Sphingolipid Biosynthesis by Rat Liver Cells: Effects of Serine, Fatty Acids and Lipoproteins

TRUDY O. MESSMER, ELAINE WANG, VICTORIA L. STEVENS AND ALFRED H. MERRILL, JR.

Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322

ABSTRACT The effects of circulating factors that might influence de novo sphingolipid biosynthesis were examined with rat liver cells by following the incorporation of [14C]serine into sphingosine and sphinganine, the predominant long-chain base backbones of hepatic sphingolipids. The rate of long-chain base formation depended on the concentration of [14C]serine in the medium and exhibited saturation kinetics. Long-chain base formation was stimulated by another precursor, palmitic acid, but stearic, oleic, linoleic and linolenic acids were inhibitory. This kinetic behavior indicates that long-chain base formation in liver is affected by the availability of the substrates of the initial enzyme of this pathway, serine palmitoyltransferase. Since liver is also exposed to sphingolipids associated with circulating lipoproteins, the effects of various lipoprotein fractions were determined and each appeared to decrease long-chain base formation. These results suggest that hepatic long-chain base biosynthesis can be stimulated by increases in the circulating levels of the precursors serine and palmitic acid whereas some other fatty acids and lipoproteins decrease the flux through this pathway. J. Nutr. 119: 534–538, 1989.

INDEXING KEY WORDS:
• sphingolipids • serine • palmitic acid
• lipoproteins • cholesterol • liver
• biosynthesis

Sphingolipids are major constituents of lipoproteins, many cellular membranes, and milk. They are formed by elaboration of a backbone moiety called a long-chain (or sphingoid) base, of which sphingosine, sphinganine, and phytosphingosine are the most common species (1). Long-chain base biosynthesis involves the condensation of serine and a fatty acyl-CoA to form 3-ketosphinganine in a pyridoxal 5'-phosphate-dependent reaction catalyzed by serine palmitoyltransferase (EC 2.3.1.50) (2–8). The 3-ketosphinganine is further modified to yield ceramides, sphingomyelin and other complex sphingolipids (7, 8).

Recent studies of [14C]serine incorporation into long-chain bases by mouse LM cells have found that the rate depends on the concentration of serine and palmitate in the culture media (9) in a manner that exactly mirrors the kinetic properties of serine palmitoyltransferase in vitro. The implication of these findings is that long-chain base biosynthesis in vivo may also be sensitive to the availability of the precursors of this pathway which are known to fluctuate during fasting and feeding (10–11). However, the validity of extrapolating these findings with a transformed cell line to an in vivo situation must await studies of other more normal cells.

Long-chain base biosynthesis also appears to be influenced by lipoproteins. The incorporation of radiolabeled serine into sphingosine has been found to be decreased by low density lipoproteins (LDL) in studies of human fibroblasts (12), Chinese hamster ovary cells (13) and renal cells (14). The mechanism of this inhibition is unknown.

To determine if these factors also affect long-chain base biosynthesis in liver, which is central to lipid and lipoprotein metabolism, similar experiments were conducted with rat hepatocytes and a liver cell line. Long-chain base biosynthesis from [14C]serine was stimu-

1Supported by National Institutes of Health grant GM33369, and by funds from the Georgia affiliate of the American Heart Association.
3Present address: Department of Dermatology, Emory University.
4To whom reprint requests should be addressed.
5The nomenclature used in this paper generally conforms to the recommendations of IUPAC/IUB. The terms sphingosine and sphinganine have been used for the long-chain bases with and without the 4-trans-double bond without specification of the alkyl chain length except where otherwise noted.

lated by the addition of serine and palmitic acid and inhibited by different lipoproteins. Hence, it is likely that hepatic sphingolipid metabolism in vivo is modulated to some extent by variations in the amounts of these factors in circulation.

MATERIALS AND METHODS

Chemicals, media, radioisotopes and lipoproteins. Ham's F12 and MCDB 301 were purchased from Gibco (Grand Island, NY). Collagenase (Type IV) and all other tissue culture reagents were obtained from Sigma [St. Louis, MO]. The [U-14C]serine was from ICN Radiochemicals (Irvine, CA, 135 mCi/mmol) and Amersham (Arlington Heights, IL, 55 mCi/mmol).

