The role of sphingomyelin in phosphatidylcholine metabolism in cultured human fibroblasts from control and sphingomyelin lipidosis patients and in Chinese hamster ovary cells

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Human fibroblasts in culture take up exogenous [*choline-Me-³H,³²P]sphingomyelin (SM) from the medium and incorporate it into cellular SM and phosphatidylcholine [Spence, Clarke & Cook (1983) *J. Biol. Chem.* **258**, 8595–8600]. The ratio of [³H]choline/[³²P]P_i is similar in SM and phosphatidylcholine, indicating that the phosphocholine (*P*-Cho) moiety is transferred intact. Similar results are obtained with Niemann–Pick (NP) cells which are deficient in lysosomal sphingomyelinase activity, suggesting that the *P*-Cho transfer may not be mediated by the lysosomal sphingomyelinase and that alternative pathways of sphingomyelin catabolism are present in cultured cells. In this study we have shown that: (1) the *P*-Cho pool in control and NP cells incubated with exogenous labelled SM has a specific radioactivity intermediate between that of SM and PtdCho; (2) expansion of the intracellular *P*-Cho pool by incubation with exogenous choline reduces the incorporation of [³H]choline from SM into PtdCho; and (3) incorporation of *P*-Cho from SM into PtdCho is decreased at the non-permissive temperature in Chinese hamster ovary cells with a temperature-sensitive mutation in the cytidyltransferase reaction. These results suggest that incorporation of *P*-Cho from SM into PtdCho involves a reaction sequence in which *P*-Cho is hydrolysed from SM by a sphingomyelinase, followed by incorporation of *P*-Cho into PtdCho via the cytidine pathway of biosynthesis (SM → *P*-Cho → CDP-Cho → PtdCho). The appreciable incorporation of *P*-Cho from SM into PtdCho in sphingomyelinase-deficient NP cells suggests a more substantial or effective lysosomal sphingomyelinase activity in intact cells than is measured *in vitro*, and/or a significant contribution by other sphingomyelinase activities in these cells.*

INTRODUCTION

Previous studies from several laboratories have shown that human fibroblasts in culture take up [³H]sphingomyelin (SM) from the medium (Beaudet & Manschrek, 1982; Maziere *et al.*, 1982; Kudoh *et al.*, 1983; Spence *et al.*, 1983). In normal fibroblasts, 50–80% of the cell-associated label is found in phosphatidylcholine (PtdCho). Studies from our laboratory using double-labelled SM ([*choline-Me-³H,³²P]SM) have shown that the phosphocholine (*P*-Cho) head group of sphingomyelin is incorporated intact into PtdCho (Spence *et al.*, 1983). In lysosomal sphingomyelinase-deficient cells from patients with Niemann–Pick (NP) disease, the incorporation of label from SM into PtdCho, although less than in control fibroblasts, has been observed to account for as much as 12–25% of the cell-associated label (Spence *et al.*, 1983; Kudoh *et al.*, 1983). We have suggested (Spence *et al.*, 1983) that appearance of the *P*-Cho moiety of SM in PtdCho in NP cells may relate to the concentration of SM in the medium, since it was not observed in studies in which the exogenous SM concentration was low (Beaudet & Manschrek, 1982; Maziere *et al.*, 1982).*

The first step in the major degradative pathway of SM metabolism in mammalian tissues is the cleavage of the phosphodiester bond to form ceramide and *P*-Cho (Barenholz *et al.*, 1966; Schneider & Kennedy, 1968; Rao & Spence, 1976) by sphingomyelinase (sphingomyelin phosphodiesterase; EC 3.1.4.12). The ability of sphingomyelinase-deficient NP cells to transfer the *P*-Cho moiety of SM to PtdCho suggested

the possibility of SM degradation via additional or alternative pathways.

