Inhibition of serine palmitoyltransferase activity in rabbit aorta by L-cycloserine

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Abstract Serine palmitoyltransferase (EC 2.3.1.50) initiates the biosynthesis of sphingolipids. Its activity is induced in the aortas of rabbits fed a Purina lab chow supplemented with 2% cholesterol (Williams, R. D., D. S. Sgoutas, and G. S. Zaatari. 1986. *J. Lipid Res.* **27:** 763–770). Induction occurs during atherogenesis in parallel with increased arterial sphingomyelin concentrations. In this study, L-cycloserine was shown to be a potent inhibitor of serine palmitoyltransferase in aortas from New Zealand White rabbits. Activity was reduced in vitro by 50% using 5 μM L-cycloserine with 50 μg of microsomal protein. To assess in vivo inhibition, L-cycloserine was administered by intraperitoneal injection to rabbits maintained on either a standard Purina laboratory chow or one supplemented with 2% cholesterol. Serine palmitoyltransferase activity was inhibited by 76% in the aortas of rabbits on the standard chow 4 hr after a single 25 mg/kg body weight dose and 52% after a 10 mg/kg dose. Activity was reduced by 36% in animals on the standard chow and by 37% in the cholesterol-fed group after 1 week of daily doses. These experiments demonstrate that L-cycloserine inhibits serine palmitoyltransferase in aorta, and thus may be used to reduce sphingomyelin concentrations during experimental atherogenesis.—**Williams, R. D., D. S. Sgoutas, G. S. Zaatari, and R. A. Santoianni.** Inhibition of serine palmitoyltransferase activity in rabbit aorta by L-cycloserine. *J. Lipid Res.* 1987. **28:** 1478–1481.

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Serine palmitoyltransferase initiates the biosynthesis of sphingolipids by catalyzing the condensation of L-serine with palmitoyl-CoA to yield the long-chain base 3-ketosphinganine (1–6). Other fatty acyl-CoA thioesters are utilized as substrates to generate the corresponding long-chain bases[2] (6, 7). It is uncertain whether these long-chain bases are directly N-acylated or are modified and then used to synthesize the ceramide precursors of complex sphingolipids (8).

Serine palmitoyltransferase activity was induced in rabbit aorta during experimental atherogenesis (9). Induction occurred prior to the detection by light microscopy of cellular proliferation or sudanophilic lipid accumulation and continued over the duration of feeding a cholesterol-supplemented diet. This induction may contribute to elevated synthesis of sphingolipids, particularly sphingomyelin, and the concurrent development of atheroma (10–14).

Sphingolipid synthesis can be inhibited in vitro and in vivo by cycloserine (4-amino-3-isoxazolidinone) through irreversible inactivation of serine palmitoyltransferase (15, 16). Herein, we describe the inhibition of serine palmitoyltransferase in rabbit aorta by L-cycloserine. The potential use of L-cycloserine in reducing aortic sphingolipid concentrations during atherogenesis is discussed.

MATERIALS AND METHODS

Chemicals

Palmitoyl-CoA was purchased from P-L Biochemicals (Milwaukee, WI) and from Sigma Chemical Co. (St. Louis, MO). Purity was determined by gas–liquid chromatography of their methyl esters (17). [G-^{3}H] L-Serine and [3-^{3}H]L-serine, purchased from Amersham (Arlington Heights, IL) and ICN Radiochemicals (Cleveland, OH), respectively, were purified by column chromatography over Dowex 50W-X8, then diluted with unlabeled L-serine to specific activities ranging from 20,000 to 50,000 cpm/nmol as previously described (4). Some of the L-cycloserine was provided by the Lilly Research Laboratories (Indianapolis, IN) and the rest was purchased from Sigma. All other chemicals were reagent grade or better.

Abbreviations and nomenclature: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal 5'-phosphate. Serine palmitoyltransferase is the name recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, but it has also been referred to as 3-ketosphinganine synthetase, 3-ketodihydrosphingosine synthetase, and the condensing enzyme.

