS. The reaction was stopped with 50 μl of 0.1% trypsin, 0.02% ethylenediamine tetraacetic acid in phosphate-buffered saline (PBS). Unless otherwise stated, HaCaT cells were maintained in keratinocyte growth medium (KGM) for the duration of the experiments. KGM was prepared from keratinocyte basal medium by addition of 10 μg/liter epidermal growth factor, 5 mg/liter insulin, 0.5 μmol/liter hydrocortisone, 50 mg/liter bovine pituitary extract, 100 μg/liter penicillin/streptomycin, and 2.5 mg/liter Fungizone. Keratinocyte basal medium and supplements were purchased from Clonetics (San Diego, CA). 25 mmol/liter HePS and 30 mmol/liter C2-ceramide stock solutions were prepared in ethanol. A 25 mmol/liter HePS stock solution was prepared in dimethyl sulfoxide and sonicated for 2 min prior to use and a 10 mmol/liter fumonisin B1 stock solution was prepared in methanol. All stock solutions were diluted with KGM to give the final concentrations. Ethanol, dimethyl sulfoxide, and methanol (vehicles) were added to controls and were present at 0.1, 0.1, and 0.5%, respectively. The addition of the vehicles did not influence viability or proliferation of HaCaT cells (23). Cytotoxicity Assay—Cytotoxicity of HePC was determined by the LDH-catalyzed conversion of lactate to pyruvate. In the second step the catalyst (diaphorase) transfers H+/H2O from NADH to 2-[4-Iodoophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride which is reduced to formazan and the reaction product is quantified photometrically at 492 nm.

The LDH assay was performed with the components of the cytotoxicity detection kit (LDH) from Boehringer Mannheim according to the manufacturer’s instructions. Confluent HaCaT cells which have been equilibrated in KGM for 24 h were incubated in KGM supplemented with different concentrations of HePC or HePS. After incubation, the cell culture supernatant was removed and clarified by centrifugation at 2000 rpm in an Eppendorf centrifuge for 5 min. 20 μl of the supernatant were diluted with 80 μl of KGM and transferred to microtiter plates. After addition of 100 μl of reaction mixture containing 2-[4-Iodoophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride, diaphorase, NAD+, and sodium lactate the samples were incubated for 5 min at room temperature. Absorbance was detected from light transmitted through microtiter plates and reagents were purchased from Schott (Germany). The purity of both products is quantified photometrically at 492 nm.

**Measurement of Apoptosis**—Apoptosis was measured using the cell death detection ELISA from Roche (Mannheim) according to the manufacturer’s instructions. Confluent HeCaT cells that had been equilibrated in KGM for 24 h were incubated in KGM supplemented with different concentrations of HePC or HePS. After 20 h of incubation, the media were removed and the cells were trypsinized using 250 μl of 0.1% trypsin, 0.02% ethylenediamine tetraacetic acid in PBS. The reaction was stopped by addition of 500 μl of RPMI medium containing 10% fetal calf serum, and the cell suspensions were combined with 50 μl of lysis buffer (component of the assay kit) for 30 min at 4 °C, and the lysate was centrifuged for 10 min at 13,000 rpm in an Eppendorf centrifuge. Then, the cytosolic nucleosomes in the supernatant were detected using the cell death detection ELISA from Roche (Mannheim). For this, 20 μl of the probe were transferred to a streptavidin-coated microtiter plate, and 80 μl of immunoreagent containing 1 part biotinylated anti-histone antibody, 1 part peroxidase-conjugated anti-DNA-antibody, and 18 parts incubation buffer were added. After 2 h of incubation at room temperature, the microtiter plate was washed three times with incubation buffer, and the amount of peroxidase retained in the immunocomplex was determined photometrically at 405 nm with 2,2’-azo-ni-di-(3-ethylbenzhiazonium sulfonate) as a substrate. The specificity of the method was checked by the use of known inducers of apoptosis, such as cell permeable ceramides, and the results of the ELISA were confirmed by typical morphological changes of apoptotic cells seen at the ultrastructural level, e.g. membrane blebbing and condensation of the chromatin.

