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RAPID TURNOVER OF SPHINGOSINE SYNTHESIZED DE NOVO FROM [^{14}C]SERINE BY CHINESE HAMSTER OVARY CELLS[1]

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SUMMARY: It has been hypothesized that complex sphingolipids may serve as another "lipid second messenger" system via their hydrolysis to free sphingosine, which inhibits protein kinase C and affects multiple cellular functions. To investigate sphingolipid turnover, Chinese hamster ovary cells were pulse labelled with [^{14}C]serine and the [^{14}C]sphingosine in cellular sphingolipids was determined over time. Much of the radiolabelled sphingosine was initially seen in ceramides and was incorporated into sphingomyelin during the 5-hour chase. A major portion of the radiolabel that was initially seen in other sphingolipids disappeared over time. Overall, about half of the total long-chain bases made during this pulse were degraded within 2 to 5 h, depending on the method of analysis. Hence, a substantial portion of the sphingosine synthesized de novo by these cells is turned over fairly quickly. Since the doubling time of these cells is 12 h, this rapid turnover may reflect the remodelling of the cell surface, or the utilization of the free sphingosine derived from sphingolipid turnover, as part of the control of cell growth and division. © 1988 Academic Press, Inc.

Sphingosine and other long-chain (sphingoid) bases have been proposed to serve as another class of "lipid second messengers" because they inhibit protein kinase C in vitro (1) and affect multiple cellular functions (see 1-6, for examples). While this has not been proven, free sphingosine is found in cells (3,7,8) and the levels can be modulated by agonists (9). The source of this sphingosine is not known. Free sphingosine does not appear in vivo as an intermediate in the biosynthesis of sphinglipids from serine and palmitoyl-CoA because the resulting long-chain bases are rapidly converted to ceramides (10). Hence, it may arise from the hydrolysis of more complex precursors (e.g., ceramides, sphingomyelin, or glycosphinglipids), as is known to occur with many of the other lipid modulators of cellular function.

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In recent studies of sphingosine biosynthesis using mouse LM cells incubated with [^{14}C]serine (10), the newly synthesized long-chain bases appeared to be degraded with a $t_{1/2}$ of approximately 8 h. This is evidence for the turnover of more complex sphingolipids; however, this earlier observation may have arisen from a number of technical artifacts. Hence, additional studies have now been conducted with Chinese hamster ovary (CHO) cells and these confirm that a substantial portion of the sphingosine that is synthesized from [^{14}C]serine is turned over rapidly.

EXPERIMENTAL PROCEDURES

Materials--Ham's F12 medium, MCDB301 medium (a formulation similar to F12) minus serine and fetal calf serum were obtained from Gibco. L-[3-^{14}C]serine was from Amersham. Sphingosine and sphinganine (dihydrosphingosine) were purchased from Sigma. The tissue culture dishes (100 mm) were obtained from Falcon.

Cell culture--Chinese hamster ovary cells (CHO-WT5) were originally obtained from Louis Simonovitch, Hospital for Sick Children, Toronto, Cananda. The cells were maintained at 37°C in 5% CO_2 and 100% humidity in Ham's F12 medium with 5% fetal calf serum supplemented with penicillin G (100 units/mL), streptomycin sulfate (100 μg/mL), and sodium bicarbonate (1.176 g/L). For subculturing, the cells were removed from the dish with 0.5 mM EDTA in Puck's saline (0.14 M NaCl, 5.4 mM KCl, 5.5 mM glucose, and 4.3 mM sodium bicarbonate). Cells were used for the experiments when they covered 80 to 90% of the dish. Unless otherwise noted, they were incubated with Ham's F12 (without fetal calf serum) 24 h prior to the experiment.

Analysis of long-chain base synthesis--The medium was removed and replaced by 2 mL of MCDB301 medium minus serine containing 0.09 mM [^{14}C]-serine (55 mCi/mmol) for 1 h. At this time, 2 mM unlabelled serine was added and the cells were incubated for up to 5 h. Long-chain bases were analyzed as follows:

1) The cells were removed from the plate by scraping and the lipids were extracted with chloroform and methanol by a modified Folch procedure (10), then hydrolyzed using methanolic HCl (11), and the long-chain bases were separated by thin-layer chromatography, visualized by autoradiography, and quantitated by liquid scintillation counting as described previously (10);

2) The cells were removed from the plate with EDTA and the lipids were extracted with chloroform and methanol (1:2 followed by 2:1) without addition of water. The extract was filtered, dried under reduced pressure, and the lipids were redissolved in chloroform. The chloroform-soluble material was centrifuged, and the clear supernatant was acid hydrolyzed and the long-chain bases were quantitated as described in 1);

