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Phytosphingosine Biosynthesis Differs from Sphingosine in Fish Leukocytes and Involves a Transfer of Methyl Groups from [^{3}H-methyl]Methionine Precursor

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We have studied the incorporation of radioactivity from either [3-^{3}H]serine as the direct or [^{3}H-methyl]-methionine as the indirect precursor into sphingoid bases of free ceramides in lymphocytes from fish. Radioactivity from serine was incorporated mostly in the sphingosine moiety of ceramides. In contrast, the radioactivity from methionine was exclusively incorporated into phytosphingosine base (i.e., 4-hydroxy-sphinganine) and the incorporation increased by about twofold in the presence of folic acid or niacinamide. Identity of the long-chain bases, phytosphingosine and sphingosine, was established chemically by thin-layer chromatography, chemical degradation, and gas–liquid chromatography.

Key Words: **phytosphingosine; sphingosine; ceramides; serine; methionine; leukocytes.**

Phytosphingosine (4-hydroxy-sphinganine) is a long-chain base found in ceramides and glycolipids of certain normal (1–4) and tumor tissues (1) and its expression changes during development (3, 5). It plays an important role in signal transduction and immunity (6). Its *de novo* biosynthesis is poorly understood and has been assumed to occur largely by hydroxylation or hydration of 4-sphingenine (7, 8). Three steps upstream, the initial condensation of serine and palmitic acid produces 3-oxosphingosine that is used in the biosynthesis of 4-sphingenine. Here we demonstrate that in lymphocytes of the fish Dicentrarchus labrax, the main precursor for the biosynthesis of phytosphingosine moiety in free ceramides is methionine and not serine. In these cells, the radioactivity from serine is incorporated mostly into sphingosine bases of free ceramides. Our data suggest the possibility of different compartmentalized intracellular pools of unknown intermediates derived from methionine or serine, and that of long chain bases and ceramide species. The significance of preferential incorporation of individual precursors and the compartmentalization of intermediates is far reaching for regulation of cellular behavior and may ultimately determine fate of cells.

METHODS

Chemicals. All solvents were of analytical grade and were purchased from SDS (Peypin, France) or from Carlo Erba (Milan, Italy). Radioactive precursors [3-^{3}H]-serine and [^{3}H-methyl]-methionine, (both with specific activity of 15 Ci/mmol) were from Isotopchim (Ganagobie, France). Tissue culture reagents and lipid standards (ceramides type III with sphingosine and normal fatty acids, ceramides type IV with sphingosine and alpha-hydroxy fatty acids, and sphingosine and phytosphingosine) and bicinchoninic acid (BCA) were purchased from SigmaChem Co. (L'Isle d'Abeau, France). Ceramide standard containing phytosphingosine and stearic acid was from Dr. E. Perrier (Coletica, France). Silica gel 60 thin-layer chromatography (TLC) plates without fluorescence indicator were from E. Merck (Darmstadt, Germany).

Incorporation of methionine and serine precursors into lipids. Blood from the sea bass *Dicentrarchus labrax* was fractionated on a discontinuous Ficoll gradient to obtain a 98% pure mononuclear population of leukocytes (9). Incorporation of radioprecursors was performed at 20°C in normal air as described (10). The cells were incubated with radioactive precursors, [^{3}H]-serine (10 microCi per ml of culture medium) or [^{3}H-methyl]methionine, (10 microCi/ml of culture medium) at the same final specific activity (15 Ci/mmol), for up to 5 h. Cellular lipids were extracted according to Folch *et al.* (11) and washed according to Chapelle *et al.* (12). Radioactivity was determined in sample aliquots at various stages of lipid analysis in

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Abbreviations used: BCA, bicinchoninic acid; TLC, thin-layer chromatography; NFA, normal fatty acids; HFA, hydroxy fatty acids; GC, gas chromatography.