**Incorporation of [methyl-3H]Choline into Phosphatidylcholine and Sphingomyelin—Incorporation of [methyl-3H]choline into PC and SM was measured as described previously (15). Confluent HaCaT cells that had been equilibrated in KGM for 24 h were incubated in KGM containing 2 μCi/ml [methyl-3H]choline supplemented with different concentrations of HePC or HePS. After incubation, cells were washed twice with ice-cold PBS, harvested, and freeze-dried. Lipids in the samples were extracted by a modified method of Bligh and Dyer (24) as described previously (15). Then, 10-μl aliquots of the chloroform phases were taken for scintillation counting, and 15-μl aliquots of the chloroform phases were separated by high performance
TLC using the solvent system chloroform/methanol/triethylamine/water (30:35:34:8, by volume). Radioactivity was quantified by radioscanning and phospholipids were identified by calibrating the scanner with PC and SM standards. For lipid quantification, all lipids were stained with a CuSO₄ solution (156 g/liter in 8.5% H₃PO₄) by the method of Touchstone et al. (25) and quantified by video densitometry. Staining was linear in the range 0.5–6 μg of lipid per band. The specific radioactivities of PC and SM were calculated from the radioactivity and the amount of lipid found in the corresponding bands.

**Incorporation of L-[3H]Serine into Sphingomyelin and Ceramide—** The incorporation of L-[3H]serine into SM and ceramide was determined as described elsewhere (23) with some modifications. Confluent HaCaT cells that had been equilibrated 24 h in serine-free minimal essential medium (Life Technologies, Inc., Karlsruhe, Germany) containing 5% fetal calf serum were radiolabeled with 2 × 10⁶ cpm/ml L-[3H]serine in the presence of different concentrations of HePC, HePS, or fumonisin B₁. After incubation, medium was removed and the cells were washed twice with ice-cold PBS, harvested, and freeze dried.

Lipids in the samples were extracted by a modified method of Bligh and Dyer (24). Then, 15-μl aliquots of the choloroform phases were separated by high performance TLC using the solvent system chloroform/methanol (9:1, v/v), and radioactivity was quantified by radioscanning. Ceramide was identified by calibrating the scanner with known standards. Additionally, an internal, radiolabeled ceramide standard was prepared by labeling cellular extracts with [3H]serine and separating the SM lipids. SM standards were prepared by incubation of serine-labeled cellular extracts with 0.1 unit/ml sphingomyelinase. The ceramide-bound radioactivity was measured as disintegrations/min ceramide/disintegrations/min total lipid, and SM-bound radioactivity of control samples was set as 100%. After radioscanning, the plates were developed using the solvent system chloroform/methanol/triethylamine/water (30:35:34:8, by volume) to separate SM from glycosphingolipids in the samples. The SM peak in the resulting radioscanned plate was identified by the use of standard SM. Additionally, it was shown that this peak was digested by sphingomyelinase treatment. The SM-bound radioactivity was measured as disintegrations/min SM/disintegrations/min total lipid, and SM-bound radioactivity of control samples was set as 100%.

**Determination of Cellular Ceramide—** Determination of cellular ceramide was carried out as described previously (26) using sn-1,2-diacylglycerol kinase. Briefly, after incubation of the cells with HePC, lipids were extracted according to Bligh and Dyer (24), and cellular ceramide in the samples was measured as described elsewhere (26).

