Two new sphingomyelin analogues inhibit phosphatidylcholine biosynthesis by decreasing membrane-bound CTP: phosphocholine cytidyltransferase levels in HaCaT cells

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The effects of two newly synthesized sphingomyelin analogues on phosphatidylcholine biosynthesis were investigated in the immortalized human keratinocyte cell line HaCaT. *N*-Acetyl-erythro-sphingosine-1-phosphocholine (AcSM) and *N*-octanoyl-erythro-sphingosine-1-phosphocholine (OcSM) inhibited the incorporation of choline into phosphatidylcholine with half-inhibitory concentrations (IC_{50}) of 6 μ g/ml and 10 μ g/ml respectively. Further experiments revealed that AcSM and OcSM interfered with the translocation of the rate-limiting enzyme of phosphatidylcholine biosynthesis, CTP:phosphocholine cytidyltransferase (EC 2.7.7.15), in HaCaT cells and

inhibited cytidyltransferase activity *in vitro*. Despite the fact that OcSM was a potent inhibitor of cytidyltransferase *in vitro*, its effects on phosphatidylcholine biosynthesis and translocation of cytidyltransferase in HaCaT cells were less pronounced as compared with AcSM. Finally, we showed that the comparatively strong effects of AcSM in cell culture experiments were due to the uptake of large amounts of this sphingomyelin analogue into the cells. The results presented demonstrate that the activity of cytidyltransferase may be negatively regulated by a high ratio of choline head group-containing sphingolipids.

INTRODUCTION

CTP:phosphocholine cytidyltransferase (CT; EC 2.7.7.15) is the rate-limiting enzyme in the biosynthesis of phosphatidylcholine (PtdCho) in higher eukaryotes [1,2]. Its activity is

regulated by translocation of the enzyme between the cytosol and the membranes [3]. The translocation process has been shown to be regulated by various lipids and it is generally accepted that the cytosolic, lipid-free form of CT is inactive, whereas the membrane-bound form is active. The data on the activation of CT by naturally occurring lipids *in vivo* and *in vitro* suggest that the process of CT-membrane interaction depends on both the surface charge of the membranes and the lipid packing of these membranes [4,5].

In 1993, Jamil, Hatch and Vance summarized the results about lipid effects on CT activation and postulated that the binding of CT to membranes is modulated by the ratio of bilayer-forming lipids to non-bilayer-forming lipids [6]. According to this hypothesis, non-bilayer-forming lipids increase membrane binding of CT, whereas bilayer-forming lipids have the opposite effect. However, the hypothesis did not fit with earlier results from our group showing that the synthetic, non-bilayer-forming phospholipid, hexadecylphosphocholine (HePC), inhibits PtdCho biosynthesis by decreasing the membrane-binding of CT [7,8]. To investigate these contradictory results in more detail, we systematically measured the effect of alkylphospholipids on PtdCho biosynthesis in Madin–Darby canine kidney cells by using analogues which differ from HePC in their alkyl chain length or in their polar head group. From the results obtained, we suggested that the binding of CT to membranes is modulated by the ratio of choline head group-containing lipids to non-choline head group-containing lipids. Choline head group-containing lipids decrease membrane binding of CT, whereas non-choline head group-containing lipids increase its binding [9]. This mechanism would be consistent with the feedback regulation of CT translocation by PtdCho which has been described in hepatocytes [10].

Figure 1 Chemical structures of SM and SM analogues

Abbreviations used: AcSM, *N*-acetyl-erythro-sphingosine-1-phosphocholine; BCA, biconchonic acid; CT, CTP:phosphocholine cytidyltransferase; HaCaT, human keratinocyte cell line; HePC, hexadecylphosphocholine; IC_{50} , half-inhibitory concentration; KGM, keratinocyte growth medium; OcSM, *N*-octanoyl-erythro-sphingosine-1-phosphocholine; PAF, platelet-activating factor; PtdCho, phosphatidylcholine; SM, sphingomyelin.

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Sphingomyelin (SM) is another choline-containing lipid which is found ubiquitously in eukaryotic cell membranes. Recently, it has been shown that SM participates in cellular signalling pathways (for a review, see [11]). However, a possible interplay between the SM content of cells and the translocation of CT has not been studied yet.