The sphingolipid standards and fatty acids were purchased from Sigma. Other reagents, including those used for assays of serine palmitoyltransferase, were as described in other reports from this laboratory (6–9, 13).

The very low density (VLDL), low density (LDL) and high density (HDL) lipoproteins were isolated from human serum by ultracentrifugation (15) with the addition of 0.1% EDTA and 0.05% α-tocopherol to minimize oxidation. The density (d) ranges used to isolate the lipoprotein fractions were: d < 1.005 g/ml for VLDL, 1.005 < d < 1.063 g/ml for LDL, and 1.063 < d < 1.25 g/ml for HDL. The lipoprotein-enriched fractions had the expected apo-lipoprotein compositions when examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (16).

Cells. Hepatocytes were prepared by the method of Moldeus, Hogberg and Orrenius (17) using livers from weaned, male, white rats [125–200 g, Kng:(SD)BR, King Animal Laboratories, Oregon, WI] fed ad libitum a nonpurified diet (Purina Rat Chow,Ralston Purina, St. Louis, MO). Cell viabilities were initially >95% as assessed by the exclusion of 0.2% trypan blue, and the data were discarded when the viabilities decreased to less than 88–91% over the course of the experiment.

The liver cell line was obtained from Ed Conrad and many of its characteristics have been published (18). One useful feature of this system is that the cells grow to confluence and stop; hence, lipid synthesis by confluent cultures reflects cell maintenance and lipoprotein synthesis rather than cell growth. The liver cell line was grown at 37°C in Ham's F12 medium supplemented with 10% fetal calf serum, penicillin-G (61 mg/l), streptomycin (100 mg/l) and sodium bicarbonate (1.176 g/l) in a humidified atmosphere of 5% CO2 in air. The medium was changed every 3–4 days and cells were routinely subcultured at approximately 60% confluence. Cell numbers were counted with a hemocytometer after the cells were released from the dishes by mild trypsin treatment.

Incubation of cells with [14C]serine. The hepatocytes were allowed to settle and the pellet was washed thrice with Dulbecco's phosphate buffered saline (PBS).

Unless otherwise noted, they were resuspended at 10^6 cells/ml in preoxygenated Krebs-Henseleit buffer, pH 7.4 [per liter: 6.9 g NaCl, 0.36 g KCl, 0.13 g KH2PO4, 0.295 g MgSO4•7H2O, 0.374 g CaCl2•H2O, and 2.0 g NaHCO3] supplemented with 2% bovine serum albumin (BSA, Sigma) and approximately 0.1 μmoles (10 μCi) of [14C]serine. The preoxygenation was conducted by bubbling the medium with 95% O2/5% CO2 for at least 30 min before adding BSA and the cells. After addition of the cells, the vials were tightly capped and incubated at 37°C for 2 h in a New Brunswick Gyrorotary shaker (Edison, NJ) at approximately 100 rpm. For experiments exceeding several hours, the cells and media were kept sterile. When fatty acids were added to the cells, they were first solubilized as the complexes with fatty acid free BSA (Sigma).

The cultured cells were incubated with [14C]serine in MCDB 301 prepared minus unlabeled serine (Gibco). This medium has essentially the same composition as the growth medium (Ham's F12); however, the lack of serine minimized dilution of the radiolabel. In experiments where the hepatocytes or cultured cells were incubated with additional components, these were added to the culture media to attain the desired final concentrations. The lipoprotein experiments involved the addition of 50 μg of each of the lipoproteins 24 h prior to replacement of the medium supplemented with 10 μCi of [14C]serine.

Lipid analyses. The 14C-long-chain bases were analyzed as described previously [8, 9]. The amount of radiolabel was corrected using an estimate for silica gel quenching obtained by adding a known amount of radiolabel to the chromatogram.

In vitro assay of serine palmitoyltransferase. Cells were disrupted by sonication and assayed as we have described previously for Chinese hamster ovary cells (13).

Statistical methods. Unless otherwise noted, the data are given as the means ± SD for triplicate measurements with a single cell preparation; the phenomena described in this report were consistently observed with several cell preparations and representative findings have been presented. Within each experiment the significance of the differences between groups was tested by the Student's t-test for unpaired data.