**Other Procedures—** Statistical comparisons were made in these studies with Student’s t test.

**RESULTS**

**Hexadecylphosphocholine Induces Apoptotic Cell Death at Nonlytic Concentrations—** Due to its amphiphilic structure the cytotoxic effect of HePC might be a simple consequence of its lytic properties. On the other hand, it has been proposed that 25 μmol/liter HePC significantly increased cytosolic nucleosomes to about 240 and 450% of control, respectively, whereas the control phospholipid HePS did not induce DNA damage (Fig. 2B). Since the procedure of the cell death detection ELISA depends on intact plasma membrane function of the cells, the increase of cytosolic nucleosomes clearly distinguished the apoptotic effect of HePC at concentrations ≥25 μmol/liter from its lytic effect at 50 μmol/liter. The apoptotic capacity of HePC was confirmed on single cell level by TUNEL staining. As shown in Fig. 3, control and HePS-treated cells showed only a weak and diffuse staining, with 2 ± 1% (n = 3) of the cell population being fluorescent (apoptotic), whereas treatment of HePC cells with 25 μmol/liter HePC significantly increased the number of fluorescent (apoptotic) cells to about 28 ± 11% (n = 3) of the cell population.

**Hexadecylphosphocholine Inhibits Cell Proliferation and Phosphatidylcholine Biosynthesis—** The antiproliferative effect of HePC has already been well documented in different tumor models (29) and cell lines (11, 30), including primary human keratinocytes (10). However, no data were available on the immortalized human keratinocyte cell line HaCaT. As shown in Fig. 4A, HePC inhibited the proliferation of HaCaT cells and 25 μmol/liter HePC significantly increased cytosolic nucleosomes to about 240 and 450% of control, respectively, whereas the control phospholipid HePS did not induce DNA damage (Fig. 2B). Since the procedure of the cell death detection ELISA depends on intact plasma membrane function of the cells, the increase of cytosolic nucleosomes clearly distinguished the apoptotic effect of HePC at concentrations ≥25 μmol/liter from its lytic effect at 50 μmol/liter. The apoptotic capacity of HePC was confirmed on single cell level by TUNEL staining. As shown in Fig. 3, control and HePS-treated cells showed only a weak and diffuse staining, with 2 ± 1% (n = 3) of the cell population being fluorescent (apoptotic), whereas treatment of HaCaT cells with 25 μmol/liter HePC significantly increased the number of fluorescent (apoptotic) cells to about 28 ± 11% (n = 3) of the cell population.
and similar results were obtained. The experiment was repeated twice.

Confluent HaCaT cells were treated with 0.1% ethanol (A), 25 μmol/liter HePC (B), 0.1% dimethyl sulfoxide (C), or 25 μmol/liter HePS (D). Cytospins were prepared after 20 h of incubation and TUNEL staining of apoptotic cells was performed as described under “Experimental Procedures.” The upper panels are phase contrast illustrations and the lower panels fluorescent illustrations of the cells at a magnitude of 400-fold. The experiment was repeated twice and similar results were obtained.

with a half-inhibitory concentration (IC₅₀) of approximately 3 μmol/liter, whereas the control phospholipid HePS had no significant effect. At the nonlytic HePC concentration of 25 μmol/liter cell proliferation was almost completely blocked (6% of control), confirming our assumption that the antiproliferative effect is not mediated by cell lysis.

The antiproliferative effect of HePC was paralleled by its ability to inhibit the incorporation of [methyl-³H]choline into PC, thereby decreasing the specific radioactivity of PC in the presence of 7.5 and 25 μmol/liter HePC after 4 h of incubation by 58 and 71%, respectively (Fig. 4B). Inhibition of PC biosynthesis was also observed after 20 h of incubation (Fig. 4C), indicating that this metabolic effect of HePC persists over a longer period of time. Consistent with the experiments described above, HePS did not significantly reduce the specific radioactivity of PC as compared with the dimethyl sulfoxide control.