In the present study, we synthesized two short-chain SM analogues (see Figure 1) which could be easily used to modify the membrane composition of cultured cells. By investigating the effect of *N*-acetyl-erythro-sphingosine-1-phosphocholine (AcSM) and *N*-octanoyl-erythro-sphingosine-1-phosphocholine (OcSM) on PtdCho biosynthesis, we could show that both analogues inhibited the incorporation of [*methyl*-³H]choline into PtdCho by decreasing the membrane-bound form of CT. Measurements of CT activity *in vitro* revealed that the analogues directly inhibited the activation of this enzyme by oleate-containing liposomes. Whereas the effects of AcSM in cell culture experiments were more pronounced compared with OcSM, OcSM was the more effective inhibitor of CT activity *in vitro*. These findings could be explained by differences in the cellular uptake of the analogues.

EXPERIMENTAL

Materials

[*Methyl*-³H]Choline chloride (2.8–3.1 TBq/mmol) and [*methyl*-¹⁴C]phosphocholine (2.04 GBq/mmol) were obtained from Amersham (Braunschweig, Germany). Silica gel 60 high-performance TLC plates, silica gel 60 (70–230 mesh), solvents and reagents (reagent grade) were purchased from E. Merck (Darmstadt, Germany) and Fluka (Neu-Ulm, Germany). The bicinchoninic acid (BCA)-kit for protein determination was obtained from Pierce (Weiskirchen, Germany). PtdCho, oleic acid, choline chloride, phosphocholine, CDP-choline and octylglucopyranoside were from Sigma (München, Germany). Hydroxyapatite was from Bio-Rad (München, Germany). For quantification of radioactivity a Berthold LB 2821 HR TLC scanner (Berthold; Wildbad, Germany) was used.

Synthesis of SM analogues

The starting material, human brain SM, was isolated according to Klenk and Huang [12]. It was then deacylated to obtain sphingosine-1-phosphocholine by the method of Kaller [13].

AcSM and OcSM were synthesized from sphingosine-1-phosphocholine by dissolving the latter in ethanol and then adding three times the stoichiometric amount of acetic anhydride or octanoic anhydride. The reaction was completed at room temperature overnight. Ethanol was removed in a rotary evaporator and the residue was extracted several times with dry acetone to remove acetic acid or octanoic acid. The remaining raw product was purified by a silicic acid column (silica gel 60, 70–230 mesh). In the procedure, a succession of eluting solvents used in the order chloroform, chloroform/methanol (4:1, v/v) and chloroform/methanol (1:4, v/v) removed all the contaminants. AcSM or OcSM was obtained from the column by eluting with methanol/aq. 25% NH₃ (4:1, v/v). Fractions were collected and purity was monitored by TLC, using the solvent system chloroform/methanol/water (65:35:8, by vol.).

Cell culture

The spontaneously immortalized human keratinocyte cell line HaCaT [14] was a gift from Dr. N. E. Fusenig (DKFZ, Heidelberg, Germany). Cells were grown in plastic culture dishes

(Nunc; Wiesbaden, Germany) containing RPMI medium supplemented with 10% (v/v) calf serum, 0.56 g/l L-glutamine, 100 000 units of penicillin and 0.1 g/l streptomycin. Media and culture reagents were obtained from Gibco (Berlin, Germany), penicillin and streptomycin from Boehringer (Mannheim, Germany). Confluent cells were subcultured every 3 days after detaching the cells with a 0.1% trypsin/0.02% EDTA solution. For experimental purposes, cells were used between passages 32 and 45. For the duration of the experiments, HaCaT cells were maintained in serum-free keratinocyte basal medium (Clonetics; San Diego, CA, U.S.A.), supplemented with 10 ng/ml epidermal growth factor, 5 µg/ml insulin, 0.5 µM hydrocortisone, 50 µg/ml bovine pituitary extract, 100 µg/ml penicillin/streptomycin and 2.5 µg/ml fungizone (all purchased from Clonetics). AcSM and OcSM were diluted into keratinocyte growth medium (KGM) as described above from 20 mg/ml stock ethanol solutions. Ethanol (vehicle) was added to controls and was present at 0.25% or less in the experiments.