3) The cells were treated as described in 2) except that the lipids were acid hydrolyzed in 0.5 N HCl and 4 M H_2O in acetonitrile for 2 h at 75° C, which is thought to result in less decomposition of the long-chain bases (12);

4) The cells were prepared in glass scintillation vials and extracted as described in Experiment 3. This enabled measurement of the total sphingolipids without losses to the medium or plate; and,

5) Since acid hydrolysis might not liberate the long-chain bases of all sphingolipids (e.g., sphingomyelin), samples were analysed essentially as in 2 and 3; however, before acid hydrolysis the lipid extracts were incubated with sphingomyelinase or endoglycoceramidase. The conditions for the incubations were: a) for sphingomyelinase, the lipid extracts were sonicated for 3 minutes and incubated with 1 unit of sphingomyelinase (from Bacillus cereus, Boehringer) in 0.1 M potassium phosphate buffer (pH 7.0) for 1 h at 37°; and, b) for endoglycoceramidase, the sonicated lipid extracts were incubated in 0.1 M Na acetate buffer (pH 6.0) with 5 munits of endoglycoceramidase (from Rhodococcus, Genzyme) at 37° for 1 h.

As has been seen before (10), no free long-chain bases were seen on autoradiograms unless the samples had been acid hydrolyzed. After acid hydrolysis, sphingosine was the major radiolabelled species seen; the cpm in sphingosine and sphinganine were added to give total long-chain bases, which are hereafter referred to by the generic name sphingosine.

The radiolabelled sphingolipids were preliminarily characterized in lipid extracts prepared as in 2, above, followed by removal of the glycerolipids by treatment with 0.1 M KOH (10), and TLC on silica gel H plates with ether:methanol (99:1,v/v) to visualize ceramides and chloroform:methanol:acetic acid:water (56:35:4:2) to separate some of the more complex sphingolipids. Since [^{14}C]serine is incorporated into long-chain bases, fatty acids, and the headgroup of sphingomyelin, the lipids were eluted from individual regions of the chromatograms and the amounts of radiolabelled long-chain bases determined by acid hydrolysis and rechromatography.

RESULTS

Turnover of newly synthesized sphingosine. Panels A and B of Fig. 1 illustrate the change in newly synthesized [^{14}C]sphingosine (expressed as the % of the cpm at time zero for easier comparisons among the different experiments) at varying times after removal of [^{14}C]serine. The decrease represents turnover of the sphingosine in more complex sphingolipids (e.g., ceramides) because free sphingosine is not detected as an intermediate of

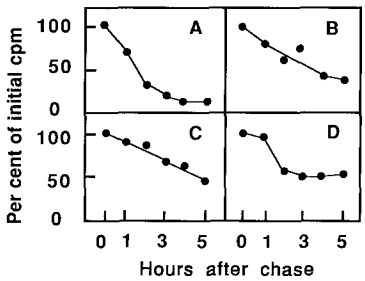


Figure 1. Relative amounts of [^{14}C]long-chain bases in CHO cells at varying times after a 1-hour pulse of [^{14}C]serine. A: Sphingolipids were recovered in the chloroform layer of a Folch extract, then hydrolyzed in acidic methanol. B: The sphingolipids were extracted without addition of an aqueous phase, then hydrolyzed in acidic methanol. C: The sphingolipids were extracted as in B, then hydrolyzed in acidic acetonitrile. D: The cells were grown in glass vials and extracted directly (cells and medium) as in C.

the de novo biosynthetic pathway. The apparent $t_{1/2}$ of the sphingolipids recovered in the chloroform layer of a standard Folch extraction was approximately 1.5 h (Fig. 1, A); whereas, the half time was 5 h for the lipids extracted by chloroform and methanol without formation of an aqueous phase (Fig. 1,B). This indicates that some of the decrease in panel A may be accounted for in more complex sphingolipids (such as gangliosides), which are lost in the aqueous phase.

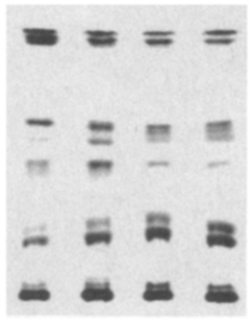
When the analyses were conducted using acidic acetonitrile to liberate the long-chain bases, the total cpm were approximately twice those obtained with methanolic HCl (not shown); however, the half time of turnover (3 h, Fig. 1, C) was similar to that in panel B. Hence, the loss of radiolabel in sphingosine does not appear to reflect the biosynthesis of sphingolipids that are refractory to analysis by acid hydrolysis. This was further supported by the studies with sphingomyelinase and endoglycoceramidase, which yielded essentially the same cpm as the untreated extracts (not shown).

