Reduction of plasma glycosphingolipid levels has no impact on atherosclerosis in apolipoprotein E-null mice

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Abstract Glycosphingolipids (GSLs) have been implicated as potential atherogenic lipids. Studies in apolipoprotein E-null (apoE$^{-/-}$) mice indicate that exacerbated tissue GSL accumulation resulting from α-galactosidase deficiency promotes atherosclerosis, whereas the serine palmitoyl transferase inhibitor myriocin (which reduces plasma and tissue levels of several sphingolipids, including sphingomyelin, ceramide, sphingosine-1-phosphate, and GSLs) inhibits atherosclerosis. It is not clear whether GSL synthesis inhibition per se has an impact on atherosclerosis. To address this issue, apoE$^{-/-}$ mice maintained on a high-fat diet were treated with a potent glucosylceramide synthesis inhibitor, D-*threo*-1-ethylendioxyphenyl-2-palmitoylamino-3-pyrrolidino-propanol (EtDO-P4), 10 mg/kg/day for 94 days, and lesion development was compared in mice that were treated with vehicle only. EtDO-P4 reduced plasma GSL concentration by approximately 50% but did not affect cholesterol or triglyceride levels. Assessment of atherosclerotic lesions at four different sites indicated that EtDO-P4 had no significant impact on lesion area. Thus, despite the previously observed positive correlations between plasma and aortic GSL concentrations and the development of atherosclerosis, and the in vitro evidence implying that GSLs may be pro-atherogenic, our current data indicate that inhibition of GSL synthesis does not inhibit atherosclerosis in vivo.—Glaros, E. N., W. S. Kim, K-A. Rye, J. A. Shayman, and B. Garner. **Reduction of plasma glycosphingolipid levels has no impact on atherosclerosis in apolipoprotein E-null mice.** ***J. Lipid Res.*** **2008.** 49: **1677–1681.**

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Previous studies have shown that plasma glycosphingolipid (GSL) concentration is elevated in patients at increased risk of developing atherosclerosis (1). It is also known that GSLs accumulate in atherosclerotic lesions in humans and in apolipoprotein E-null (apoE$^{-/-}$) mice (2, 3). Several in vitro studies have revealed potential atherogenic properties for specific GSLs. These include the findings that lactosylceramide (LacCer) promotes cholesterol accumulation in macrophage foam cells (4), inhibits cellular cholesterol removal via the ABCA1/apoA-I pathway (5), induces monocyte adhesion to endothelial cells (6), and stimulates vascular smooth muscle cell proliferation (7). Other studies have reported that ganglioside GM3 accelerates LDL uptake by macrophages, which results in the generation of lipid-laden foam cells (8). Studies in apoE$^{-/-}$ mice indicate that increased accumulation of tissue GSLs, induced by α-galactosidase A deficiency, accelerates atherosclerosis (9).

The abovementioned research raised the possibility that GSL synthesis inhibition may represent a therapeutic target for the treatment of atherosclerosis. Data from our group and others indicates that inhibition of serine palmitoyl transferase (SPT, which catalyses the initial step in sphingolipid biosynthesis) using myriocin results in dramatically reduced development of atherosclerotic lesions in apoE$^{-/-}$ mice (10–13). Although it is clear that the myriocin-mediated inhibition of the development of atherosclerosis is associated with decreased GSL synthesis (12, 13), the fact that SPT inhibition may have an impact on numerous members of the sphingolipid family that could theoretically regulate lesion development (14) led us to examine the inhibition of glucosylceramide synthase, which catalyses the initial step in GSL biosynthesis, as a potential modulator of atherosclerosis in apoE$^{-/-}$ mice. Previous work indicates that D-*threo*-1-ethylendioxyphenyl-2-palmitoylamino-

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Abbreviations: 2-AB, 2-aminobenzamide; apoE$^{-/-}$, apolipoprotein E-null mice; CTH, ceramide trihexoside; EtDO-P4, D-*threo*-1-ethylendioxyphenyl-2-palmitoylamino-3-pyrrolidino-propanol; GlcCer, glucosylceramide; GSL, glycosphingolipid; LacCer, lactosylceramide; NP-HPLC, normal-phase HPLC; PC, phosphatidylcholine; PLV, phospholipid vesicle; SPT, serine palmitoyl transferase; TG, triglyceride.