Cytotoxicity assays

Cytotoxicity of the different analogues to HaCaT cells was measured by the Trypan Blue exclusion assay. After incubation of confluent cells for 6 h with different concentrations of the respective SM analogue, the media were removed and the cells were washed with PBS. Then a Trypan Blue solution (0.1% in PBS) was added and blue (dead) and unstained (living) cells were counted under a Diavert microscope (Leitz; Wetzlar, Germany). At least 200 cells were counted per experiment.

Additionally, cytotoxicity of the different analogues to HaCaT cells was determined by measurement of the alkaline phosphatase activity of the cells as described elsewhere [9].

Uptake assay

HaCaT cells were grown to confluence. Medium was removed and 1×10^6 cells were incubated for 0.5 h, 1 h, 2 h and 3 h with 2 ml of KGM containing 5 µg/ml of AcSM and OcSM. After incubation, cells were washed three times with ice-cold PBS, harvested in 400 µl of 20 mM ammonium acetate, pH 7.0, and freeze-dried. Lipids were extracted using 60 µl of chloroform/methanol (1:2, v/v) and separated by high-performance TLC as described below. Lipids were stained with cupric sulphate reagent (156 g/l in 8.5% phosphoric acid) and quantified by video-densitometry. Staining was linear in the range 0.2 to 2 µg of lipid per band.

Measurement of PtdCho biosynthesis

Incorporation of [*methyl*-³H]choline into PtdCho was measured essentially as described previously [8]. Confluent HaCaT cells were incubated with pulse-medium (KGM containing 7.4×10^4 Bq/ml [*methyl*-³H]choline) supplemented with different amounts of AcSM and OcSM. After incubation, cells were washed twice with ice-cold PBS (pH 7.2) and harvested using a cell lifter (Costar; Cambridge, MA, U.S.A.) followed by a modified lipid extraction as described by Bligh and Dyer [15]. After freeze-drying of the cells, 50 µl of methanol, 25 µl of chloroform and 20 µl of water were added. Samples were stirred for 2 min on a vortex-mixer and centrifuged at 13 000 g for 10 min. Phase separation was accomplished by addition of 25 µl of chloroform and 25 µl of water. The suspension and centrifugation steps were repeated. Then 10 µl aliquots of the chloroform phases were taken for scintillation counting and 15 µl samples were applied to silica gel 60 high-performance TLC plates using a high-performance TLC applicator (Camag; Berlin, Germany). Lipids

were separated as described elsewhere [16], using the solvent chloroform/methanol/triethylamine/water (30:35:34:8, by vol.). Radioactivity was quantified by radioscanning. Phospholipids were identified by calibrating the scanner with known standards. Approximately 95% of the radioactivity co-chromatographed with the PtdCho standard.

Permeabilization of cells by digitonin

Confluent HaCaT cells were incubated for 3 h in KGM containing 5 µg/ml AcSM, 5 µg/ml OcSM and 0.025% ethanol as a control. Cells were then washed twice with ice-cold PBS, and all the following steps were carried out at 4 °C. Aliquots (300 µl) of buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 0.025% NaN₃, 1 mM PMSF and 0.5 mg/ml digitonin were added to the cells. After 3 min the digitonin supernatant was removed. Undissolved digitonin was pelleted at 13000 *g* for 2 min and the remaining cell ghosts were washed with 1 ml of PBS, harvested in 50 µl of buffer as described above (the harvesting buffer contained 0.03% Triton X-100 and no digitonin) and then homogenized using a Dounce homogenizer. CT activity was measured in the digitonin supernatants and in the cell ghost homogenates as described below. From the data, the ratio of cytosolic to membrane-bound CT activity was calculated for each dish.

Purification of rat liver CT

CT was partially purified from rat liver cytosol as described [17] using acetic acid precipitation and octylglucoside extraction followed by hydroxyapatite column chromatography. Purified CT was stored at -70 °C.