RESULTS

Long-chain base biosynthesis by isolated hepatocytes. Shown in Table 1 are the dpm found in 3-keto-, sphinganine, sphinganine, and sphingosine when isolated hepatocytes were incubated with [14C]serine followed by acid hydrolysis to liberate the free long-chain bases of more complex sphingolipids. The amount of total radiolabel after 2 h was almost double that after 1 h. The amount in sphinganine appeared to approach a steady state in the first hour whereas sphingosine continued to increase during the second hour. This
probably reflects the precursor/product relationship between these two long-chain bases [4, 8]. No free [i.e., not amide-linked] long-chain bases were detected, which suggests that these intermediates are rapidly converted to amide-linked sphingolipids such as dihydroceramides, ceramides, sphingomyelin, etc., as has been observed in LM cells [8].

**Effects of varying the concentration of [14C]Serine.** The rate of incorporation of [14C]serine into sphingosine and sphinganine was affected by the concentration of serine in the medium (Fig. 1). A relatively simple saturation curve was obtained with an apparent $K_{1/2}$ of 0.3 mM and a $V_{max}$ of approximately 1.2 pmol/[min·10^6 cells], assuming that the cellular specific activity of [14C]serine is comparable to that of the added precursor. One possible explanation for the kinetic behavior of serine in Figure 1 is that varying the amount of extracellular serine alters the intracellular concentration and, thus, the activity of serine palmitoyltransferase which has a $K_m$ in this same range [6]. To test this hypothesis, the concentration of serine in the cells was determined. As shown in Table 2, intracellular serine increased by about twofold when the extracellular serine concentration was increased to 0.5 or 1.0 mM. Since the volume of hepatocytes was approximately 5 µL/mg protein (D. P. Jones, personal communication), this corresponds to serine concentrations of about 0.38 mM (for 1.9 nmol) to 0.7 mM (for 3.5 nmol).

**Effects of fatty acids.** To determine if other precursors of long-chain base formation could alter the flux through this pathway in intact hepatocytes, cells were assayed in media containing fatty acids. The addition of 0.3 mM palmitic acid (16:0, added as the complex with BSA) increased the incorporation of radiolabeled serine into total long-chain bases by about 40% (Fig. 2). This stimulation was not observed with saturated fatty acids of other chain lengths [e.g., myristic (14:0) and stearic (18:0) acids] or with unsaturated fatty acids [oleic, 18:1; linoleic, 18:2; or linolenic, 18:3]. In many instances, the other fatty acids decreased the incorporation of radiolabel relative to the control (no added fatty acid). Addition of fatty acid–free BSA alone had no effect [not shown]. These results indicate that long-chain base formation can be stimulated fairly exclusively by palmitic acid, which is the same specificity that has been seen with serine palmitoyltransferase in vitro [6, 19] and with intact LM cells [8]. The cause of the inhibition by other fatty acids is not known, but may be due to their competition with endogenous palmitic acid for CoA.

**Serine palmitoyltransferase activities in vitro.** To compare the rate of long-chain base synthesis in intact cells with the activity of serine palmitoyltransferase, this enzyme was assayed using disrupted hepatocytes. Under the optimal in vitro assay conditions, the activity was 6.0 ± 1.1 pmol/[min·mg cells]. This is equiv-

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Long-chain base formed</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Ketosphinganine</td>
<td>&lt; 150</td>
<td>&lt; 150</td>
</tr>
<tr>
<td>Sphinganine</td>
<td>2380 ± 25</td>
<td>3010 ± 250</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>2440 ± 244</td>
<td>5580 ± 682</td>
</tr>
<tr>
<td>Total</td>
<td>4820</td>
<td>8590</td>
</tr>
</tbody>
</table>

*1Mean ± SD (n = 3). Values reflect the dpm in long-chain bases after acid hydrolysis to cleave more complex sphingolipids. No dpm above background were detected if the acid hydrolysis step was omitted.

*2The minimum dpm that could have been detected above background.*

**FIGURE 1** Long-chain base synthesis by hepatocytes in culture media containing different concentrations of [14C]serine. The long-chain bases were isolated and quantitated after acid hydrolysis; the dpm [mean ± SD, n = 3] in sphinganine and sphingosine were added to give total long-chain bases. The insert shows a replot of data.