The present study was undertaken to determine how the *P*-Cho moiety of SM is transferred to diacylglycerol to form ceramide and PtdCho. Our previous studies showing transfer of the complete *P*-Cho portion (Spence *et al.*, 1983) had ruled out a substantial contribution by phospholipase D or base exchange (Kanfer, 1980) and so we considered three other possibilities. First, a reversal of the direct transfer pathway of sphingomyelin biosynthesis may occur. Studies from several laboratories have shown a precursor–product relationship between PtdCho and SM in mammalian cells (Marggraf *et al.*, 1972; Voelker & Kennedy, 1982; Cook *et al.*, 1988), but the reverse pathway has not been demonstrated. Second, there may be a reversal of the CDP-Cho pathway with CMP as an acceptor of *P*-Cho from SM. Such pathways with PtdCho as the *P*-Cho donor have been shown in rat liver and in rabbit brain neuronal nuclei (Sundler & Åkesson, 1975; Baker & Chang, 1984). The third possibility is that SM is hydrolysed by residual sphingomyelinase activity in the lysosomes or by some other unidentified sphingomyelinase, with recycling of the released *P*-Cho to PtdCho via the conventional CTP-dependent biosynthetic pathways (Kennedy & Weiss, 1956). These possibilities are outlined below.

- (1) SM + diacylglycerol → ceramide + PtdCho
- (2) SM + CMP → CDP-Cho + ceramide
CDP-Cho + diacylglycerol → PtdCho + CMP

Abbreviations used: SM, sphingomyelin; PtdCho, phosphatidylcholine; *P*-Cho, phosphocholine; CDP-Cho, CDP-choline; GroPCho, glycerophosphocholine; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdEtn, phosphatidylethanolamine; NP, Niemann–Pick; CHO cells, Chinese hamster ovary cells; MEM, minimal essential medium.

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- (3) $\text{SM} \rightarrow \text{P-Cho} + \text{ceramide}$
 $\text{P-Cho} + \text{CTP} \rightarrow \text{CDP-Cho} + \text{PP}_i$
 $\text{CDP-Cho} + \text{diacylglycerol} \rightarrow \text{PtdCho} + \text{CMP}$

MATERIALS AND METHODS

Materials

[Me-³H]Choline (80 Ci/mmol), [³H]methyl iodide and H₃³²PO₄ were purchased from Dupont Canada Ltd. (New England Nuclear), Lachine, Quebec, Canada. Non-radioactive choline, P-Cho, glycerophosphocholine (GroPCho), CDP-Cho, choline kinase, alkaline phosphatase (bovine intestinal mucosa), snake venom phosphodiesterase (from *Crotalus adamanteus*), ATP, Dowex-1-X8 (400 mesh), sphingomyelin and PtdCho were from Sigma, St. Louis, MO, U.S.A. Solvents (h.p.l.c. grade) and silica G Rediplates were from Fisher Scientific, Dartmouth, NS, Canada. Eagle's minimal essential medium (MEM), Ham's F12 medium and fetal bovine serum were from Gibco, Burlington, ON, Canada.

Control fibroblasts were cultured from biopsies from the forearm of normal volunteers or from human foreskin. Cells from patients with NP disease Type I were supplied by Human Genetic Mutant Cell Repository, Camden, NJ, U.S.A., and by Dr. J. A. Lowden, Hospital for Sick Children, Toronto, ON, Canada. Chinese hamster ovary (CHO) cells (mutant 58) were generously donated by Dr. C. R. H. Raetz, University of Wisconsin, Madison, WI, U.S.A. (Esko & Raetz, 1980; Esko *et al.*, 1981).

Methods

[Me-³H]SM and [Me-³H, ³²P]SM and [Me-³H]PtdCho and [Me-³H, ³²P]PtdCho were prepared from precursor lipids and [³H]methyl iodide, or were prepared biosynthetically (Spence *et al.*, 1983). Fibroblasts were grown in MEM and CHO cells were grown in Ham's F12 medium. All culture media were supplemented with 10% fetal bovine serum and cells were maintained in a humidified atmosphere of air/CO₂ (19:1) at 37 °C.