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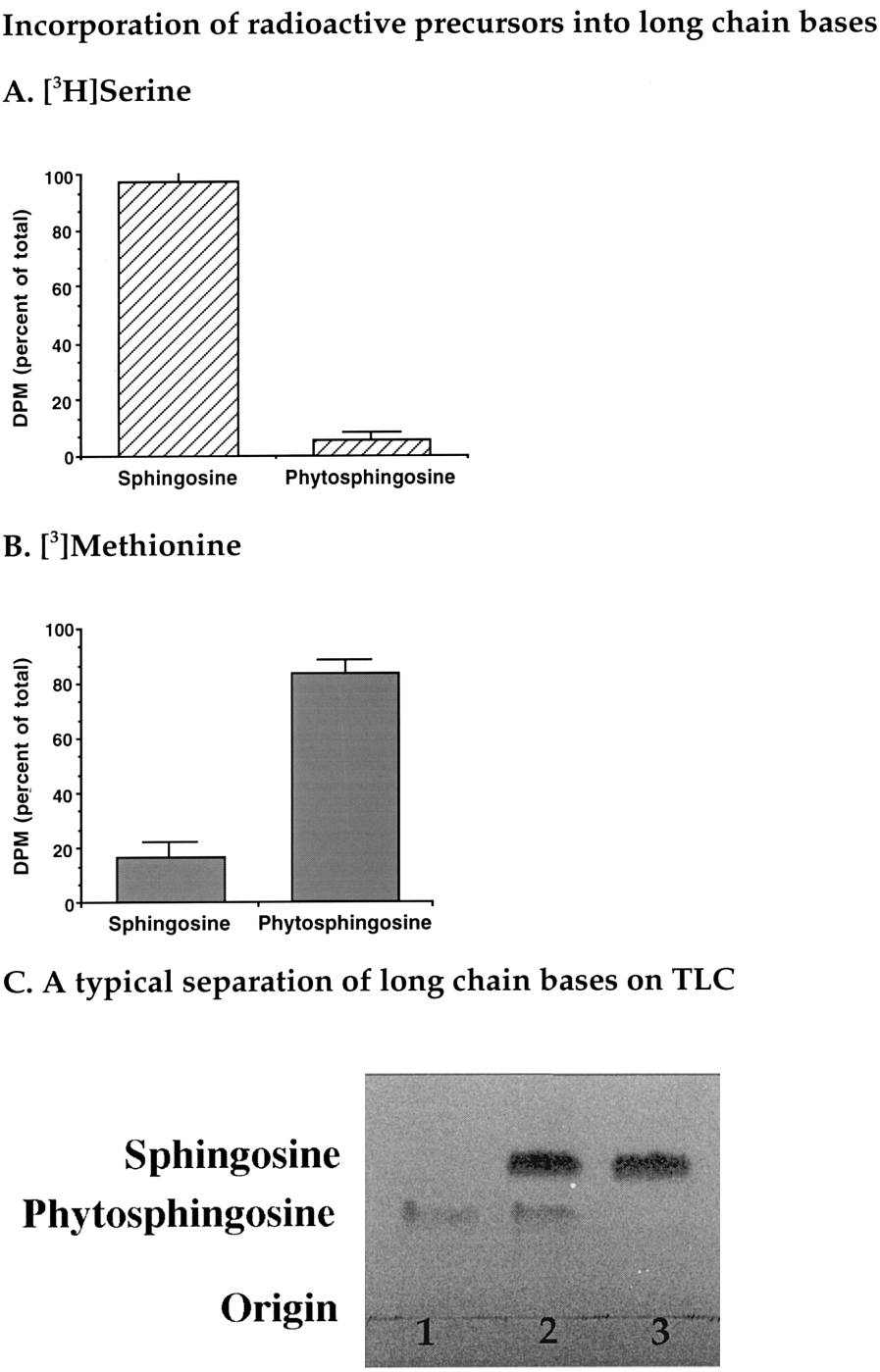

FIG. 1. Analysis of precursor incorporation into free ceramides of fish leukocytes. (A) Incorporation of [^{3}H]-serine into free ceramides. (B) Incorporation of [^{3}H-methyl]methionine into free ceramides. (C) A typical TLC separation of HFA- and NFA-containing ceramides. Lane 1, standard HFA-ceramide; lane 2, ceramides of fish leukocytes; lane 3, standard NFA-ceramide. Cells were incubated for 2 h in presence of the radiolabeled precursor, and lipids were extracted and analyzed as described in Materials and Methods.

a Packard beta spectrometer (Packard Instruments, Downers Grove, IL). Total protein in the cells was determined in the residues after extraction of lipids, by the bicinchoninic acid micro-assay procedure (13, 14). Six individual fish were used in each experiment, and data collected from three separate experiments were statistically evaluated.

Isolation of sphingolipids. Non-labeled carrier lipids, sphingomyelin, non-hydroxy fatty acid (NFA-), and hydroxy fatty acid (HFA-) containing ceramides, and synthetic ceramide containing phytosphingosine and C18:0 fatty acid were added to the labeled lipid extracts before processing. Carrier lipids were not added to labeled samples when processed for gas chromatography (GC) (see below).