Hexadecylphosphocholine Inhibits Sphingomyelin Biosynthesis and Increases Cellular Ceramide—SM is synthesized by the transfer of a phosphocholine head group from PC to ceramide, a reaction catalyzed by the enzyme phosphatidylcholine:ceramide phosphocholine transferase. Although the exact mechanism of the enzyme reaction remains unclear, more than 95% of overall SM synthesis is attributed to this pathway (31). Due to this precursor-product relationship, biosynthesis of SM might be influenced by inhibition of PC biosynthesis. First of all, we measured the time-dependent incorporation of [methyl-³H]choline into SM and found that the percentage of label in SM increased with incubation time (after 4 h of incubation only 1.5% of total radioactivity were SM-bound, whereas after 20 h of incubation SM-bound radioactivity reached 5.7% of total radioactivity), indicating that the choline head group originates from PC. This time course was also observed when the cells were treated with HePC; whereas HePC had already inhibited PC biosynthesis after 4 h, incorporation of [methyl-³H]choline into SM was not significantly influenced after 4 h (data not shown). However, incubation of HaCaT cells with 25 μmol/liter HePC for 20 h decreased the specific radioactivity of SM by 52% (Fig. 5A), whereas the control lipid HePS was inactive. From the specific radioactivities of PC and the radioactive counts in SM the actual synthesis of SM was calculated, and we found that 1.38 nmol of SM/10⁶ cells were formed in control cells after 20 h of incubation and 0.81 nmol of SM/10⁶ cells in HePC-treated cells (59% of control).

In a second approach, we metabolically labeled the lipid backbone of sphingolipids using radiolabeled serine. In accordance with the choline-labeling experiments, incorporation of L-[³H]serine into SM was inhibited in the presence 25 μmol/liter HePC after 20 h of incubation by 35% (Fig. 5B). The decrease of label in SM after HePC treatment was accompanied by a significant increase of L-[³H]serine incorporation into ceramide of 72% (Fig. 5C). Both HePC-induced effects on sphingolipid metabolism were not observed when the cells were treated with the control lipid HePS. However, the HePC-induced increase of ceramide was confirmed by direct enzymatic assay using sn-1,2-diacylglycerol kinase, and it was found that 25 μmol/liter HePC increased cellular ceramide by 53% (5.2 ± 0.4 pmol of ceramide/nmol of lipid phosphate in HePC-treated and 3.4 ± 0.3 pmol of ceramide/nmol of lipid phosphate in control cells).

The Apoptotic Effect of Hexadecylphosphocholine Is Enhanced by C₂- ceramide and Inhibited by Fumonisin B₁.—In the last few years it has become obvious that increasing cellular ceramide leads to the induction of apoptosis in different cell types (19, 28, 32). Thus, we tested the ability of exogenously added C₂-ceramide to induce apoptosis in HaCaT cells. The results clearly showed that treatment of HaCaT cells with C₂-ceramide concentration-dependently induced apoptosis (Table I). In the following experiments, we used the submaximal but readily effective C₂-ceramide concentration of 15 μmol/liter together with different concentrations of HePC. As shown in Fig. 6, the apoptotic effect of HePC was enhanced by C₂-ceramide at HePC concentrations of 2.5 and 7.5 μmol/liter, whereas at the maximal HePC concentration of 25 μmol/liter no additional effect was observed. On the other hand, when HaCaT cells were simultaneously treated with 25 μmol/liter HePC and 20 or 50 μmol/liter fumonisin B₁, HePC-induced apoptosis was totally abolished (Fig. 7). Together with the finding that 20 μmol/liter fumonisin B₁ was sufficient to suppress ceramide synthesis from L-[³H]serine by 74 ± 10% (n = 3) as compared with controls, the antagonistic action of fumonisin B₁ supports the hypothesis that ceramide plays a crucial role in HePC-induced apoptosis.
DISCUSSION

