Effect of Myriocin on Plasma Sphingolipid Metabolism and Atherosclerosis in apoE-deficient Mice*

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Sphingolipids play a very important role in cell membrane formation, signal transduction, and plasma lipoprotein metabolism, all of which may well have an impact on the development of atherosclerosis. To investigate the relationship between sphingolipid metabolism and atherosclerosis, we utilized myriocin to inhibit mouse serine palmitoyl-CoA transferase (SPT), the key enzyme for sphingolipid biosynthesis. We injected 8-week-old apoE-deficient mice with myriocin (0.3 mg/kg every other day, intraperitoneal) for 60 days. On a chow diet, myriocin treatment caused a significant decrease (50%) in liver SPT activity \( (p < 0.001) \), significant decreases in plasma sphingomyelin, ceramide, and sphingosine-1-phosphate levels \( (54, 32, \text{ and } 73\% , \text{ respectively}) \) \( (p < 0.0001) \), and a significant increase in plasma phosphatidylcholine levels \( (91\% ) \) \( (p < 0.0001) \). Plasma total cholesterol and triglyceride levels demonstrated no significant changes, but there was a significant decrease in atherosclerotic lesion area \( (42\% \text{ in root and } 36\% \text{ in en face assays}) \) \( (p < 0.01) \). On a high fat diet, myriocin treatment caused marked decreases in plasma sphingomyelin, ceramide, and sphingosine-1-phosphate levels \( (59, 66, \text{ and } 81\% , \text{ respectively}) \) \( (p < 0.0001) \), and a marked increase in plasma phosphatidylcholine levels \( (100\%) \) \( (p < 0.0001) \). Total cholesterol and triglyceride demonstrated no significant changes, but there was a significant decrease in atherosclerotic lesion area \( (39\% \text{ in root and } 37\% \text{ in en face assays}) \) \( (p < 0.01) \). These results indicate that, apart from cholesterol levels, sphingolipids have an effect on atherosclerotic development and that SPT has proatherogenic properties. Thus, inhibition of SPT activity could be an alternative treatment for atherosclerosis.

Sphingolipids have many biological functions, including cell membrane formation, signal transduction, and lipid metabolism, and all of these may be related to the development of atherosclerosis. Serine palmitoyl-CoA transferase (SPT) is the rate-limiting enzyme in the biosynthesis of sphingolipids. It has long been known that SPT plays an important role in the metabolism of sphingolipids, but its role in other lipid metabolisms and atherosclerosis has not been unequivocally determined. When SPT activity is increased in rat liver \( (2) \) and lung \( (3) \), sphingolipid formation is likewise increased. The activity of SPT is heightened in the aortas of rabbits fed a high cholesterol diet \( (4) \).

Two candidate cDNAs for yeast SPT, termed LCB1 and LCB2, have been cloned \( (5, 6) \), and the translated sequences indicate that their gene products have a 21% amino acid sequence identity \( (6) \). The lack of SPT activity in a yeast strain defective in LCB1 or LCB2, together with the protein similarity data, suggest that the two genes encode subunits of SPT \( (6) \). Mouse and human LCB1 and LCB2 cDNA homologues have also been cloned \( (7, 8) \). In mouse, the two mRNAs have the same tissue distribution (lung, kidney > brain > cartilage, skin > heart > liver > muscle), and the ratio of the amounts of the two transcripts remains approximately constant in all tissues \( (8) \). The tissue distribution of LCB2 mRNA parallels the distribution of SPT activity \( (9) \).

Isaria sinclairii is a fungus traditionally used in Chinese medicine in an effort to attain eternal youth \( (10) \). From it, a specific SPT inhibitor named myriocin has been isolated \( (10) \) and proven to have a molecular structure similar to that of sphingosine \( (11) \). Using myriocin-based affinity chromatography, two proteins, LCB1 and LCB2, can be purified from an interleukin-2-dependent mouse cytotoxic T cell line \( \text{(CTLL-2)} \) \( (12) \). This result indicates that LCB1 and LCB2 are myriocin-binding proteins and confirms the fact that they are responsible for SPT activity \( (12) \).