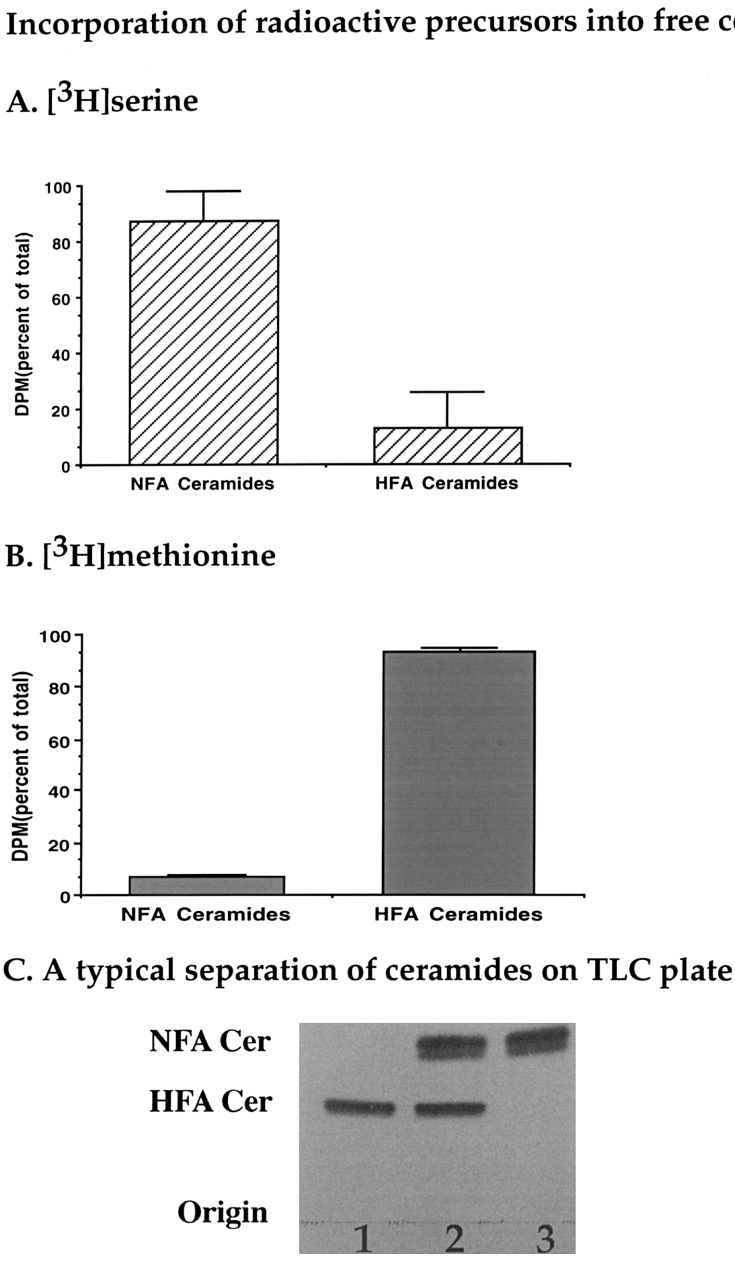

FIG. 2. Analysis of precursor incorporation into sphingoid bases of free ceramides of fish leukocytes. (A) Incorporation of [^{3}H]-serine into sphingoid bases of free ceramides. (B) Incorporation of [^{3}H-methyl]methionine into sphingoid bases of free ceramides. (C) TLC of sphingosine and phytosphingosine bases. Lane 1, standard phytosphingosine; lane 2, sphingoid bases of ceramides; lane 3, standard sphingosine. Cells were incubated for 2 h in presence of the radiolabeled precursor, and lipids were extracted and analyzed as described in Materials and Methods.

The lipids were taken up in 6 ml chloroform and subjected to mild alkaline hydrolysis by addition of 6 ml of 0.6 N methanolic sodium hydroxide at room temperature for 1 h (15). The mixture was acidified with 0.7 ml concentrated HCl and hydrolysis continued for another hour. 4.8 ml of distilled water were added and after thorough shaking and centrifugation, the aqueous upper phase was discarded. The lower phase was washed two times with theoretical upper phase, dried and taken up in a minimal volume of chloroform. The lipids were applied to an aminopropyl-bonded silica gel cartridge column (Supelchem, Paris, France) pre-conditioned with hexane (16). The neutral lipid fraction containing free ceramides was eluted with 3 ml of chloroform-isopropanol (2:1, v/v), then the free fatty acid fraction was recovered with 1.4 ml of isopropylether-acetic acid (98:2, v/v), and finally the phospholipid fraction was eluted with 4 ml of methanol.