Lipid substrates were prepared for the cell culture experiments as described previously (Spence *et al.*, 1983). Following incubation with cells, the medium was removed and in some cases analysed further to determine the identity and amounts of lipid-soluble and water-soluble radioactive materials (Spence *et al.*, 1983; Morash *et al.*, 1988). The cells were harvested by scraping into 0.15 M-NaCl (Spence *et al.*, 1983) or 2 ml of methanol/water (5:4, v/v; Morash *et al.*, 1988), and protein was determined by the method of Lowry *et al.* (1951). Following extraction with chloroform/methanol (2:1, v/v) and partition with aqueous solvent mixtures (Folch *et al.*, 1957), the lipids in the lower organic phase were filtered, dried under N₂, and dissolved in chloroform/methanol (2:1, v/v). The lipid mixture was resolved by t.l.c. and radioactivity was determined by scraping the gel into vials followed by liquid scintillation spectrometry, or by using a Bioscan Imaging Scanner System 200-IBM (Bioscan Inc., Washington, DC, U.S.A.). Phospholipid mass was determined by measuring phosphorus after separation by t.l.c. (Morash *et al.*, 1988). Radioactivity and mass of the water-soluble choline-containing compounds in the upper phase were determined by t.l.c., or by a choline kinase assay as described previously (Morash *et al.*, 1988).

RESULTS

The lysosomal sphingomyelinase activity of the control fibroblasts used in these studies ranged between 30 and 160 nmol of SM cleaved/h per mg of cell protein. By contrast, the activity in

Fig. 1. Phospholipid composition of normal and NP fibroblasts cultured in the presence or the absence of exogenous SM

Confluent cell monolayers in 60 mm dishes were cultured in MEM with 10% fetal bovine serum for 48 h in the presence (▨, ▩) or absence (■, □) of 1 mM exogenous SM. Following harvest and extraction of cell lipid, individual phospholipids were resolved and quantified by t.l.c. and phospholipid phosphate measurement. ■, Control; ▨, control plus SM; ▩, NP; ▩, NP plus SM (mean ± s.d., *n* = 3). The following pairs were significantly different at *P* < 0.01: control versus NP for SM and PtdEtn; control versus control plus SM for SM and PtdIns; NP versus NP plus SM for SM. All other pairs were not significantly different (*P* > 0.05).

the NP cells was less than 5% of that in controls cultured under similar conditions. Incubation of control and NP cells with exogenous SM did not appreciably alter the sphingomyelinase activity measured subsequently *in vitro*. As shown in Fig. 1, NP fibroblasts were characterized by a 2-fold elevation in SM, a 35% increase in phosphatidylethanolamine (PtdEtn), and a slight decrease in phosphatidylserine (PtdSer) and phosphatidylinositol (PtdIns) compared with control cells. Consistent elevations in the concentration of SM and variable changes in other phospholipids have been reported in visceral tissues of patients with sphingomyelinase-deficient forms of NP disease (Fredrickson & Sloan, 1972). When both cell types were incubated with exogenous SM (1 mM) for 48 h, there was a significant increase in SM, and this was more marked in the NP cells. In control cells, the increase was balanced in part by a decrease in PtdCho, and although a similar decrease was seen in the NP cells, it did not offset the SM accumulation.

As documented previously (Spence *et al.*, 1983), both control and NP cells took up exogenous SM from the medium, and a portion of the incorporated lipid was converted into other lipids (Fig. 2) and into water-soluble degradation products (Table 1). In the present experiments, double-labelled [choline-Me-³H, ³²P]SM (Figs. 2a and 2b) and [choline-Me-³H, ³²P]PtdCho (Figs. 2c and 2d) were used at a higher specific radioactivity, achieved by decreasing the unlabelled carrier from 1 mM used previously (Spence *et al.*, 1983) to a trace (~1 μM). This was done to increase the specific radioactivity of the metabolic products of the degradation of these lipids and hence provide more reliable ³H/[³²P]P_i ratios (Table 1), but it also resulted in substantially less label appearing in PtdCho (Fig. 2b) in both control and NP cells. PtdCho was also taken up by both cell types (Fig. 2c) and in these experiments uptake was less than for SM. Uptake of both lipids tended to be variable; in other experiments, we have noted similar rates of uptake for both lipids (M. Spence, unpublished work). There was a modest transfer of label from PtdCho to SM (Fig. 2d) which amounted to < 10% of the total label uptake.

