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DIHYDROSPHINGOSINE GROWTH INHIBITION AND REPRESSION OF 3-KETODIHYDROSPHINGOSINE SYNTHETASE ACTIVITY IN BACTEROIDES MELANINOGENICUS

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SUMMARY. Addition of dihydrosphingosine or 3-ketodihydrosphingosine to growing cultures of Bacteroides melaninogenicus markedly reduced the activity of 3-ketodihydrosphingosine synthetase in extracts. Neither compound reduced the activity of a previously solubilized preparation of the enzyme. Dihydrosphingosine and synthetic acetyl ceramides inhibited the growth of the microorganism; 3-ketodihydrosphingosine was not inhibitory and reversed the growth inhibition caused by dihydrosphingosine. This reversal may indicate a preferential utilization of 3-ketodihydrosphingosine over dihydrosphingosine by the cells for the biosynthesis of complex sphingolipids.

In the scheme for sphingolipid synthesis in eukaryotic systems the first step involves the condensation of an acyl CoA with serine to form 3-ketodihydrosphingosine (3KDS) (1,2). This product is then reduced to dihydrosphingosine from which sphingosine is formed by dehydrogenation. The latter compound is then acylated to ceramide.

Morell and Radin (3) have shown that synthesis of 3-ketoceramide occurs in a system using mouse-brain microsomes, 3-ketodihydrosphingosine and stearoyl CoA. The role of 3-ketoceramide as an intermediate was studied by Shoyama and Kishimoto (4) who demonstrated conversion of 3-ketoceramide to ceramide in rat liver.

Sphingolipids are rare in bacteria but do occur in certain anaerobes such as Bacteroides melaninogenicus and B. thetaiotaomicron where they have been characterized in detail (5,6,7,8). During an investigation of sphingolipid biosynthesis in B. melaninogenicus, we had observed that dihydrosphingosine was inhibitory to the growth of the microorganism. This has led to the present study of the effect of dihydrosphingosine and other compounds on 3KDS synthetase activity.

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MATERIALS AND METHODS

Bacterial Culture. B. melaninogenicus (ATCC 29147) was grown in the basal medium used in previous studies (9) consisting of 3% Trypticase (BBL), 0.3% yeast extract (Difco) and 0.5% NaCl, pH 7.2. This was supplemented with 0.025% hemolized red blood cells and 0.1 μg/ml vitamin K_1.

To determine the growth inhibiting capacity of a sphingolipid, 0.1 ml of a 10 mg/ml ethanolic solution of the lipid was treated with 2 ml of hot (80°C) water to form a homogeneous emulsion. Aliquots of this emulsion were added to tubes containing 10 ml medium supplemented with vitamin K and with lysed red cells. The culture was seeded with a small inoculum and incubated (37°C, 20 hr) in an anaerobic jar containing 95% H_2/5% CO_2. Turbidities were read in a Klett-Sommerson colorimeter using a red filter.

3-ketodihydrosphingosine preparation. 3KDS was synthesized enzymatically as described previously (10). The enzyme preparation used was the 20,000 xg supernatant of sonicated B. melaninogenicus cells. The reaction mixture (37°C, 1 hr) consisted of 2 μmoles L-serine and 800 nmoles palmitoyl CoA in 0.5 ml supernatant. Unreacted acyl CoA and other water soluble components were removed by a modification of the partitioning procedure of Folch et al (11). The yield was approximately 70 nmoles 3KDS assayed in a separate experiment with 3-[^{14}C]-L-serine and palmitoyl CoA as described below.

Assay of 3KDS synthetase activity in extracts of B. melaninogenicus.