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[2]Several different long-chain bases exist, but the most common are sphinganine, sphingosine, and phytosphingosine.

Animals

Young, male New Zealand White rabbits weighing 1.5–2.5 kg were divided into groups of three animals and placed on either a standard Purina laboratory chow containing approximately 0.006% cholesterol or the same formula supplemented with 2% cholesterol w/w). The cholesterol-supplemented diet was prepared from Purina chow within weeks of its delivery to us, then maintained fresh by refrigeration at 4°C. The rabbits on the cholesterol diet were fed ad libitum, and the controls were pair-fed. Both groups received water ad libitum. Animals were killed under ether anesthesia.

Administration of L-cycloserine

L-Cycloserine was dissolved in 5 ml of phosphate-buffered saline at pH 7.2 immediately before its use and was administered by intraperitoneal injection. The activity of serine palmitoyltransferase was measured in microsomes from aortas of animals fed standard chow 4 hr after a single injection of 10 or 25 mg per kg body weight, and in animals fed either standard chow or a cholesterol-supplemented chow after 1 week of daily single injections of 10 mg per kg body weight.

Isolation of microsomes

Each aorta, from the valve to the bifurcation, was removed, rinsed with cold phosphate-buffered saline, and blotted dry. The adventitia was removed. The remaining tissue consisting of intima and media was weighed and used for the isolation of microsomes. The tissue was placed in a cold 20% (w/v) solution containing 0.25 M sucrose, 50 mM HEPES, pH 7.4, at 4°C, 5 mM EDTA, and 5 mM DTT, and minced with scissors, then homogenized at 4°C in three 15-sec treatments with a Brinkman Polytron PT 10. The homogenates were centrifuged for 10 min at 22,000 *g* in a Beckman L5-75B ultracentrifuge (50Ti rotor) yielding supernatant fractions that were centrifuged for 40 min at 150,000 *g*. The resulting microsomal pellets were suspended in 20% glycerol, 5 mM HEPES, pH 7.4 (at 25°C), 5 mM EDTA, and 5 mM DTT on a 500 μl/g original tissue basis using a ground-glass hand-held homogenizer and immediately frozen in aliquots at −60°C (9). Protein was measured by a modification of the Lowry method with bovine serum albumin as the standard (18).

Serine palmitoyltransferase assay

The incorporation of aqueous-soluble [^{3}H]serine into the chloroform-soluble long-chain base 3-ketosphinganine was used to measure serine palmitoyltransferase activity (9). Each tube (100 μl, final volume) contained 0.1 M HEPES (pH 8.3, at 25°C), 5 mM DTT, 2.5 mM EDTA, 1 mM [^{3}H]L-serine, 50 μM PLP, and from 50 to 200 μg of microsomal protein. The reaction was initiated with the addition of palmitoyl-CoA to an assay concentration of 0.2 mM. Control tubes contained all of the assay constituents except for palmitoyl-CoA which was substituted with water. The tubes were placed in a water bath maintained at 37°C and allowed to incubate with gentle agitation. The reactions were performed in dim light to protect the PLP. Assays were terminated after 10 min of incubation with the addition of 1.5 ml of chloroform–methanol 1:2 (v/v). One ml of chloroform and 2 ml of water were added and the products were then separated from reactants by extraction using the method of Bligh and Dyer (19). Sphinganine (20 μg) was added during the extraction to facilitate recovery. The chloroform phase was washed a total of three times with water made basic to remove unreacted [^{3}H]serine. Radioactivity (cpm) from the blanks was subtracted from experimental group radioactivity (cpm) in the calculations.