To determine if radiolabelled sphingolipids were being lost to the medium or left on the petri dishes, the cells, medium, and dish were extracted together. Even under these conditions, turnover was rapid (c.f. Fig. 1, C and D); hence, such losses do not account for the rapid disappearance of [^{14}C]sphingosine.

Examination of the radiolabelled sphingolipids over this time course. The identification of some of the labelled sphingolipids is shown in Fig. 2. After one hour of incubation with [^{14}C]serine, CHO cells contain a substantial amount of radiolabel in ceramides (at Rf 0.95) and smaller amounts in sphingomyelin (Rf of 0.24 to 0.29) and other polar compounds (this identification of the ceramides and sphingomyelin was confirmed by chromatography in other solvent systems, data not shown). The most obvious changes that occurred during the chase were the disapperance of radiolabel from ceramides and an unidentified compound at Rf 0.65, and an increase in both of the doublet bands of sphingomyelin.

Since these lipids may be labelled in the fatty acid or headgroup moieties, as well as in the long-chain base backbone, different regions of the chromatogram were acid hydrolyzed and the long-chain bases were quantitated by TLC and scintillation counting. Changes in [^{14}C]ceramides and [^{14}C]sphingomyelin are shown in Fig. 3; there appeared to be a stoichiometric relationship between the synthesis of sphingomyelin and ceramide disappearance, which is consistent with the precursor/product relationship between these compounds.

Similar analyses with other regions of the chromatogram shown in Fig. 2 (and others) found either no change or losses in the radiolabelled long-chain bases. The bands at Rf 0.65 and 0.58 decreased from 754 and 383 cpm



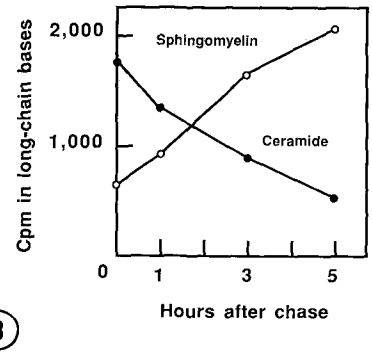


Figure 2. Radiolabelled lipids after incubation of CHO cells with [^{14}C]serine for 1 hour followed by a chase with unlabelled serine. The lipid extracts were incubated in base to cleave the glycerolipids and chromatographed on silica gel thin-layer plates developed with chloroform-methanol-acetic acid-water (56:35:4:2, v/v). The radiolabelled lipids were visualized by autoradiography and the ceramides and sphingomyelin were identified by comparison with authentic standards.

Figure 3. Comparison of the amounts of radiolabelled long-chain bases in ceramides and sphingomyelin after after incubation of CHO cells with [^{14}C]serine for 1 hour followed by a chase with unlabelled serine. These analyses were conducted as described in Fig. 2; however, the lipids were extracted from the TLC plates, acid hydrolyzed to release the free long-chain bases, which were identified by TLC and quantitated by scintillation counting as described in the text.

at time zero to 159 and 211 cpm after 5 h, respectively. About half of the sphingolipids that remained at the origin also turned over during this period (from 4072 to 1888 cpm). Although none of these species were further characterized, they illustrate that several sphingolipids are radiolabelled in the backbone moiety and degraded during this time course.

DISCUSSION

These results establish that approximately half of the [^{14}C]sphingosine that is incorporated into more complex sphingolipids upon incubating CHO cells with [^{14}C]serine for 1 h is turned over surprisingly quickly. Some of the changes reflect a redistribution of radiolabel among different sphingolipid types (as seen in the conversion of ceramide to sphingomyelin); however, a significant number of sphingolipids are degraded with a net loss of total [^{14}C]sphingosine. This probably does not reflect the overall rate of degradation of cellular sphingolipids; but rather, the turnover of a subpopulation that is both synthesized and degraded rapidly. These sphingolipids are probably hydrolyzed to [^{14}C]sphingosine, which is degraded via formation of sphingosine 1-phosphate and lytic cleavage to hexadecenal and ethanolamine phosphate (13).

The rapid turnover of sphingosine may be related to the growth and division of these cells, which divide every 12 h. One can envision that

the sphingosine is incorporated into a number of complex sphingolipids that are only utilized during a portion of the cell cycle. Consistent with this concept, Bremer et al. (14) and Usuki et al. (15) have suggested that gangliosides modulate the responsiveness of cells to growth factors. Furthermore, since sphingolipid turnover will proceed via sphingosine and/or lysosphingolipids, which are potent modulators of protein kinase C (1) and growth factor receptors (6), the purpose of the turnover of complex sphingolipids may be to liberate these potential "lipid second messengers.

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