Cultures were grown in anaerobic jars modified so that sample addition and removal can be made without disturbance of the reduced atmosphere (12). Synthetase activity was determined in extracts of cells from 20 ml culture. The cells were sedimented, washed in phosphate buffer (0.05M, pH 7.4), resuspended (0.05g/1.6 ml) and sonicated 3 times for 10 sec with 1 min intervals. The sonicated cells were centrifuged (20,000 xg/5 min) and the sediment discarded. The reaction mixture contained 3-[^{14}C]-L-serine (2 μCi; 2 μmoles) palmitoyl CoA (200 nmoles) and 20,000 xg supernatant, final volume 0.5 ml. This was incubated 37°C, 20 min) with vigorous stirring and the reaction stopped by the addition of 9.5 ml $CHCl_3$: CH_3OH (2:1). The lipids were then extracted and chromatographed. 3KDS was located by radioautography after which the spots were scraped off the plates and counted (10).

Dihydrosphingosine, sphingosine and n-palmitoyl dihydrosphingosine (ceramide) were purchased from Miles-Yeda; n-acetyl erythro sphingosine and n-acetyl threo sphingosine were gifts from Dr. M. Sribney.

RESULTS

The inhibition of growth of B. melaninogenicus by dihydrosphingosine is shown in Fig. 1. At a concentration of 4 μM inhibition is complete and at 2 μM, approximately 30% inhibition occurred. Similar results were obtained using erythro sphingosine or the synthetic ceramides n-acetyl erythro sphingosine and n-acetyl threo sphingosine. However 3-ketodihydrosphingosine and n-palmitoyl dihydrosphingosine did not inhibit growth at 8 μM (Table 1). Because the growth inhibitory effect of dihydrosphingosine could conceivably be the result of inhibition of 3KDS synthetase, the activity of this enzyme was determined in ex-

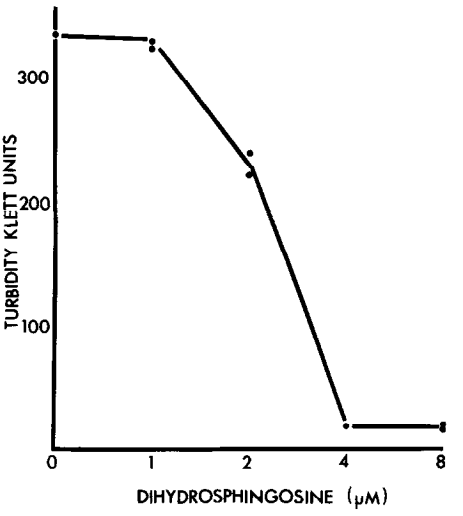

Fig. 1. Growth inhibition of B. melaninogenicus by dihydrosphingosine. Dihydrosphingosine was added as an emulsion at the concentrations indicated to tubes of medium supplemented with a dilution of red cells and vitamin K_1. They were seeded with 0.1 ml of a 1/10 dilution of a 2 day culture of the microorganism and turbidities read after 20 hr anaerobic incubation.

Table 1. Growth Inhibition of B. melaninogenicus by certain Sphingolipids.

*Inhibitory	**Non-Inhibitory
erythro dihydrosphingosine	3-ketodihydrosphingosine
erythro sphingosine	n-palmitoyldihydrosphingosine
n-acetyl erythro sphingosine	
n-acetyl threo sphingosine	

* These compounds were added as an ethanolic emulsion prepared as described in Materials and Methods. They completely inhibited growth at a level of 4 μM.
** These compounds were not inhibitory at 8 μM.

tract of cells incubated in the presence of 4 μM dihydrosphingosine.

In the first experiment, synthetase activity was determined in extracts from cells incubated for a short period of time following inoculation and which were incubated with and without 4 μM dihydrosphingosine. The media were seeded with a large inoculum (c. 50 Klett units initial turbidity) to obtain sufficient cells for subsequent enzyme determinations. The control culture showed a significant increase in enzyme activity during the 3-4.5 hr of incubation, whereas, in ex-

tracts from the culture containing dihydrosphingosine there was no increase in enzyme activity during the same period (Fig. 2). The growth rate of the culture was not affected during the limited growth (approximately 2 x doubling) of the cells.