RESULTS

Inhibition of serine palmitoyltransferase

L-Cycloserine was found to be an inhibitor of serine palmitoyltransferase in aorta. Its in vitro activity was reduced by about 50% using 5 μM L-cycloserine and 50 μg of microsomal protein. A 90% inhibition was achieved with a tenfold increase of L-cycloserine. In vivo inhibition was demonstrated with a 76% reduction in activity in animals killed 4 hr after a single 25 mg/kg body weight dose and 52% inhibition after a 10 mg/kg dose **(Table 1)**. More than 80% of the enzyme present in the microsomes after the 25 mg/kg dose was holo enzyme based on activity measurements with and without the addition of PLP to the assays, suggesting that there was not a substantial removal of the cofactor in vivo by cycloserine through oxime formation (20).

Serine palmitoyltransferase activity was inhibited by 36% in animals fed Purina chow and injected daily with 10 mg/kg doses of L-cycloserine for 1 week **(Table 2)**. Ac-

TABLE 1. Activity of serine palmitoyltransferase in aorta 4 hr after intraperitoneal injection of L-cycloserine

No Cycloserine	Cycloserine (10 mg/kg)	Cycloserine (25 mg/kg)	Cycloserine (25 mg/kg) − PLP[a]
6.5	3.6	2.0	1.8
5.9	2.0	0.9	0.7
2.7	1.5	0.6	0.4
5.0 ± 1.18[b]	2.4 ± 0.63	1.2 ± 0.43	1.0 ± 0.43
	$P < 0.10$	$P < 0.10$	$P < 0.20$[c]

All rabbits were fed a standard Purina lab chow. There were three rabbits in each group, and each value is from a single animal. Values are expressed as pmol product per min per mg microsomal protein.

[a] Assays were identical to the rest except PLP was not included.

[b] Mean ± SEM.

[c] Compared to the group with 25 mg/kg cycloserine and PLP (column 3).

TABLE 2. Activity of serine palmitoyltransferase after 1 week of daily intraperitoneal injections of L-cycloserine

Purina Chow No Cycloserine	Purina Chow Cycloserine (10 mg/kg)	Purina Chow + 2% Cholesterol No Cycloserine[a]	Purina Chow + 2% Cholesterol Cycloserine (10 mg/kg)
5.8	3.0	8.1	4.8
4.0	3.0	4.4	3.3
3.7	2.8	4.2	2.4
4.5 ± 0.66[b]	2.9 ± 0.07	5.5 ± 1.26	3.5 ± 0.70
	$P < 0.10$[c]		$P < 0.10$[c]

There were three rabbits in each group. Each value is from a single animal. Values are expressed as pmol product per min per mg microsomal protein.

[a] Data from reference 9.

[b] Mean ± SEM.

[c] Compared to group with Purina chow–no cycloserine (column 1).

tivity was reduced by 22% in animals treated similarly, but on the cholesterol-supplemented diet, when compared to controls on the standard chow. Less inhibition in the cholesterol-fed group is due to concurrent induction of serine palmitoyltransferase as a result of the diet, i.e., activity increases in proportion to atherogenesis over the duration of feeding cholesterol (9). When compared to cholesterol-fed controls, activity was reduced by 37%.

DISCUSSION

L-Cycloserine was demonstrated to be a potent inhibitor of serine palmitoyltransferase, the initial enzyme of sphingolipid synthesis in aorta. The 76% inhibition of activity in microsomes from aorta of rabbits killed 4 hr after a single intraperitoneal dose of 25 mg/kg reveals a relatively rapid distribution of the drug into constituent cells (endothelial and smooth muscle) of the aortic intima and media. Similarly, Sundaram and Lev (16) reported that a single 100 mg/kg dose of L-cycloserine inhibits mouse brain serine palmitoyltransferase by 80% at 2 hr followed by a 75% recovery at 16 hr. We found a 36% inhibition of microsomal activity in aortas from rabbits fed standard Purina chow and injected daily with 10 mg/kg doses for 1 week. Sundaram and Lev (16) found a 15% inhibition in mouse brain after 1 week of daily 100 mg/kg doses concurrent with a reduction in the concentrations of gangliosides, cerebrosides, and sulfatides. The rapid return of serine palmitoyltransferase activity after a single dose of L-cycloserine, coupled with reduced inhibition after daily doses, may reflect compensatory synthesis for the amounts of enzyme rendered inactive.