Fig. 2. Metabolism of [*choline-Me-³H, ³²P]SM and [*choline-Me-³H, ³²P]PtdCho by cultured normal (□) and NP (■) fibroblasts**

Cells were incubated in MEM containing 10% heat-denatured fetal bovine serum containing the following. (a) and (b) [*choline-³H, ³²P]SM [1×10^6 d.p.m. (as ³H)/ml; ³H/³²P = 2.7; 1 μ Ci of ³H/nmol]. (c) and (d) [*choline-³H, ³²P]PtdIns [2×10^6 d.p.m. (as ³H)/ml; ³H/³²P = 2.7; 1 μ Ci of ³H/nmol]. The cell lipids were resolved by t.l.c. following extraction. (a) SM; (b) PtdCho following incubation with labelled SM; (c) PtdCho; (d) SM following incubation with labelled PtdCho.**

Table 1. Metabolism of exogenous [*choline-Me-³H, ³²P]SM and [*choline-Me-³H, ³²P]PtdCho by control and NP cells**

Confluent cells in 60 mm dishes were incubated for 24 and 48 h in MEM + 10% heat-denatured fetal calf serum containing either 1×10^6 d.p.m. (as ³H) of [*choline-Me-³H, ³²P]SM (³H/³²P = 2.7)/ml (1 μ Ci of ³H/nmol) or 2×10^6 d.p.m. (as ³H) of [*choline-Me-³H, ³²P]PtdCho (³H/³²P = 2.7)/ml of medium (1 μ Ci of ³H/nmol). Results are given as means $\pm$ one-half range for two separate dishes. In cases where single values are given, duplicate dishes were pooled to provide sufficient material for accurate analysis. Values in parentheses are ³H/³²P ratios $\pm$ one-half range.**

Cell type	Labelled substrate	Incubation time (h)	Specific radioactivity (d.p.m./nmol)			
			SM	PtdCho	P-Cho	GroPCho
Control	SM	24	9089 $\pm$ 64 (2.7 $\pm$ 0.0)	256 $\pm$ 34 (2.7 $\pm$ 0.0)	334 $\pm$ 65 (2.0 $\pm$ 0.1)	166 $\pm$ 36 (2.5 $\pm$ 0.1)
		48	13214 $\pm$ 2420 (2.7 $\pm$ 0.0)	464 $\pm$ 2 (2.9 $\pm$ 0.1)	409 $\pm$ 6 (2.6 $\pm$ 0.1)	513 $\pm$ 27 (2.7 $\pm$ 0.0)
	PtdCho	24	127 $\pm$ 27 (2.6 $\pm$ 0.1)	465 $\pm$ 47 (2.6 $\pm$ 0.1)	190 $\pm$ 27 (5.1 $\pm$ 0.0)	647 $\pm$ 0.1 (2.4 $\pm$ 0.1)
		48	285 $\pm$ 47 (2.9 $\pm$ 0.1)	912 $\pm$ 49 (2.9 $\pm$ 0.0)	478 $\pm$ 7 (6.1 $\pm$ 0.2)	1124 $\pm$ 199 (2.5 $\pm$ 0.0)
	SM	24	10661 $\pm$ 1704 (2.7 $\pm$ 0.0)	124 $\pm$ 25 (2.8 $\pm$ 0.0)	59 (2.3)	82 (2.5)
		48	10317 $\pm$ 133 (2.7 $\pm$ 0.0)	190 $\pm$ 11 (2.9 $\pm$ 0.0)	96 (2.4)	100 (2.1)
NP	PtdCho	24	96 $\pm$ 7 (2.7 $\pm$ 0.1)	837 $\pm$ 6 (2.7 $\pm$ 0.0)	256 (14.6)	513 (2.2)
		48	174 $\pm$ 18 (2.7 $\pm$ 0.1)	1128 $\pm$ 141 (2.9 $\pm$ 0.0)	262 $\pm$ 5 (13.7 $\pm$ 1.8)	387 $\pm$ 62 (1.8 $\pm$ 0.2)