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3-pyrrolidino-propanol (EtDO-P4) potently inhibits GSL synthesis in mouse plasma and tissues (15). In the present study, we used EtDO-P4 to inhibit GSL synthesis in apo$E^{-/-}$ mice and evaluated the potential impact on atherosclerotic lesion development.

MATERIALS AND METHODS

Materials

All organic solvents were of analytical grade and were purchased from Ajax Finechem (Sydney, Australia). Purified leech (*Macrobdella decora*) ceramide glycanase (E.C.3.2.1.123) was from V-Labs (Covington, LA) and phosphatidylcholine (PC) (850457P) was from Avanti Polar Lipids (Alabaster, AL). EtDO-P4 was synthesized by the Mannich reaction from 2-*N*-acylaminoacetophenone, paraformaldehyde, and pyrrolidine followed by reduction with sodium borohydride as detailed previously (16). Four enantiomers were produced during the synthesis. Because only the D-*threo* enantiomers are active in inhibiting glucosylceramide synthase, resolution of the active D-*threo* inhibitors was performed by chiral chromatography. All other reagents were of the highest purity available and were purchased through standard commercial suppliers.

Animals and diet

Male apo$E^{-/-}$ mice were supplied by the Animal Resources Centre (Canning Vale, WA, Australia). From 8 weeks of age, two groups of 10 mice were fed high-fat chow (22% w/w fat, 0.15% w/w cholesterol; Diet No. SF00-219, Specialty Feeds, Glen Forest, WA, Australia) for 94 days. One group received daily intraperitoneal injections of EtDO-P4 (10 mg/kg) incorporated into phospholipid vesicles (PLVs) as a delivery vehicle, alongside a control group, which received the vehicle alone. This study was approved by the University of New South Wales Animal Care and Ethics Committee (approval No. ACEC05/39A) and conforms to the US Public Health Service Policy on Humane Care and Use of Laboratory Animals.

PLVs

Using a Hamilton syringe, 40 mg (400 μl of a 100 mg/ml hexane stock solution) of PC was transferred to a 20 ml scintillation vial and dried under nitrogen gas. Ten milliliters of PBS was added, and the vial was vortexed until the solution became opalescent. Sixteen milligrams of EtDO-P4 was added where appropriate, and the mixture was sonicated for 3 min on ice using a Branson Sonifier 250 (Branson Ultrasonics; Danbury, CT) (duty cycle: constant; intensity: 5). Sonication was repeated three times, after which the solution became translucent. The solution was centrifuged at 1,850 *g* for 10 min, and the supernatant was filter-sterilized through a 0.45 μm filter.

Assessment of atherosclerotic lesions

All mice were fasted overnight; plasma was collected, perfusion-fixed hearts and aortas were dissected, and the sinus, arch, intercostal (third branch), and abdominal (at the celiac branch point) sections of the aorta were prepared for assessment of lesion area as described previously (13). Morphometric data were collected for the four sites after sections were subjected to Verhoeff staining. Animals were euthanized ~24 h after the final intraperitoneal injection of EtDO-P4 or vehicle.