With an actively growing culture, 3KDS synthetase activity increased proportionally to an increase in turbidity of the culture (Fig. 3) whereas 1 hr following addition of 4 μM dihydrosphingosine, there was a drop in synthetase activity in the extracts; this persisted for the 2 hr experimental period. The growth rate of this actively growing culture was only minimally affected by the dihydrosphingosine. A similar drop in synthetase activity was found following addition of 3-ketodihydrosphingosine to an actively growing culture.

While in most experiments synthetase activity was determined on 20,000 xg supernatant of sonicated cells, an enzyme determination on the crude sonicate of a dihydrosphingosine treated culture showed a depressed synthetase activity similar to that found with the supernatant.

In further experiments, the effect of dihydrosphingosine on the activity of the synthetase solubilized by sonication (10) was determined. Dihydrosphingosine added at 5 times the concentration required for complete growth inhibition (20 μM) (Fig. 1) to a 20,000 xg supernatant of the sonicate did not affect activity of the soluble 3KDS synthetase assayed as described in Materials and Methods. 3KDS added at a similar concentration also did not inhibit activity of solubilized synthetase

Reversal of Dihydrosphingosine Growth Inhibition

Dihydrosphingosine might be exerting its growth inhibiting effect by its depression of 3KDS synthetase activity and subsequent cessation of sphingolipid biosynthesis. If 3KDS is utilized more efficiently than dihydrosphingosine for sphingolipid synthesis, then the growth inhibition by dihydrosphingosine should be relieved by 3KDS. Dihydrosphingosine (4μM) was added to a culture of B. melaninogenicus and dihydrosphingosine plus an equimolar concentration of 3KDS, to a second culture. 3KDS was added as an unpurified lipid extract of the biosynthetic reaction mixture since we have found that 3KDS is not stable following

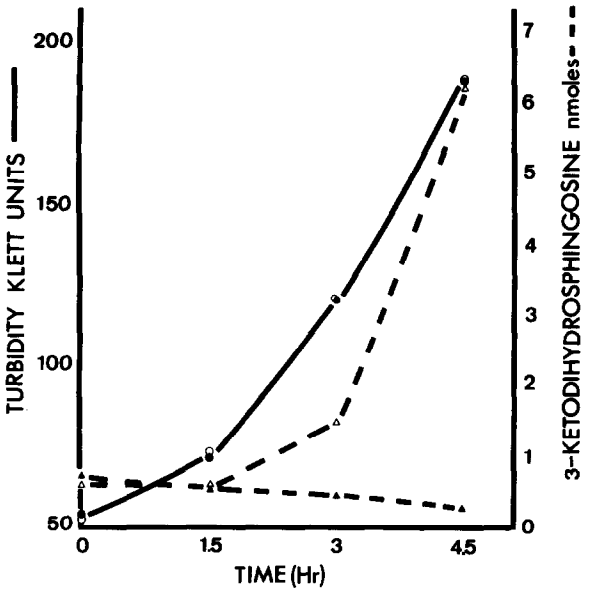

Fig. 2. Inhibition of 3KDS synthetase activity with dihydrosphingosine. A large inoculum of cells of B. melaninogenicus was placed in 2 flasks and incubated anaerobically with and without 4 μM dihydrosphingosine. Enzyme determinations were performed on cells from 20 ml medium removed at the times indicated as described in Materials and Methods.

△ Control culture ▲ plus dihydrosphingosine

thin layer chromatography. In order to eliminate the possibility that compounds other than 3KDS in lipid extracts could reverse the dihydrosphingosine inhibition, a control tube containing dihydrosphingosine plus a lipid extract of a 20,000 xg supernatant of sonicated cells was included. Following incubation for 72 hr, growth occurred in the culture supplemented with the 3KDS containing lipid extract; no growth was found in the culture to which dihydrosphingosine alone was added or in the control.