CT activity assay

CT activity was measured by a modified method of Sohal and Cornell [18]. The reaction mixture contained 50 mM Tris/HCl, pH 7.4, 0.03% Triton X-100, 100 mM NaCl, 10 mM MgCl₂, 3 mM CTP, 1.5 mM [*methyl*-¹⁴C]phosphocholine (specific radioactivity 20 Bq/nmol), liposomes (100 µM PtdCho/100 µM oleic acid or 400 µM PtdCho/400 µM oleic acid) and 10 µl of enzyme preparation in a final volume of 55 µl. After incubating for 30 min at 37 °C the reaction was stopped by freezing the samples in liquid nitrogen and CDP-choline was analysed by high-performance TLC as described [19]. One unit of enzyme activity is defined as 1 nmol of CDP-choline formed per min.

Other procedures

Protein was determined by the BCA assay [20], with BSA as standard. Liposomes were prepared as described [18] by sonication for 3 min at 4 °C using a 375 W sonicator (Heat Systems; Plainview, NY, U.S.A.) at 20% power output. All solvents and vehicles (ethanol) were evaporated prior to sonication. Statistical comparisons were made in these studies with Student's *t*-test.

RESULTS

Chemical synthesis of SM analogues

The truncated SM analogues, AcSM and OcSM, were synthesized by a two-step procedure from human brain SM. The yields after column chromatography were usually 30%.

The products were characterized by MS, ¹H-NMR and high-performance TLC.

The MS of AcSM and OcSM revealed molecular masses of 507 [*M* + H]⁺ and 591 [*M* + H]⁺ respectively.

Figure 2 High-performance TLC of SM analogues

Different amounts of AcSM and OcSM were applied to silica gel 60 high-performance TLC plates. Lipids were separated using the solvent system chloroform/methanol/triethylamine/water (30:35:34:8, by vol.) and subsequently stained with cupric sulphate reagent. The relative mobility of standard SM is indicated by an arrowhead.

Employing ¹H-NMR, the following chemical shifts in parts per million using tetramethylsilane as internal standard were detected: δ (p.p.m.) 0.85 (3 H, t, 1-CH₃); 1.3 (24 H, m, [2-13]-CH₂); 2.0 (4 H, m, 20-CH₃ and O-H); 3.3 [9 H, s, N-(CH₃)₃]; 5.4 (1 H, q, 14-CH); 5.7 (1 H, m, 15-CH); 7.2 (1 H, d, N-H).

The purity of AcSM and OcSM was checked by high-performance TLC, using the solvent systems chloroform/methanol/water (65:35:8, by vol.) and chloroform/methanol/triethylamine/water (30:35:34:8, by vol.). Figure 2 shows that both analogues had a purity of more than 95%. Furthermore, naturally occurring SM was not found in the samples of the purified SM analogues.

Effect of SM analogues on the viability and the alkaline phosphatase activity of HaCaT cells

The cytotoxic effects of AcSM and OcSM were investigated in confluent HaCaT cells by Trypan Blue exclusion and measurement of the alkaline phosphatase activity of the cells. When cytotoxicity was determined by Trypan Blue exclusion a dose-dependent decrease of the viability of HaCaT cells occurred for both substances, which might be due to lytic effects (Figure 3a). At concentrations of 5 µg/ml or less, AcSM and OcSM did not show any cytotoxicity in this assay (viability ≥ 95%). These results were confirmed by measurement of the alkaline phosphatase activity of the cells. At concentrations of 10 µg/ml or less, AcSM and OcSM did not significantly reduce the alkaline phosphatase activity of the cells (Figure 3b). On the other hand, incubation of cells with 50 µg/ml of the analogues decreased the alkaline phosphatase activity by approximately 60%, thereby demonstrating their unspecific, cytotoxic effects at concentrations above 10 µg/ml.

Dose- and time-dependent inhibition of PtdCho biosynthesis by SM analogues

It has been demonstrated that the *de novo* synthesis of PtdCho is influenced by different lipids, such as fatty acids, ether-lipids and alkylphosphocholines (for a review, see [3]). We therefore investigated the effects of AcSM and OcSM on the incorporation of [*methyl*-³H]choline into PtdCho in HaCaT cells. After 6 h of incubation, AcSM dose-dependently inhibited the incorporation

Figure 3 Concentration-dependent effects of SM analogues on the viability and alkaline phosphatase activity of HaCaT cells