When cells were incubated with double-labelled SM or PtdCho, the specific radioactivity of the cell SM and PtdCho, and the ratio of ³H/³²P, were consistent with those expected for a precursor-product relationship for SM and PtdCho when labelled SM was used, and vice versa when labelled PtdCho was used (Table 1). In both control and NP cells, the ³H/³²P ratio for P-Cho was close to that of SM and PtdCho when SM was the

precursor, a finding compatible with an intermediary role for P-Cho in label movement from SM to PtdCho. However, the specific radioactivity of P-Cho did not always exceed that of PtdCho, suggesting that if P-Cho is the immediate source of PtdCho labelling it may be derived from a higher specific radioactivity pool than is reflected by specific radioactivity measurements of the entire intracellular P-Cho pool. In contrast,

Table 2. Concentrations of choline, *P*-Cho and GroPCho in control and NP fibroblasts following incubation with or without added SM or choline

Confluent cells (60 mm dishes) were incubated for 48 h in MEM + 10 % heat-denatured fetal bovine serum with additions as noted. After incubation, the medium was removed and the cells were washed and harvested as described in the Materials and methods section. Choline, *P*-Cho and GroPCho were measured in the upper phase of the chloroform/methanol extract.

Cell type and conditions	<i>n</i>	Concentration (nmol/mg of protein)		
		Choline	<i>P</i> -Cho	Gro <i>P</i> Cho
Control cells:				
No additions	4	0.3±0.1	15.3±2.8	2.7±1.2
+ Choline (4 mM)	2	23.9±12.2	37.9±4.7	9.0±3.2
+ SM (1 mM)	4	0.3±0.1	20.6±5.1	1.9±0.9
+ SM + choline	2	34.9±10.0	58.2±5.4	8.8±4.5
NP cells:				
No additions	4	1.1±1.0	24.3±6.2	2.4±3.5
+ Choline (4 mM)	2	22.3±3.2	36.8±3.3	10.4±13.3
+ SM (1 mM)	4	0.8±0.7	26.4±3.1	1.8±1.7
+ SM + choline	2	29.9±3.3	62.8±14.7	4.0±2.0

the $^3\text{H}/^{32}\text{P}$ ratio in *P*-Cho was 2–6-fold higher than in PtdCho or SM when PtdCho was the exogenous precursor, suggesting that *P*-Cho is not an intermediate in this label transfer. The $^3\text{H}/^{32}\text{P}$ ratio and specific radioactivity of GroPCho roughly paralleled that of PtdCho. These findings were similar in control and NP cells, indicating that the transfer of label in either direction is similar in normal and mutant cells.

The results suggest that movement of label from SM to PtdCho involves a sphingomyelinase reaction, which liberates free *P*-Cho, with subsequent incorporation into PtdCho. We reasoned that if this were so, increasing the intracellular *P*-Cho concentration should cause a decrease in labelling of PtdCho from SM. The intracellular levels of choline, *P*-Cho and GroPCho were not altered significantly by incubation in 1 mM-SM in either control or NP cells, but were increased by 1.5–20-fold by incubation in 4 mM-choline (Table 2). The increase in intracellular *P*-Cho was even more marked in the presence of 1 mM-SM, rising to 2–4 times the levels with added choline only (Table 2). In the presence of 4 mM-choline, total lipid radioactivity in the cells (lower phase, which is SM + PtdCho) was slightly decreased in control but not in NP cells (Fig. 3). Incorporation of [^3H]choline from SM into PtdCho was decreased in both control and NP cells. The incorporation of SM label into PtdCho was lowered to 60–80 % of that in the absence of added exogenous choline, which is a change in the appropriate direction, but is less than might be predicted from the expansion in the intracellular *P*-Cho pool. This may reflect an intracellular compartmentation of *P*-Cho so that the total expanded pool is not available (George *et al.*, 1989). Alternatively, the expansion of the *P*-Cho pool may occur gradually over the 48 h period of the experiment, so that the mean expansion may be less than that measured at 48 h.