The cellular targets of the anticancer drug HePC have been intensively investigated in several studies and different modes of action, such as inhibition of protein kinase C (3, 33, 34) or CTP:phosphocholine cytidylyltransferase (8) and modulation of the enzyme activities of phospholipase C (34), phospholipase D (35), or acyltransferase (36), have been proposed. Protein kinase C seemed to be the most promising of these targets because this enzyme plays a key role in various growth factor-signaling pathways and is involved in the regulation of cellular proliferation (37, 38). However, using cell lines with different susceptibilities toward phospholipid analogs, it was demonstrated that inhibition of protein kinase C did not correlate with the antiproliferative effect of HePC (39). Furthermore, some doubts about the inhibitory effects of choline-containing phospholipid analogs on protein kinase C were raised by Hesbeen et al. (40), who reported an activation of this enzyme in the presence of 1-O-octadecyl-2-O-methylglycerol-3-phosphocholine. The hypothesis that HePC-induced effects are mediated by protein kinase C seemed to be more and more unlikely. On the other hand, we and others have clearly shown that inhibition of PC biosynthesis at the level of the rate-limiting enzyme, CTP:phosphocholine cytidylyltransferase, is consistently observed in different cell lines and correlates with the antiproliferative effects of choline-containing lipid analogs (11–15). Two further hints that PC biosynthesis might be a main target of these compounds came from the observations that (i) restoring PC synthesis by supplementation with lyso-PC overcame the cytotoxic effect of 1-O-octadecyl-2-O-methylglycerol-3-phosphocholine (16), and (ii) phospholipid analog-resistant leukemic cell lines express a mutant form of CTP:phosphocholine cytidylyltransferase (41). This mutation occurs in the lipid binding domain and influences the lipid modulation of cytidylyltransferase activity. Although these data were quite convincing and suggested that membrane phospholipid content may be monitored by the mammalian cell cycle apparatus, the direct consequences of the inhibition of PC biosynthesis still remained to be elucidated. Because 95% of the choline head groups of newly formed SM originate from PC, one possible mechanism providing a link between the inhibition of PC biosynthesis and cell death was the HePC-induced inhibition of SM biosynthesis which should result in increased ceramide levels. Using two different experimental procedures to label the polar head group and the lipid backbone of SM, it was demonstrated for the first time that HePC inhibits SM biosynthesis.

![FIG. 4. Inhibition of cell proliferation and phosphatidylcholine biosynthesis by HePC. Subconfluent HaCaT cells were incubated with different concentrations of HePC and HePS as control. Proliferation was determined after 24 h by crystal violet staining (A) and the absorbance at 570 nm ± S.D. of the samples is given (n = 4). Additionally, PC biosynthesis of confluent HaCaT cells was measured as described under “Experimental Procedures” after 4 h (B) and 20 h (C) of incubation in the presence of different concentrations of HePC and HePS. Values of incorporation of [methyl-3H]choline into PC are given as disintegrations/min/µg of PC ± S.D. (n = 3). ***Significantly different from vehicle-treated controls at p < 0.01.

![FIG. 5. Inhibition of sphingomyelin formation and elevated ceramide levels induced by HePC. Confluent HaCaT cells were incubated with different concentrations of HePC and HePS as control and the incorporation of [methyl-3H]choline (A) or L-[3H]serine (B) into SM was determined after 20 h. Additionally, incorporation of L-[3H]serine into ceramide was measured after 20 h (C). For the choline-labeling experiments, values are given as disintegrations/min/µg of SM ± S.D. (n = 3). For the serine-labeling experiments, values are given as percent of control ± S.D. (n = 3). In control samples, SM was 22.6% of total radioactivity and ceramide was 7.5% of total radioactivity. Total radioactivity was not significantly influenced by treatment with HePC and HePS and normally reached 26,500 dpm/10⁶ cells. ***Significantly different from vehicle-treated controls at p < 0.01.]}
Ceramide Mediates Hexadecylphosphocholine-induced Apoptosis

Confluent HaCaT cells were incubated with different concentrations of C\textsubscript{16}-ceramide for 20 h. Then, cytosolic nucleosomes were determined as described under “Experimental Procedures,” and the absorbance was measured at 405 nm. Absorbance of control cells was set as 100%, and values are given as percent of control ± S.D. (n = 4).