In this study, we utilized myriocin to investigate the impact of SPT inhibition on lipid metabolism and atherosclerosis development in the apoE-deficient mouse, a well known atherosclerosis animal model. We found that myriocin administration caused a decrease in plasma sphingomyelin (SM), ceramide (Cer), sphingosine (Sph), and sphingosine-1-phosphate (S1P) levels, caused an increase in plasma phosphatidylcholine (PC) levels, and caused a decrease in atherosclerotic lesions in apoE knock-out (apoE KO) mice on both chow and high fat, high cholesterol diets.

EXPERIMENTAL PROCEDURES

Animals and Myriocin Treatment—Eight-week-old apoE KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Myriocin \( (0.3 \text{ mg/kg}) \) (Biomol Research Laboratories Inc.) or phosphate-buffered saline was injected intraperitoneally every other day for 8 weeks. The animals were on Purina Rodent Chow (catalog number 5001) or a high fat, high cholesterol diet \( (20% \text{ milk fat and } 0.15\% \text{ cholesterol}) \) (Harlan Teklad, Madison, WI).

Lipid and Lipoprotein Measurements—Fasting plasma was collected for fast protein liquid chromatography (FPLC) separation and lipid
measurement. Total cholesterol, phospholipids and triglyceride in plasma, and lipoproteins were assayed by enzymatic methods (Wako Pure Chemical Industries Ltd., Osaka, Japan). Plasma sphingomyelin was measured as described previously (13). PC concentration was obtained by subtracting SM from total phospholipid concentration. Apolipoprotein analysis using SDS-PAGE was also done as described previously (14).

**Sphingolipid Analysis by Mass Spectrometry**—Plasma sphingosine bases, sphingoid base-1-phosphates, and ceramide species were performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer operating in a multiple reaction monitoring, positive ionization mode at the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, on a fee-for-service basis. Briefly, 250 μl of mouse plasma was fortified with the internal standards (IC₄, base n-erythro-sphingosine (17C-Sph), C₁₄ sphingosine-1-phosphate (17C-S1P), N-palmitoyl-e-erythro-C₁₃ sphingosine (13C-Cer), and heptadecanoyl-e-erythro-sphingosine (17C-Cer)) and extracted with ethyl acetate/iso-propanol/water (60:30:10) (v/v) solvent system. After evaporation and reconstitution in 100 μl of methanol, samples were injected onto the Surveyor/TSQ 7000 liquid chromatography/mass spectrometry system, and gradient was eluted from a BDS Hypersil C8, 150 × 3.2-mm, 3-μm particle size column with a 1 mM methanolic ammonium formate, 2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and internal standards were collected and processed using the Xcalibur software system. Quantitative analysis was based on the calibration curves generated by spiking an artificial matrix with the known amounts of the target analyte synthetic standards and an equal amount of the internal standards. The target analyte/internal standard peak area ratios were plotted against analyte concentration. The target analyte/internal standard peak area ratios from the samples were similarly normalized to their respective internal standards and compared with the calibration curves using a linear regression model.

**Atherosclerosis**—At the end of the myriocin treatment period, the mice were sacrificed, and the hearts and proximal aortas as well as the whole aortas were removed, dissected, and photographed. An aorta root

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**FIG. 1.** Myriocin treatment dramatically decreased plasma SM levels and increased plasma PC levels but had no effect on plasma cholesterol levels in apoE KO mice on a chow diet. Aliquots of 200 μl of pooled plasma from mice (n = 7) with or without myriocin treatment were analyzed by FPLC (V: VLDL; L: LDL; and H: HDL). An aliquot of each fraction was used for the determination of SM (A), PC (B), and cholesterol (C).

**TABLE I**

|        | SM | Chol | TG | PC/SM |
|--------|----|------|----|-------|
| Control| 71 ± 8| 209 ± 23| 591 ± 73| 65 ± 17| 2.9 ± 0.5 |
| Myriocin| 33 ± 3α| 399 ± 59α| 660 ± 105| 75 ± 19| 12.1 ± 0.2α |

α p < 0.001, n = 7.