We have also examined the effects of various manipulations designed to alter intracellular concentrations of acceptors and other participants in the various putative transfer reactions (Table 3). Such studies are complicated by effects on the uptake of [^3H]SM from the medium, in addition to any direct effects on the transfer reaction. Since the amount of transfer to PtdCho is dependent on the amount of [^3H]SM taken up by the cells, we have calculated the amount of *P*-Cho transfer predicted from the amount of [^3H]SM uptake, determined in other experiments, to

Fig. 3. Effect of exogenous choline on the uptake (total lipid label) and metabolism (PtdCho) of [^3H]-*Me*- ^3H]SM by control and NP cells in culture

Confluent cells were incubated in MEM plus 10 % fetal bovine serum for 48 h with 1 mM-[*choline-Me*- ^3H]SM (1473 d.p.m./nmol) in the presence or absence of 4 mM-choline. Results are given for: total lipid label incorporated (nmol/mg of protein) in the absence (■) and presence (▨) of choline; SM in the absence (□) and presence (▩) of choline; PtdCho in the absence (□) and presence (■) of choline (means ± s.d., *n* = 4). For PtdCho minus choline versus PtdCho plus choline, *P* = 0.03 (control) and 0.08 (NP). All other pairs were not significantly different (*P* > 0.1).

aid in interpretation of the results (see footnote to Table 3). Diacylglycerol (dioleoyl), either as a mixed micelle with the [^3H]SM or as pure micelles added separately, decreased overall label incorporation by 80 %, but did not stimulate appreciably transfer of label to PtdCho, in either the presence or the absence of exogenous choline and *P*-Cho. The addition of linoleic acid and insulin, to increase neutral lipid formation and possibly the diacylglycerol availability, was without effect on transfer to PtdCho. Cytidine and CMP were also without effect. These results must be interpreted cautiously, since it is possible that some of these exogenous compounds were not taken up by the cells or may not have reached the site(s) of the reactions.

Although these experiments with normal and mutant human fibroblasts suggested that the most probable route for *P*-Cho transfer from SM to PtdCho was hydrolysis by sphingomyelinase and subsequent utilization of the released *P*-Cho through the usual pathways of PtdCho biosynthesis, the possibilities of a direct transfer reaction, or of reversal of the choline phosphotransferase reaction (Introduction, reactions 1 and 2) were not excluded. The latter two reactions, however, should be relatively independent of factors that affect either the choline kinase reaction and/or the CTP:phosphocholine cytidyltransferase reaction. Esko and co-workers (Esko & Raetz, 1980; Esko *et al.*, 1981) have described temperature-sensitive mutant CHO cells that are defective in cytidyltransferase activity at the non-permissive temperature. We studied the transfer of label from SM to PtdCho in these cells (Table 4). When cells were incubated at 33 °C for 48 h and subsequently harvested and sphingomyelinase activity measured *in vitro*, both mutant and wild-type cells had similar sphingomyelinase activities. Growth for 48 h at 40 °C had no effect on wild-type cells, but decreased activity in mutant cells by 40 %. The reasons for this decrease are not known. The incorporation of [*Me*- ^{14}C]choline into PtdCho and SM was not altered in the wild-type cells by growth at 40 °C compared with 33 °C. However, as expected, incorporation of choline into PtdCho was decreased by 75 % in the mutant cells by incubation at 40 °C. Incorporation into SM was decreased by

Table 3. Incorporation of [³H]choline from [³H]SM into PtdCho in control cells in culture

Cells were grown in 60 mm dishes in MEM + 10 % fetal bovine serum as described in text. At time zero, the medium was changed to medium containing 1 mM-[³H]SM (2000 d.p.m./nmol) and additions as noted. Cells were incubated for 48 h. Single figures are the means of two dishes; for 3 dishes, means ± s.d. are given.