Isolation of long chain bases from free ceramides. The neutral lipid fraction obtained from the aminopropyl column, as described above, was spotted on a silica gel 60 TLC plate and the plate developed in chloroform-methanol (50:5, v/v) (17). The plate was exposed

TABLE 1

Incorporation of Radioactivity into Free Ceramides and Their Constitutive Sphingoid Bases in Fish Lymphocytes

Fraction	Precursor	
	[^{3}H]serine	[^{3}H-methyl]methionine
Free ceramides		
Dihydroxy-	11,386 ± 1,834	4,030 ± 1,580
Trihydroxy-	1,058 ± 112	51,440 ± 13,830
Free dihydroxy-ceramides		
Containing phytosphingosine	0	0
Containing sphingosine	9,866 ± 100	3,000 ± 1,180
Free trihydroxy-ceramides		
Containing phytosphingosine	0	42,940 ± 1,280
Containing sphingosine	952 ± 100	8,490 ± 250

Note. Radioactive ceramides and long-chain bases were isolated from fish lymphocytes incubated for 2 h in the presence of either [^{3}H]serine or [^{3}H-methyl]methionine. The radioactivity is expressed as dpm in free ceramides or long-chain bases per milligram of recovered cell protein. Values are means ± SEM of data from six different fish used in one representative experiment. Data variation in three such separate experiments was 5–7%.

to iodine vapor to visualize lipid spots. Of the two spots seen on the plate, the fast-moving one (dihydroxy-type) co-migrated with authentic standards of NFA-ceramides, and the slow-moving spot (trihydroxy-type) co-migrated with standard HFA-ceramides mixture that contained ceramides with phytosphingosine, and ceramides with sphingosine and hydroxy-fatty acids. Each of these two spots was scraped, either into scintillation vials for counting of radioactivity (see Fig. 1), or into screw-capped tubes for hydrolysis in a mixture of concentrated HCl-water-methanol (1:2:6, v/v/v) for 18 h at 80°C (18) to release sphingoid bases. Free fatty acids and their methyl esters were removed by partitioning the mixture with hexane. The methanolic phase containing sphingoid bases was concentrated to a small volume under nitrogen, and made alkaline with 2 ml of 7 N NaOH. The sphingoid bases were extracted from this mixture with an equal volume of diisopropylether. The last step was repeated three times. The pooled ether phase was dried down and re-dissolved in a minimal amount of chloroform-methanol (1:1, v/v). The extracts were applied on a TLC plate and developed in chloroform-methanol-2N ammonia (40:10:1, v/v/v) (19). The long-chain bases phytosphingosine (lower spot) and sphingosine (upper spot) were visualized by ninhydrin spray (see Fig. 2), and scraped into scintillation vials for counting of radioactivity.

Analysis of sphingoid bases by gas chromatography (GC). Ceramides obtained from lymphocytes were extracted and hydrolysed, without adding any carrier lipids, to obtain the long chain bases as described above. The ether phase containing sphingoid bases was evaporated under nitrogen and re-dissolved in 1 ml methanol. 0.2 ml of 0.2M sodium periodate was added to the sample and the long-chain bases were oxidized at room temperature for 1 hour in the dark. Then, 0.6 ml of water and 2.4 ml of 1,2-dichloroethane were added. After vigorous mixing, the lower phase of 1,2-dichloroethane containing the aldehydes derived from long-chain bases was removed and dried under nitrogen at 15°C. The aldehydes were redissolved in a minimal amount of hexane and an aliquot was injected into an 0V 17-01 capillary column (25 m × 0.32 mm), in a Hewlett Packard gas chromatograph type 5890 series II equipped with a split injector and a flame ionization detector. The run was programmed for temperature to rise from 170 to 220°C at the rate of 2°C/min (20). The flow of carrier gas (nitrogen) was at 15 ml/min. Data were recorded and integrated on a CR3-A integrator (Shimadzu, Kyoto, Japan). Chromatograms were compared to those obtained from aldehyde derivatives of authentic standards of phytosphingosine, DL-erythro dihydrosphingosine and sphingosine.