Confluent HaCaT cells were incubated with different concentrations of AcSM (○) and OcSM (△). After 6 h of incubation, viability of the cells was measured using a Trypan Blue exclusion assay (a). Values are given as percentages of living cells in the absence of SM analogues (means ± S.D., $n = 3$). Additionally, the alkaline phosphatase activity of the cells was determined (b) after 6 h of incubation with different concentrations of AcSM (○) and OcSM (△). Values are given as percentages of alkaline phosphatase activity in the absence of SM analogues (means ± S.D., $n = 4$).

of [*methyl*-³H]choline into PtdCho with a half-inhibitory concentration (IC_{50}) of 6 µg/ml (Figure 4). OcSM was slightly less effective with an IC_{50} of 10 µg/ml. However, both SM analogues inhibited PtdCho biosynthesis at non-toxic concentrations of 1 µg/ml and 5 µg/ml (Figure 4).

In order to avoid cytotoxic side-effects, the following experi-

Figure 4 Concentration-dependent effects of SM analogues on choline incorporation into PtdCho in HaCaT cells

Confluent HaCaT cells were incubated in KGM containing 7.4×10^4 Bq/ml [*methyl*-³H]choline supplemented with different concentrations of AcSM (○) and OcSM (△). After 6 h of incubation, the cells were mechanically harvested and PtdCho was analysed as described in the Experimental section. Values are given as percentages of incorporated radioactivity in the absence of SM analogues (means ± S.D., $n = 3$). Incorporated radioactivity in control experiments was 142 000 d.p.m./mg of cellular protein.

Figure 5 Effects of SM analogues on the time-dependent incorporation of choline into PtdCho in HaCaT cells

Confluent HaCaT cells were incubated in KGM containing 7.4×10^4 Bq/ml [*methyl*-³H]choline supplemented with 5 µg/ml AcSM (○, a), 5 µg/ml OcSM (△, b) and 0.025% ethanol (●, a and b) as control. After different incubation times, as indicated in the Figure, the cells were mechanically harvested and PtdCho was analysed as described in the Experimental section. The values of incorporated radioactivity are given in d.p.m./mg of cellular protein (means ± S.D., $n = 3$).

ments were carried out using non-toxic concentrations of each analogue. When cells were treated with 5 µg/ml AcSM for different time periods an inhibition of the incorporation of [*methyl*-³H]choline into PtdCho was clearly observed after 1 h of incubation (Figure 5a). In contrast, OcSM seemed to exert its

Table 1 Effects of SM analogues on the distribution of CT in HaCaT cells

The translocation of CT in HaCaT cells was measured by digitonin-mediated release of cytosolic CT. Cells were incubated for 3 h in KGM containing 5 $\mu\text{g/ml}$ AcSM, 5 $\mu\text{g/ml}$ OcSM and 0.025% ethanol as control. After incubation, CT activity was measured in the digitonin-treated supernatants released after 3 min and in the cell ghost homogenates as described in the Experimental section. Liposomes (400 μM PtdCho/400 μM oleic acid) were used to fully activate CT. At this concentration of activating lipids AcSM and OcSM did not interfere with the assay *in vitro*. From the data, the distribution (%) of cytosolic and membrane-bound CT activity was calculated for each dish ($n = 4$; *significantly different from controls at $P < 0.01$).

Treatment	Distribution (%)		Total activity of CT (units per 10^6 cells)
	Cytosol	Membranes	
Control	22.6 $\pm$ 2.9	77.4 $\pm$ 7.3	1.37 $\pm$ 0.14
AcSM (5 $\mu\text{g/ml}$)	50.4 $\pm$ 4.8*	49.6 $\pm$ 4.0*	1.25 $\pm$ 0.11
OcSM (5 $\mu\text{g/ml}$)	28.6 $\pm$ 6.7	71.4 $\pm$ 7.6	1.19 $\pm$ 0.17

effects much slower and a significant inhibition of PtdCho biosynthesis was first seen after 3 h of incubation (Figure 5b).