Migration of medial smooth muscle cells to the intima, coupled with their proliferation, are considered primary events of arteriosclerosis. Parker and Odland (21) reported a substantial increase in plasma membrane, its vesicles, and intracellular organelles of intimal smooth muscle cells from atherosclerotic rabbits. These cells displayed increased phospholipid synthesis (22). Since the phospholipid sphingomyelin, as well as other sphingolipids, participate in several membrane-associated events (23–26) and the concentrations of these sphingolipids increase in aorta during atherogenesis (10–14, 27), inhibition of the activity of serine palmitoyltransferase may alter some of the processes the sphingolipids are involved in such as medial smooth muscle migration and/ or division.

Preliminary studies from this laboratory have shown that an inhibition of medial smooth muscle cell migration towards the intima accompanies the inhibition of aortic serine palmitoyltransferase in rabbits fed a 2% cholesterol diet and receiving daily intraperitoneal injections of L-cycloserine for 1 week. Thus it seems prudent to study the long-term effects of L-cycloserine on atherogenesis in this experimental model. If an inhibitory action is sustained biochemically and/or morphologically, the results may be used towards the development of a protocol with clinical significance. For example, coronary occlusions redevelop in up to 30% of individuals subjected to transluminal angioplasty within a few months of the procedure (28). The cells present appear to be of smooth muscle origin (29, 30). If administration of L-cycloserine reduces smooth muscle cell migration and division during this period, its use could complement the effectiveness of angioplasty in reducing coronary stenosis. Consideration of potential side effects, e.g., neurological changes resulting from dopa and glutamate decarboxylase inhibition, would be necessary (20). Additional therapeutic applications of L-cycloserine (e.g., sphingolipidoses) have been discussed by Radin (8).

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REFERENCES

1. Brady, R. O., and G. J. Koval. 1958. The enzymatic synthesis of sphingosine. *J. Biol. Chem.* **233:** 26–31.
2. Snell, E. E., S. J. Di Mari, and R. N. Brady. 1970. Biosynthesis of sphingosine and dihydrosphingosine by cell-free systems from *Hansenula ciferri*. *Chem. Phys. Lipids.* **5:** 116–138.
3. Stoffel, W. 1970. Studies on the biosynthesis and degradation of sphingosine bases. *Chem. Phys. Lipids.* **5:** 139–158.
4. Williams, R. D., E. Wang, and A. H. Merrill, Jr. 1984. Enzymology of long-chain base synthesis by liver: characterization of serine palmitoyltransferase in rat liver microsomes. *Arch. Biochem. Biophys.* **228:** 282–291.
5. Williams, R. D., D. W. Nixon, and A. H. Merrill, Jr. 1984. Comparison of serine palmitoyltransferase in Morris hepatoma 7777 and rat liver. *Cancer Res.* **44:** 1918–1923.
6. Merrill, A. H., Jr., D. W. Nixon, and R. D. Williams. 1985. Activities of serine palmitoyltransferase (3-ketosphinganine