RESULTS AND DISCUSSION

Alkali-stable free ceramides were isolated from leukocytes that had been labeled for 2 h with either L-[3-^{3}H]serine or [^{3}H-methyl]methionine. Free ceramides were separated into dihydroxy- and trihydroxy-types that co-migrated on TLC with authentic NFA- and HFA-ceramides (see Fig. 1) respectively. The lipid spots were scraped into scintillation vials and counted for radioactivity. The data are shown in Fig. 1. When labeled methionine was used as the precursor, 93% of radioactivity in the alkali-stable free ceramides fraction was obtained in the trihydroxy-ceramide spot (that contains ceramides with phytosphingosine) on TLC, and about 7% in dihydroxy-ceramide spot containing sphingosine. In contrast, when serine was used as the precursor, more than 91% of the radioactivity was obtained in the dihydroxy-ceramide spot on TLC and only about 8.5% in the trihydroxy-ceramide spot (Fig. 1). To determine precursor incorporation into individual long chain bases, phytosphingosine and sphingosine, total free ceramides and each fraction of the free ceramides were acid-hydrolyzed. The phytosphingosine and sphingosine bases were purified and separated on TLC (see Fig. 2). When labeled methionine was used as the precursor, about 83% of the radioactivity was found in the trihydroxy-ceramide(phytosphingosine moiety) and 17% in sphingosine. In contrast, when labeled serine was used as the precursor, only a negligible amount of radioactivity was incorporated in phytosphingosine of trihydroxy-ceramides (Fig. 2). All the radioactivity from serine that labeled the dihydroxy-ceramide spot appeared to be in its sphingosine moiety (Table 1). The data therefore suggest that a certain amount of the label from methyl groups of methionine is converted to serine, the known precursor of sphingosine. However,

TABLE 2

Influence of Folic Acid (1 mg per Liter of Medium) and Niacinamide (1 mg per Liter of Medium) on the Incorporation of Radioactive [^{3}H-methyl]Methionine into Free Ceramides and Their Constitutive Sphingoid Bases of Fish Lymphocytes

Fraction	Incubation medium		
	Control	Folic acid	Niacinamide
Free ceramides			
Dihydroxy-	6,120 ± 2,399	5,894 ± 1,328	6,340 ± 568
Trihydroxy-	57,230 ± 10,487	99,466 ± 8,921	106,162 ± 9,895
Free dihydroxy-ceramides			
Containing phytosingosine	0	0	0
Containing sphingosine	4,520 ± 892	4,328 ± 542	4,780 ± 295
Free trihydroxy-ceramides			
Containing phytosphingosine	53,132 ± 4,230	94,575 ± 6,524	103,873 ± 4,520
Containing sphingosine	6,072 ± 384	6,852 ± 452	5,682 ± 234

Note. Radioactivity (expressed as dpm per milligram of cell protein) was recovered in free ceramides and long-chain bases after 2 h incubation of fish lymphocytes in the presence of [^{3}H-methyl]methionine. Values are means ± SEM of data from one representative experiment using six fish. Three separate experiments showed variations in a 5–7% range.

the newly synthesized serine (from methionine) is either localized in a separate compartment, different from that occupied by the direct incorporation of exogenous serine precursor label, or the extent of conversion of methionine to sphingosine and/or phytosphingosine is regulated by mechanisms not yet understood. It is interesting to note that incorporation of label from methionine into sphingosine was unaffected by folate or niacinamide.

These data clearly indicate that methionine is the preferred precursor of phytosphingosine in trihydroxy-type of ceramides, and serine is the preferred precursor for sphingosine in dihydroxy-type of ceramides. Addition of folic acid or niacinamide at the concentration of 1 mg per liter in the incubation medium increased the incorporation of radioactivity from methionine into phytosphingosine by 86 and 74%, respectively (Table 2). These data suggest that glycine may be the immediate intracellular precursor of phytosphingosine synthesis, and experiments are in progress to confirm this hypothesis.

In our experiments, trihydroxy- and dihydroxy-ceramides were isolated from alkali stable lipids. Long-chain bases were obtained by acid hydrolysis of purified ceramides. Phytosphingosine and sphingosine were identified by their comigration with standards on TLC plates (19) (Fig. 2), and their characteristic coloration with ninhydrin spray that gives orange color with phytosphingosine and pink with sphingosine (not shown). These bases are well resolved on TLC from their O-methyl and N-methyl derivatives (19) which can also be labeled with methyl group of methionine precursor, as reported by Igarashi and Hakomori (20). Identification of the bases was further assessed by gas chromatography (GC) after periodate oxidation (21). Since the periodate-oxidized derivatives of phytosphingosine and sphingosine clearly separate from each other by GC, phytosphingosine in the samples derived from ceramides could be identified. Trace amounts of dihydrosphingosine were also detected by GC. The apparent pool sizes calculated from GC data indicate that the amount of phytosphingosine bases represents about 10% of the pool of sphingoid bases of free ceramides. Although long chain bases can also be identified by mass spectrometry, such data would not add any new information about their identity or radioactive labeling already established by chemical, TLC and GC methods. Our data at this time do not provide a definitive indication about the intracellular intermediates for synthesis of ceramide containing phytosphingosine. However, since we have observed incorporation of these ceramides into sphingomyelin and glycolipids, the preferential fate of such labeled long chain bases is being actively pursued.

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