It is generally accepted that the biosynthesis of PtdCho is mainly regulated by translocation of the rate-limiting enzyme, CT, from cytosol to membranes. By permeabilizing cells with digitonin, it is possible to distinguish between the cytosolic and the membrane-bound form of CT. To examine the effect of AcSM and OcSM on the translocation process, we compared the distribution of CT in control, AcSM- and OcSM-treated cells by measurement of its activity in the digitonin supernatant and in the remaining cell ghosts. Table 1 shows that treatment of HaCaT cells for 3 h with 5 $\mu\text{g/ml}$ AcSM significantly decreased the amount of membrane-bound CT by 41% as compared with controls. On the other hand, a 100% increase of the amount of cytosolic CT was found in AcSM-treated cells. Consequently, the total amount of CT activity was not affected by treatment of HaCaT cells with AcSM. Similar to the choline incorporation experiments, OcSM (5 $\mu\text{g/ml}$) was less effective and decreased the amount of membrane-bound CT by approximately 20% (Table 1). However, this effect of OcSM was not significant.

SM analogues inhibit CT activity *in vitro*

The effects of AcSM and OcSM on translocation of CT in HaCaT cells suggested that inhibition of PtdCho biosynthesis occurs directly at the level of the rate-limiting enzyme of this pathway. When the activity of purified CT in the presence of liposomes containing 100 μM PtdCho, 100 μM oleic acid and different concentrations of AcSM and OcSM was measured both analogues inhibited liposome-activated CT *in vitro* (Figure 6). In contrast to the results in living cells, OcSM was more effective than AcSM in this cell-free assay. Whereas 10 $\mu\text{g/ml}$ OcSM inhibited CT activity by 65%, AcSM at the same concentration reduced CT activity by only 24%. It is interesting to note that the inhibitory effects did not increase when concentrations above 10 $\mu\text{g/ml}$ were added to the liposomes. This observation is in accordance with an earlier study on the inhibition of CT activity by the alkylphosphocholine, HePC [8].

Cellular uptake of SM analogues into HaCaT cells

It is obvious that the effects of the SM analogues were disparate in intact cells and in cell-free assays. Whereas AcSM was more effective in cell culture experiments, OcSM was the more potent inhibitor of CT activity *in vitro*. One reason for this disparity might be a greater cellular uptake of AcSM compared with

Figure 6 Effects of SM analogues on CT activity *in vitro*

CT was purified from rat liver and CT activity was measured in the presence of liposomes containing 100 μM PtdCho, 100 μM oleic acid and different concentrations of AcSM ($\circ$) and OcSM ($\triangle$). After 30 min the reaction was stopped and the formation of CDP-choline was analysed as described in the Experimental section. Values are given as percentages of activity in the absence of SM analogues (mean $\pm$ S.D., $n = 3$). Activity in control experiments was 40.4 units/ml.

Figure 7 Time-dependent uptake of SM analogues into HaCaT cells

Confluent HaCaT cells were incubated in KGM containing 5 $\mu\text{g/ml}$ AcSM and 5 $\mu\text{g/ml}$ OcSM. After different incubation times, as indicated in the Figure, cells were mechanically harvested and the uptake of AcSM ($\circ$) and OcSM ($\triangle$) were determined as described in the Experimental section. Values of uptake are given in $\mu\text{g}/10^6$ cells. Each point represents the mean of two determinations with an error less than 10%. The experiment was repeated and similar results were obtained.

OcSM. Indeed, after 3 h of incubation with KGM containing 5 $\mu\text{g/ml}$ AcSM, the uptake was $2.8 \pm 0.7 \mu\text{g}/10^6$ cells, thereby exceeding by about 3-fold the uptake of OcSM which was $1.0 \pm 0.5 \mu\text{g}/10^6$ cells ($n = 3$). Furthermore, Figure 7 illustrates

that the uptake of AcSM into HaCaT cells was faster compared with OcSM and did not reach a plateau after 3 h of incubation. In contrast, the uptake of OcSM reached a plateau after 3 h of incubation.

DISCUSSION

Synthetic phospholipids, such as alkylphosphocholines and alkyl lysophospholipids, comprise a relatively new class of agents which have been shown to possess anti-neoplastic properties *in vitro* and *in vivo* [21,22]. Since the structural features of these synthetic lipids, namely their choline head group bound to a hydrophobic alkyl side chain, very much resemble the chemical structures of lyso-PtdCho and platelet-activating factor (PAF) [23] it was tempting to speculate whether they would have an effect on PtdCho biosynthesis. Indeed, it was shown that the alkylphosphocholine HePC inhibits PtdCho biosynthesis by decreasing the membrane-bound CT level [8]. Further studies revealed that inhibition of PtdCho biosynthesis by synthetic phospholipids depends on the nature of the polar head group and it was shown that a choline head group is necessary for the inhibitory effect [9].