synthase) in microsomes from different rat tissues. *J. Lipid Res.* **26:** 617–622.
7. Merrill, A. H., Jr., and R. D. Williams. 1984. Utilization of different fatty acyl-CoA thioesters by serine palmitoyltransferase from rat brain. *J. Lipid Res.* **25:** 185–188.
8. Radin, N. S. 1984. Biosynthesis of the sphingoid bases: a provocation. *J. Lipid Res.* **25:** 1536–1540.
9. Williams, R. D., D. S. Sgoutas, and G. S. Zaatari. 1986. Enzymology of long-chain base synthesis by aorta: induction of serine palmitoyltransferase activity in rabbit aorta during atherogenesis. *J. Lipid Res.* **27:** 763–770.
10. Portman, O. W., and R. D. Illingworth. 1976. Arterial metabolism in primates. *Primates Med.* **9:** 145–223.
11. Zilversmit, D. B., M. L. Shore, and R. F. Ackerman. 1954. The origin of aortic phospholipid in rabbit athermatosis. *Circulation.* **9:** 581–585.
12. McCandless, E. L., and D. B. Zilversmit. 1956. The effect of cholesterol on the turnover of lecithin, cephalin, and sphingomyelin in the rabbit. *Arch. Biochem. Biophys.* **62:** 402–410.
13. Buck, R. C., and R. J. Rossiter. 1951. Lipids of normal and atherosclerotic aorta. *Arch. Pathol.* **48:** 224–237.
14. Breckenridge, W. C., J. L. Halloran, K. Kovacs, and M. D. Silver. 1975. Increase of gangliosides in atherosclerotic human aorta. *Lipids.* **10:** 256–259.
15. Sundaram, K. S., and M. Lev. 1984. Inhibition of sphingolipid synthesis by cycloserine in vitro and in vivo. *J. Neurochem.* **42:** 577–581.
16. Sundaram, K. S., and M. Lev. 1985. Inhibition of cerebroside synthesis in the brains of mice treated with L-cycloserine. *J. Lipid Res.* **26:** 473–477.
17. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride–methanol. *J. Lipid Res.* **5:** 600–608.
18. Bensadoun, A., and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **70:** 241–250.
19. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
20. Holtz, P., and D. Palm. 1964. Pharmacological aspects of vitamin B_6. *Pharmacol. Rev.* **16:** 113–178.
21. Parker, F., and G. F. Odland. 1966. A correlative histochemical, biochemical and electron microscopic study of experimental atherosclerosis in the rabbit aorta with special reference to the myo-intimal cell. *Am. J. Pathol.* **48:** 197–239.
22. Parker, F., J. W. Ormsby, N. F. Peterson, G. F. Odland, and R. H. Williams. 1966. In vitro studies of phospholipid synthesis in experimental atherosclerosis. *Circ. Res.* **19:** 700–710.
23. Barenholz, Y., and T. E. Thompson. 1980. Sphingomyelins in bilayers and biological membranes. *Biochim. Biophys. Acta.* **604:** 129–158.
24. Cheng, S., and D. Levy. 1979. The effects of cell proliferation on the lipid composition and fluidity of hepatocyte plasma membranes. *Arch. Biochem. Biophys.* **196:** 424–429.
25. Hakomori, S. 1983. Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. *In* Handbook of Lipid Research: Sphingolipid Biochemistry. J. N. Kanfer and S. Hahomori, editors. Plenum Press, New York and London. **3:** 327–379.
26. Critchley, D. R., and I. A. Macpherson. 1973. Cell density dependent glycolipids in NIL2 hamster cell lines. *Proc. Natl. Acad. Sci. USA.* **69:** 872–876.
27. Adams, C. W., and O. B. Bayless. 1963. Histochemical observations on the localization and origin of sphingomyelin, cerebroside and cholesterol in the normal and atherosclerotic artery. *J. Pathol. Bacteriol.* **85:** 113–119.
28. Gruentzig, A. 1982. Results from coronary angioplasty and implications for the future. *Am. Heart J.* **103:** 779–783.
29. Grunwald, J., and C. C. Haudenschild. 1984. Intimal injury in vivo activates vascular smooth muscle cell migration and explant outgrowth in vitro. *Arteriosclerosis.* **4:** 183–188.
30. Campbell, G. R., and J. H. Campbell. 1985. Smooth muscle phenotypic changes in arterial wall homeostasis: implications for the pathogenesis of atherosclerosis. *Exp. Mol. Pathol.* **42:** 139–162.