Analysis of plasma lipids

Plasma cholesterol, triglyceride (TG), and SM analysis was determined by enzymatic methods described previously (13, 17). Plasma GSL quantification was achieved by normal-phase HPLC analysis of the 2-aminobenzamide (2-AB)-labeled glycans released by ceramide glycanase as described previously (3, 13). In brief, mice were fasted overnight, and 40 μl of plasma was extracted in 4 ml chloroform-methanol (2:1; v/v), and the crude lipid fraction was dried and redissolved in 200 μl chloroform. The sample was then passed over a silicic acid column, the neutral glycolipids were eluted with 1.8 ml of methanol-acetone (1:9; v/v), and gangliosides were eluted with 1.8 ml of methanol. The neutral GSLs and gangliosides were evaporated to dryness, redissolved in 50 μl of 50 mM sodium actetate buffer (pH 5) containing 1 mg/ml sodium cholate and 0.1 units ceramide glycanase, and incubated for 16 h at 37°C to release the glycans from ceramide. The glycans were then fluorescently labeled with 2-AB and analyzed by normal-phase HPLC (NP-HPLC) using an Agilent 1100 system equipped with an Agilent G1321A fluorescence detector set at Ex 360 nm and Em 425 nm and a 4.6 × 250 mm TSK gel Amide 80 column (Tosoh Bioscience; Montgomeryville, PA). Glycan separation was achieved using a gradient of 50 mM ammonium formate, pH 4.4, in acetonitrile as previously described (3, 13). The major murine plasma GSL detected using this method is *N*-glycolyl GM2, which accounts for ~90% of total plasma gangliosides (18).

Because free glucose interferes with the HPLC assay for glucosylceramide (GlcCer) (18), plasma samples were pooled (from 10 mice, to give a sample volume of 0.3 ml), and the isolated GSLs were analyzed by TLC to assess changes in GlcCer concentration. The total cholesterol content of the two pooled plasma samples was also measured and found to vary by <3%. For the TLC analysis, the isolated neutral GSL fraction was dissolved in 20 μl chloroform-methanol (2:1; v/v) and loaded on Silica Gel 60 TLC plates (Merck; Damstadt, Germany), and bovine brain GlcCer standard (Avanti) was run in a parallel lane. The samples were separated in chloroform-methanol-water (65:25:4; v/v/v), and GSLs were visualized by spraying with 0.2% (v/v) orcinol in 1 M sulphuric acid, followed by drying for 20 min at 80°C. The plates were scanned, and GlcCer and LacCer were quantified densitometrically using National Institutes of Health ImageJ software.

Statistical analysis

Data are presented as means ± SE (n = 10) unless stated otherwise. Statistical significance for differences in lesion areas and plasma lipid concentrations was determined using the Mann-Whitney U test and Student's *t*-test, respectively. Differences were considered significant where $P < 0.05$.

RESULTS

Previous studies have shown that EtDO-P4 (10 mg/kg twice daily for up to 8 weeks) potently reduces GSL synthesis in mice when injected intraperitoneally as a liposomal suspension (15). In the present study, a similar protocol of (once) daily administration of EtDO-P4 (10 mg/kg) for 94 days was used. All mice were weighed at the start of the study and at the time of euthanization, and, as predicted (13), both groups gained weight during the study (**Fig. 1**). Unexpectedly, the average weight of mice receiving EtDO-P4 was significantly higher than that of control mice (28.98 ± 0.22 g versus 32.92 ± 0.78 g, mean ± SE, $P = 0.00013$) after 94 days (Fig. 1).

Treatment with EtDO-P4 resulted in a highly significant 48.6% reduction in plasma GSL levels as determined by

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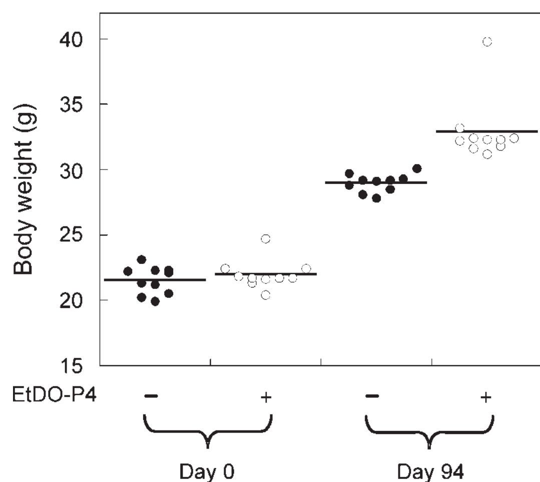

Fig. 1. Total body weight of mice. Two groups of 10 male apolipoprotein E-null (apoE$^{-/-}$) mice were maintained on a high-fat diet for 94 days. One group (+) received daily intraperitoneal injections of D-*threo*-1-ethylendioxyphenyl-2-palmitoylamino-3-pyrrolidino-propanol (EtDO-P4; 10 mg/kg) incorporated into phospholipid vesicles (PLVs) as a delivery vehicle; the other group (−) received the vehicle alone. Body weight was measured when the mice were 8 weeks old (day 0) and again after 94 days, at the conclusion of the study (day 94). Mean values are indicated by the horizontal bars. Significance was assessed by Student's *t*-test.