Among the lipid families found in eukaryotic cell membranes there are two groups which contain choline head groups: (i) glycerophospholipids, such as PtdCho and PAF, and (ii) SM. Recently, sphingolipids have emerged as another class of signal-transducing lipids [24] and a cycle for SM has been described [25] paralleling the well known phosphatidylinositol cycle [26] and PtdCho cycle [27]. Whereas SM does not seem to have signalling capacity by itself, sphingolipid breakdown products, such as ceramide and sphingosine, exert a variety of cellular effects finally leading to inhibition of cell growth or apoptosis [11]. In the present study, we synthesized and characterized two short-chain SM analogues with an *N*-acetyl- and an *N*-octanoyl-side chain (AcSM and OcSM respectively). In contrast to other sphingolipids, e.g. *N*-acetylsphingosine and sphingosylphosphocholine which have been used by Hudson et al. [28], AcSM and OcSM still possess all the features of naturally occurring SM. However, the truncated *N*-acyl side chains render the SM analogues cell-permeable. AcSM and OcSM should therefore be useful tools to investigate the influence of choline head group-containing sphingolipids on PtdCho biosynthesis in cell culture experiments. Using the human keratinocyte cell line HaCaT, we could show that both SM analogues inhibited the incorporation of [*methyl*-³H]choline into PtdCho at non-toxic concentrations. The IC₅₀ values of AcSM and OcSM were 6 µg/ml and 10 µg/ml respectively. Due to differences in the cellular uptake of the analogues, inhibition of PtdCho biosynthesis by AcSM occurred as early as 1 h after addition to the culture medium, whereas inhibition by OcSM was first observed after 3 h of incubation. In accordance with earlier studies on the uptake of HePC into Madin-Darby canine kidney cells [29] we could show that the uptake of AcSM and OcSM into HaCaT cells seems to be a prerequisite for their inhibitory effects in cell culture experiments. These results further substantiate the hypothesis that synthetic phospholipids are membrane-active substances [30].

CT is the rate-limiting enzyme of PtdCho biosynthesis and the membrane composition of cells has been shown to be crucial for regulation of enzyme activity by translocation. Fatty acids [31] and diacylglycerol [32] stimulate translocation of CT to membranes. Conversely, choline head group-containing ether-lipids [33] and HePC [8] decrease membrane-bound CT. In the studies presented here, incubation of HaCaT cells with AcSM and OcSM led to decreased incorporation of [*methyl*-³H]choline into PtdCho, which was accompanied by a slight increase of in-

corporation of radioactivity into the phosphocholine pool and a decrease of incorporation of radioactivity into the CDP-choline pool (results not shown), suggesting that CT is a target of inhibition. This was examined in more detail using two experimental approaches: (i) the digitonin-mediated release of cytosolic CT, and (ii) the measurement of liposome-activated CT *in vitro*. In both systems, AcSM and OcSM inhibited translocation of CT. Interestingly, OcSM was an effective inhibitor of CT activity *in vitro*. In contrast, the distribution of CT was only slightly affected by treatment of cells with 5 µg/ml OcSM for 3 h, thereby emphasizing the role of the cellular uptake of OcSM for its effectiveness in whole cells. In this context, an inhibition of CT activity *in vitro* has also been described for sphingosine [18]. However, sphingosine has never been shown to interfere with PtdCho biosynthesis in living cells.

It has been reported that CT is a nuclear protein which translocates to membranes of the nuclear envelope in different cell lines [34,35]. However, immunofluorescence studies did not reveal an intranuclear localization for CT in HaCaT cells (results not shown). Therefore, the possibility that AcSM and OcSM interfere with the nuclear localization of CT in our experimental system is unlikely.

In conclusion, the inhibition of PtdCho biosynthesis and translocation of CT by two synthetic SM analogues confirms the hypothesis that the translocation process in cultured cells is, in part, regulated by the ratio of choline head group-containing to non-choline head group-containing lipids. Furthermore, SM analogues represent a new class of promising agents which might be used in future studies of the role of PtdCho cycles and SM cycles in cellular proliferation and differentiation.

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