**TABLE II**

|        | C18:1Cer | C14Cer | C16Cer | C18:1Cer | C20Cer | C22:1Cer | DHSph | DHSph-1P | Sph | S1P |
|--------|----------|--------|--------|----------|--------|----------|-------|----------|-----|-----|
| Control| 9 ± 2    | 10 ± 3 | 24 ± 2 | 87 ± 9   | 61 ± 8 | 2123 ± 201| 1461 ± 209| 46 ± 12   | 173 ± 21| 226 ± 50 |
| Myriocin| 2 ± 1α   | 12 ± 2α| 11 ± 3α| 51 ± 8α  | 35 ± 9α| 1321 ± 22α| 854 ± 144α| 30 ± 3    | 9 ± 5α  | 127 ± 8α| 58 ± 9α |

α p < 0.01, n = 7.
assay and an en face assay were performed as described previously (15, 16).

**Statistical Analysis**—Differences between groups were tested by Student’s t test. Data are presented as mean ± S.D. A p value of <0.05 was considered significant.

**RESULTS**

Two groups of 8-week-old apoE KO mice were utilized. Group 1 (n = 7) and group 2 (n = 7) animals were injected with 100 μl of myriocin (0.3 mg/kg) or phosphate-buffered saline, respectively, every other day for 8 weeks. As expected, myriocin-treated mice had 50% less SPT activity in the liver than the controls.

As shown in Table I, plasma SM levels were significantly decreased (54%) (p < 0.001) and plasma PC levels were significantly increased (91%) (p < 0.0001) after myriocin administration, whereas total cholesterol and triglyceride levels were not significantly changed. It should be emphasized that the PC/SM ratio was dramatically increased (317%) (p < 0.0001) in the myriocin-treated group as compared with control, indicating that lipoprotein composition was changed.

To investigate the lipid distribution among the lipoproteins with or without myriocin treatment, we utilized FPLC to fractionize lipoproteins and measured SM, PL, and cholesterol in each fraction. We found that myriocin significantly decreased SM and increased PC levels but had no significant effect on cholesterol (Fig. 1). SDS-PAGE revealed that there were no significant changes of the levels of apolipoproteins, including apoB100, apoB48, and apoA-I (data not shown).

To investigate whether myriocin treatment has any impact on other sphingolipid levels, including Cer, Sph, and S1P, mass spectrometry was utilized. We found that after myriocin treatment Cer, Sph, and S1P were significantly decreased (Table II), indicating that myriocin treatment not only influences plasma SM levels but also those of Cer, Sph, and S1P, three important second messengers in signal transduction. The following two findings are also worth noting: 1) The major ceramides in apoE KO mouse plasma are Cer24:0, Cer24:1, Cer18:0, and C16:0 (Table II). 2) The S1P and Sph concentrations in apoE KO mice are ~200 nM (Table II).

For further evaluation of the myriocin effect on plasma lipid levels, 2-month-old mice were challenged with a high fat, high cholesterol (Western type) diet for 8 weeks with or without

![Fig. 2. Myriocin treatment dramatically decreased plasma SM levels and increased plasma PC levels but had no effect on plasma cholesterol levels in apoE KO mice on a high fat diet.](image)

**TABLE III**

Plasma lipid measurement after myriocin administration in apoE KO mice on a high fat diet

|          | SM (mg/dl) | PC (mg/dl) | Chol (mg/dl) | TG (mg/dl) | PC/SM |
|----------|------------|------------|--------------|------------|-------|
| Control  | 114 ± 11   | 397 ± 93   | 1827 ± 306   | 95 ± 19    | 3.5 ± 0.5 |
| Myriocin | 47 ± 14*   | 795 ± 97*  | 1807 ± 342   | 107 ± 27   | 16.9 ± 0.2* |

*p < 0.001, n = 7.

![Fig. 3. (A) The SM concentration (OD490) in different fractions of pooled plasma from mice with or without myriocin treatment. (B) The PC concentration (OD490) in different fractions of pooled plasma from mice with or without myriocin treatment. (C) The cholesterol concentration (OD490) in different fractions of pooled plasma from mice with or without myriocin treatment.](image)