**TABLE 2**

<table>
<thead>
<tr>
<th>Extracellular serine concentration</th>
<th>Amounts of cellular serine after different periods of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>0 min</td>
</tr>
<tr>
<td>None</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>0.50</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0 ± 0.2</td>
</tr>
</tbody>
</table>

*1Means ± SD, n = 3. Means designated by a superscript were significantly different (P < 0.05) from the time zero control.*
This report presents the first description of long-chain base synthesis by isolated liver cells. In agreement with previous studies with liver microsomes [6, 7] and mouse LM cells [8], the incorporation of [14C]serine into the sphingosine and sphinganine backbones of more complex sphingolipids could be demonstrated. Furthermore, as has also been observed previously [8], none of the free intermediates (3-keto-sphinganine, sphinganine, or sphingosine) accumulate. This implies that the first step of long-chain base formation is rate limiting, the next crucial step probably occurs immediately after ceramide formation when other enzymes partition this intermediate toward different complex sphingolipids.

This kinetic behavior may protect the cells from free long-chain bases which have potent effects on cellular function and can be toxic to cells at concentrations as low as 10 μM [13, 20–22]. Sphingosine and other long-chain bases are potent inhibitors of protein kinase C [20–22] which mediates the effects of a wide variety of hormones, growth factors and tumor promoters. Liver cells can theoretically form up to 4 pmol of long-chain bases/[min·10⁶ cells] based on the serine palmitoyltransferase activity. Hence, based on a hepatocyte volume of approximately 5 μl/[10⁶ cells], the concentration of sphingosine could increase by about 1 μM every minute if removal of these compounds by acylation did not occur rapidly.

These studies have identified several possible associations between nutrition and sphingolipid metabolism. The influence of the extracellular serine concentration on long-chain base synthesis may indicate that sphingolipid formation in vivo fluctuates pre- and postprandially since plasma serine increases from 0.32 mM at fasting to 0.58 mM in rats fed 9% casein [10]. Furthermore, plasma and liver serine levels fluctuate over a 30-fold range under other dietary conditions [11]. The mechanism of the effects of serine on long-chain base formation could be that serine transport and/or synthesis de novo is limiting, or that the intracellular concentra- tion affects serine palmitoyltransferase in parallel to the affinity of this enzyme for serine [as occurs in LM cells] [8]. Whichever is the case, the stimulation of sphingolipid synthesis by serine could facilitate sphingomyelin synthesis postprandially when it is needed as a constituent of VLDL and other lipoproteins.

The ability of serine to increase long-chain base biosynthesis accounts for the observation of Bjerve [23] that the incorporation of [14C]serine into sphingomyelin by hepatocytes increased from 11% of the total label in phospholipids at 0.2 mM serine to 42% at 2.25 mM. Our finding of a 2.4-fold increase in radiolabeled sphingomyelin upon increasing the serine concentration from 0.1 to 1 mM indicates that the phenomenon observed by Bjerve is due to increased long-chain base formation relative to the other lipid classes that would contain radiolabel from serine [i.e., phosphatidylserine fol-
allowed by phosphatidylethanolamine and phosphatidylcholine.

The further stimulation of long-chain base formation by palmitic acid indicates that this pathway can also be affected by dietary fat; however, this is likely to be complex because other saturated fatty acids (particularly stearic acid) and unsaturated fatty acids appear to have an opposite effect. Since palmitic acid is only about 20% of the fatty acids in plasma of mammals [see 24 for data for rats], the effects of other fatty acids are likely to be manifested in vivo. Considering the possible differences between stearic acid versus palmitic acid in atherogenesis, this association between different fatty acids and sphingolipid formation could be important to the regulation of other aspects of lipid metabolism. There appears to be an association between sphingomyelin and cholesterol [25]; we have observed that incubating liver cells in 1 mM serine increases the amount of radiolabel incorporated into cholesterol from [14C]acetate [26].

In addition to circulating free fatty acids, serum lipoproteins have an effect on long-chain base formation. These investigations found that VLDL decrease [14C]serine incorporation into long-chain bases and, to a lesser extent, LDL was also inhibitory as has been seen previously with other cell types [12–14]. The mechanism of this inhibition is unknown but could involve receptor-mediated down regulation of this pathway, feedback inhibition by sphingolipids taken up from the lipoproteins, or modulation by other factors (for example, the uptake of unsaturated fatty acids from the lipoproteins).

The nutritional modulation of sphingolipid metabolism has previously received little attention. It is hoped that these initial observations that serum, fatty acids and lipoproteins affect this pathway will encourage investigations in this area.

**LITERATURE CITED**