Addition	Total cell label (% of control)	PtdCho	
		(% of total cell label)	(% predicted)*
Control	100	60.0 ± 8.6	58 ± 6
Diacylglycerol (0.2 mM)	25	51.1 ± 2.4	58 ± 6
Choline (1 mM) + <i>P</i> -Cho (1 mM)	63	36.4 ± 1.9	51 ± 4
Choline (1 mM) + <i>P</i> -Cho (1 mM) + diacylglycerol (0.2 mM)	16	40.9 ± 3.7	69 ± 11
Cytidine (0.5 mM)	127	62	58 ± 6
CMP (0.5 mM)	134	63	58 ± 6
Linoleic acid (100 mM) + insulin (5 µg/ml)	128	55	58 ± 6

* The amount of [³H]choline that should have been incorporated into PtdCho based on the amount of [³H]SM taken up by the cell. This value was calculated from experiments in which the concentration of [³H]SM in the medium was varied with a resulting variation in [³H]SM uptake by the cells, and a change in the percentage of the total cell label in PtdCho.

Table 4. Sphingomyelinase activity and the incorporation of [¹⁴C]choline and [³H]SM into mutant and wild-type CHO cells

Wild-type and mutant CHO cells (2×10^6 cells) were subcultured into 25 cm² flasks in Ham's F12 medium plus 10 % fetal bovine serum. After 72 h, 3 ml of fresh medium containing 0.1 mM-[*Me*-¹⁴C]choline chloride (1.35 µCi/µmol) and 2 mM-[*choline-Me*-³H]SM (0.7 µCi/µmol) was added. Cells were incubated at 33 °C or 40 °C for 48 h, and harvested as described in the Materials and methods section. Means ± s.d. for determinations on three separate flasks are given.

	Mutant		Wild-type	
	33 °C	40 °C	33 °C	40 °C
Sphingomyelinase (nmol/h per mg of protein)	68.1 ± 5.0	40.3 ± 2.5	69.1 ± 5.1	66.0 ± 5.8
Incorporation into lipid (nmol/mg of protein):				
[¹⁴ C]Choline into PtdCho	48.9 ± 3.0	11.7 ± 3.0	77.9 ± 13.6	87.6 ± 8.6
[¹⁴ C]Choline into SM	9.9 ± 1.1	4.8 ± 1.1	8.1 ± 1.1	10.5 ± 1.4
[³ H]SM into PtdCho	28.3 ± 0.8	2.7 ± 0.8	21.0 ± 5.7	17.6 ± 3.1
[³ H]SM into SM	42.8 ± 8.9	51.6 ± 2.1	28.5 ± 10.6	28.9 ± 3.2

50 %. The uptake of exogenous [³H]SM was similar at 33 and 40 °C in the wild-type cells, and the incorporation of [*Me*-³H]choline from SM to PtdCho was unaffected. The mutant cells, in contrast, showed increased uptake of SM at both temperatures. Although the transfer of label from SM was substantial at 33 °C, it was decreased by 90 % at 40 °C. Since the decrease in sphingomyelinase activity in the mutant cells at 40 °C is only 40 %, this result strongly suggests that the label transfer is proceeding via the cytidyltransferase reaction. Modifications of these experiments have been done several times with qualitatively similar results with respect to transfer of SM label to PtdCho.