**TABLE IV**

Plasma sphingolipid measurement after myriocin administration in apoE KO mice on a high fat diet

|          | C18:1Cer | C14Cer | C16Cer | C18Cer | C20Cer | C22Cer | C24:1Cer | DHSph | DHSph-1P | Sph | S1P |
|----------|----------|--------|--------|--------|--------|--------|----------|-------|----------|-----|-----|
| Control  | 61 ± 2   | 22 ± 3 | 95 ± 19| 205 ± 32| 100 ± 5| 5551 ± 911| 2423 ± 277| 29 ± 3| 55 ± 12  | 172 ± 21| 218 ± 55 |
| Myriocin | 9 ± 1*   | 20 ± 2 | 12 ± 6*| 36 ± 14*| 45 ± 11*| 1708 ± 426*| 1009 ± 134*| 20 ± 1*| 10 ± 2*  | 114 ± 19*| 42 ± 19* |

*p < 0.01, n = 7.
myriocin treatment. As shown in Table III, plasma SM levels were dramatically decreased (59%), whereas plasma PC levels and the PC/SM ratio were dramatically increased (100% and 380%, respectively) \( p < 0.0001 \) after myriocin administration. Total cholesterol and triglyceride levels were not significantly changed, with FPLC administration producing the same results (Fig. 2). Again, SDS-PAGE revealed that there were no significant changes of the levels of apolipoproteins, including apoB100, apoB48, and apoA-I (data not shown). We also measured other sphingolipid levels, finding that Cer, Sph, and S1P were dramatically decreased after myriocin treatment (Table IV). Basically, a profound myriocin effect was observed when a high fat, high cholesterol diet was used.

It is reported that myriocin treatment (1 mg/kg but not 0.3 mg/kg) reduces T-lymphocyte populations in mice (17). We utilized FAS to evaluate myriocin effect on T cell counts in the
These results indicate that myriocin possesses important anti-atherosclerotic properties.

**DISCUSSION**

In this study we demonstrated for the first time that intraperitoneal myriocin administration in apoE KO mice caused the following: 1) dramatic decreases in plasma SM, Cer, S1P, and Sph levels; 2) dramatic increases in plasma PC levels, thus increasing the PCS/SM ratio; and 3) significant decreases in atherosclerotic lesions.

There are two methods of myriocin delivery in vivo, intraperitoneal injection and oral administration. Because the latter was shown to inflict serious gastrointestinal toxicity (18) and may have had an impact on cholesterol absorption during the high fat, high cholesterol loading experiment, we chose the former, as have other investigators (19, 20). Indeed, intraperitoneal injection of myriocin did not change mouse plasma cholesterol levels on the chow or high fat diets (Tables I and III). In a most recent report, Park et al. showed that oral myriocin administration causes significant reduction of plasma cholesterol and SM levels, thus causing a dramatic reduction of atherosclerotic lesions in apoE KO mice on a high cholesterol diet (21). The different outcome of that study and ours, in terms of plasma cholesterol levels, might be due to the different methods of myriocin delivery.

There was a profound induction of plasma PC levels after myriocin treatment (Tables I and III). This result was consistent with a previous report indicating that administration of L-cycloserine, another inhibitor of SPT, stimulated CTP-choline-phosphate cytidylyltransferase (CT; a key enzyme for PC biosynthesis) activity by 74% (22). This effect might have been due to the decrease of Sph (Tables II and IV), a specific inhibitor of CT activity (23).

There is some question as to why myriocin treatment caused fewer atherosclerotic lesions in apoE-deficient mice. The decrease of SM and the increase of PC contents in non-HDL particles might be one of the mechanisms. Substantial evidence now supports the role of lipoprotein SM and arterial SMatrix in atherosclerosis. SM carried into the arterial wall on atherogenic lipoproteins, where HDL is the major carrier of plasma cholesterol levels, might be due to the different methods of myriocin delivery.

It is reported that myriocin treatment (1 mg/kg but not 0.3 mg/kg) reduces T-lymphocyte populations in mice (37). We utilized phycoerythrin-labeled anti-CD3 antibodies and flow cytometry to evaluate the effect of myriocin on T cell counts in the circulation, and we did not find any difference (data not shown), confirming that 0.3 mg/kg myriocin administration has no effect on T cell populations (37).

In summary, SPT inhibition mediated by myriocin dramatically decreases plasma SM, Cer, S1P, and Sph levels and has anti-atherogenic properties. Because the treatment has no or little effect on cholesterol metabolism, the inhibition of SPT activity could well be an important alternative treatment for atherosclerosis.

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