DISCUSSION

White and co-workers (5,6,7) have shown that the long chain bases in the sphingolipids of B. melaninogenicus consist of dihydrosphingosines (sphinganines) some with branch chains but a significant proportion (18%) contained C_{18} straight chain bases. Stoffel et al (8) reported that dihydrosphingosine is incorporated into the sphingolipids of B. thetaiotaomicron, and we have shown previously that in B. melaninogenicus incorporation of 3KDS and dihydrosphingosine occurs ex-

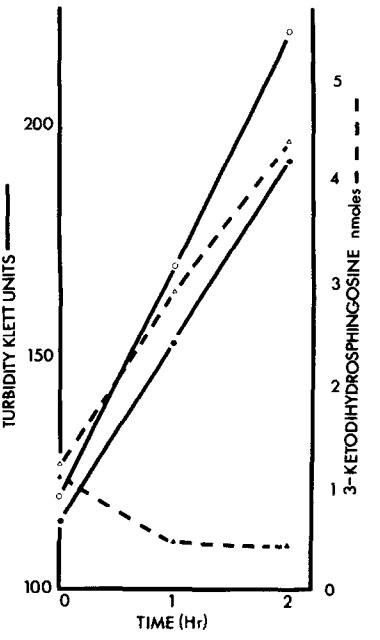

Fig. 3. Dihydrosphingosine inhibition of 3KDS synthetase activity in an actively growing culture. Dihydrosphingosine (4 μM final concentration) was added to a flask containing a mid log phase culture of B. melaninogenicus. Samples (20 ml) were removed at intervals from this and a control culture for enzyme determinations.

Δ Control culture ▲ plus dihydrosphingosine

clusively in ceramide phosphorylethanolamine and ceramide phosphorylglycerol - no other phospholipids were labeled (9).

Dihydrosphingosine exerts a dramatic effect on the activity of 3KDS synthetase in intact cells. For example, in a culture examined after inoculation where initial synthetase activity is low, dihydrosphingosine prevents any increase in activity and with an actively growing culture, synthetase activity drops to a very low level following dihydrosphingosine addition. 3KDS exerted a similar effect on synthetase activity. This decreased enzyme activity is not related to a change in extractability of the enzyme since crude sonicates showed activities similar to 20,000 xg supernatants.

Following solubilization, the activity of the enzyme was not depressed by dihydrosphingosine. 3KDS synthetase is a membrane associated enzyme and the dihydrosphingosine taken up by the cells presumably exerts its effect following

permeation of the membrane. With a solubilized enzyme preparation, however, the concentration in the water phase of dihydrosphingosine (added as an emulsion) is probably too low to influence enzyme activity.

A reduction in synthetase activity would result in a corresponding reduction in sphingolipid synthesis and we have indicated in previous experiments (13) that there is a relationship between sphingolipid synthesis and cell division. However, the addition of dihydrosphingosine to cultures at growth inhibitory concentrations did not affect the growth rate for 2 doublings.

Since dihydrosphingosine and 3KDS depress synthetase activity in a similar manner, the difference in growth inhibitory properties of the two compounds is of interest. The inhibitory nature of dihydrosphingosine but not 3KDS may indicate that 3KDS is utilized for sphingolipid synthesis whereas dihydrosphingosine is not utilized to a significant degree. Further evidence for this view is the reversal of dihydrosphingosine growth inhibition with 3KDS. The experiments in which low levels of dihydrosphingosine are incorporated into ceramide phosphoryl ethanolamine and ceramide phosphoryl glycerol (9) do not rule out its conversion to 3KDS prior to ceramide formation. Our results, therefore, would argue against the participation of dihydrosphingosine in sphingolipid biosynthesis in B. melaninogenicus and would so favor an alternative scheme where 3KDS → 3-ketoceramide → ceramide.

ACKNOWLEDGMENT

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