DISCUSSION

The present study was undertaken to determine the metabolic pathways by which the *P*-Cho moiety of exogenous SM is

transferred to diacylglycerol to form PtdCho in cultured human fibroblasts. Knowledge of the existence, number and types of such pathways is important for at least two reasons. First, the observation of regulatory roles for diacylglycerol and sphingosine in protein kinase C activity (Hannun *et al.*, 1986; Merrill *et al.*, 1986) and the evidence that PtdCho and SM may be important precursors of those regulatory molecules (Bocckino *et al.*, 1985; Kolesnick, 1987; Cabot *et al.*, 1988; Silfe *et al.*, 1989), make knowledge of pathways which contribute to the supply of these molecules of great interest. Second, the presence of other pathways that could contribute to SM catabolism might be of practical significance for the development of treatment for the sphingomyelin lipidoses which affect man (Spence & Callahan, 1989).

We have shown that: (1) labelled intracellular *P*-Cho in control and NP cells incubated with SM has a specific radioactivity intermediate between that of SM and PtdCho, which is compatible with a precursor-product relationship for *P*-Cho and PtdCho; (2) expansion of intracellular *P*-Cho by incubation with exogenous choline decreases the transfer of [³H]choline from SM to diacylglycerol; and (3) transfer of *P*-Cho to diacylglycerol from [³H]SM is decreased at the non-permissive temperature in CHO cells with a temperature-sensitive mutation in the cytidyltransferase reaction. Each of these observations strongly supports a reaction sequence in which *P*-Cho is cleaved from SM by a sphingomyelinase, followed by incorporation of *P*-Cho into PtdCho by the Kennedy pathway of biosynthesis (SM → *P*-Cho → CDP-Cho → PtdCho). The similar [³H]choline/[³²P]P_i ratios in SM, *P*-Cho and PtdCho do not support the occurrence of a phospholipase D reaction or base exchange. The clear involvement of the cytidyltransferase in the SM-to-PtdCho head-group transfer in CHO mutant cells, and the failure to appreciably affect the transfer with diacylglycerol, cytidine-containing compounds or other measures, suggests that reversal of the choline phosphotransferase reaction is not a major route for SM → PtdCho head-group transfer, and that the supply of intracellular diacylglycerol probably is not rate-limiting to the process. The possibility of direct transfer from SM to diacylglycerol, in analogy to the PtdCho:ceramide cholinephosphotransferase reaction (Marggraf *et al.*, 1972; Voelker & Kennedy, 1982), cannot be excluded completely. However, the evidence is most compatible with a pathway in which most of the SM head group cycles to PtdCho through the free *P*-Cho intermediate.

If the source of PtdCho labelling from [³H]SM is the *P*-Cho

pool, it follows that cells and tissues demonstrating this activity must have sphingomyelinase activity. What is the source of this activity in sphingomyelinase-deficient NP cells? It should be noted that SM-to-PtdCho transfer in these cells is most readily demonstrated at high concentrations of exogenous SM (Kudoh *et al.*, 1983; Spence *et al.*, 1983) and it is possible that the activity of the lysosomal sphingomyelinase at 2–3 % of normal in these cells may be adequate to provide some hydrolysis, particularly at higher substrate concentrations. Alternatively, the hydrolytic activity may be provided by the other sphingomyelinases described in mammalian tissues. Two of these are possible candidates. The neutral Mg^{2+} -stimulated sphingomyelinase is at particularly high levels in brain, but has also been described in peripheral tissues (Spence *et al.*, 1979). Levels of this activity are very low in cultured fibroblasts and are not increased after incubation with exogenous SM (M. W. Spence, unpublished work). The Zn^{2+} -stimulated enzyme found in fetal bovine serum might also play a role (Spence *et al.*, 1989). Its cellular source is not known, and it may be present in a variety of tissues. Alternatively, the fetal serum enzyme may provide a source of activity to cells cultured in serum-containing medium. These possibilities require further investigation.

It is clear, from this study and others, that there is a close relationship between PtdCho and SM metabolism. Although this relationship has been known for some time insofar as the biosynthetic pathways are concerned, a close relationship, or a scavenger pathway relationship between the degradation and synthesis, has been less apparent. The present study suggests that this latter type of reaction may be important in the normal metabolism of choline-containing phospholipids.

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