| Concentration of C\textsubscript{16}-ceramide | Cytosolic nucleosomes |
|---------------------------------------------|-----------------------|
| μmol/liter                                  | % of control          |
| 0                                          | 100 ± 8               |
| 6                                          | 100 ± 7               |
| 15                                         | 140 ± 17              |
| 30                                         | 191 ± 5               |
| 60                                         | 370 ± 23              |
| 120                                        | 238 ± 13              |

Fig. 6. Additive effect of HePC and C\textsubscript{2}-ceramide on apoptosis. Confluent HaCaT cells were incubated with different concentrations of HePC in the presence or absence of 15 μmol/liter C\textsubscript{2}-ceramide for 20 h. Then, cytosolic nucleosomes were determined as described under “Experimental Procedures,” and the absorbance at 405 nm ± SD of the samples is given (n = 4). **Significantly different from HePC-treated control at p < 0.02.

This metabolic effect of HePC led to the accumulation of ceramide, which is a known inducer of apoptosis (19). In this context, elevation of ceramide as a possible mechanism for the antiproliferative activity of different drugs has also been observed after treatment of cells with retinoic acid (42) and daunorubicin (43). Interestingly, in both cases elevated ceramide levels did not arise from SM breakdown via sphingomyelinsase but were due to enhanced ceramide synthesis. Besides its effect on cellular ceramide HePC also induced a slight increase of diacylglycerol (141% of control), which is in accordance with an earlier study (8). Since the activation state of a cell might be conversely influenced by different lipid second messengers (44), it is likely that the induction of apoptosis by HePC depends on the ratio of ceramide versus diacylglycerol. This might explain that a critical concentration of HePC is necessary to induce apoptosis.

In principle, two forms of eukaryotic cell death have been described: (i) apoptosis, or programme cell death, which is a highly regulated process for elimination of unwanted or damaged cells from multicellular compartments, and (ii) necrosis, which occurs by accidental destruction (for review, see Thompson (45)). Necrotic cell death is observed when a cell is severely injured, for example by a harsh physical insult, the cell begins to swell and the plasma membrane ruptures. In contrast, the plasma membrane remains intact and the chromatin frequently breaks into fragments when cells are driven into apoptosis. HePC and other phospholipid analogs are membrane-active compounds which are rapidly internalized by cells (46, 47), and the lytic properties of these substances should be taken into account. However, we demonstrated that HePC inhibited PC and SM biosynthesis and induced apoptosis at nonlytic concentrations ≥25 μmol/liter. In agreement with other studies on the cytotoxic effects of phospholipid analogs (16, 27, 48), HePC-treated HaCaT cells died by apoptosis and unspecific destruction of the plasma membrane seemed to play a minor role, at least at low concentrations. This was confirmed by the use of HePS, a biologically inactive phospholipid analog with a serine head group which is also internalized by cells (11), and it was shown that treatment of cells with HePS did not significantly interfere with PC and SM biosynthesis or the apoptotic program. In additional experiments, the proapoptotic effect of HePC was further characterized and the hypothesis of a ceramide-mediated mechanism was substantiated: (i) the apoptotic effect of HePC was enhanced by simultaneous treatment with C\textsubscript{2}-ceramide and (ii) treatment of cells with fumonisin B\textsubscript{1}, an inhibitor of ceramide synthesis (20), totally blocked HePC-induced apoptosis.

In summary, strong experimental evidence is provided that the antineoplastic phospholipid analog, HePC, induces apoptosis and that ceramide may be one intracellular mediator connecting the inhibition of PC biosynthesis at the level of CTP:phosphocholine cytidylyltransferase with the apoptosis program. The role of SM biosynthesis in this process might lead to the development of new, antiproliferative lipid analogs directly interfering with this metabolic pathway.

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