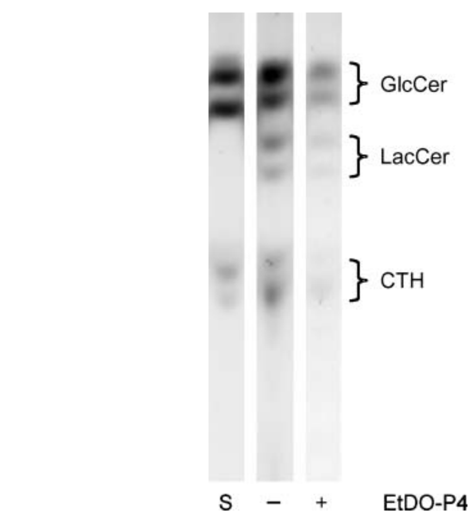

Fig. 2. TLC analysis of neutral glycosphingolipids (GSLs). Two groups of 10 male apoE$^{-/-}$ mice were maintained on a high-fat diet for 94 days. One group (+) received daily intraperitoneal injections of EtDO-P4 (10 mg/kg) incorporated into PLVs as a delivery vehicle; the other group (−) received the vehicle alone. Plasma samples were pooled from each group of mice, and the isolated neutral GSL fraction was analyzed by TLC as described in Materials and Methods. GlcCer, glucosylceramide; LacCer, lactosylceramide; CTH, ceramide trihexoside; S, bovine brain GlcCer standard.

NP-HPLC (**Table 1**). The major plasma GSL detected by this method is *N*-glycolyl GM2 (3). TLC was also used to confirm the inhibition of GSL synthesis by EtDO-P4. **Figure 2** shows that plasma GlcCer and LacCer levels were also significantly reduced by EtDO-P4 administration. Densitometric analysis of the TLC plate indicated that plasma GlcCer and LacCer concentrations were reduced by 49% and 56%, respectively, in the EtDO-P4-treated mice. The levels of ceramide trihexoside (CTH) were also clearly reduced by EtDO-P4 administration (Fig. 2). However, because the CTH levels were close to the lower limit of detection in the EtDO-P4-treated animals, accurate quantification was not achievable. Overall, these data confirm the potent action of this compound as a GSL inhibitor and further indicate that EtDO-P4 is effective and well-tolerated for extended periods in mice. EtDO-P4 administration did not result in significant changes in plasma cholesterol or TG levels, whereas plasma SM levels were reduced by 23.7% (Table 1).

TABLE 1. Plasma lipids

	GSL	Chol	TG	SM
	μM	*mM*	*mM*	*mM*
Control (n = 10)	99.30 ± 3.57	25.71 ± 1.19	1.67 ± 0.26	1.31 ± 0.10
EtDO-P4 (n = 10)	51.00 ± 5.60 $P < 0.0001$	23.30 ± 1.37	1.61 ± 0.15	1.00 ± 0.04 $P = 0.011$

GSL, glycosphingolipid; Chol, cholesterol; TG, triglyceride. Two groups of 10 male apolipoprotein E-null mice were maintained on a high-fat diet for 94 days. One group (EtDO-P4) received daily intraperitoneal injections of D-*threo*-1-ethylendioxyphenyl-2-palmitoylamino-3-pyrrolidino-propanol (10 mg/kg) incorporated into phospholipid vesicles as a delivery vehicle; the other group (Control) received the vehicle alone. Fasted plasma lipids were determined for all groups as described in Materials and Methods. Data are means ± SE. Significance was assessed by Student's *t*-test.

To investigate whether the GSL synthesis inhibition induced by EtDO-P4 has the potential to inhibit the development of atherosclerosis, lesion area was assessed at the aortic sinus, arch, third intercostal branch, and celiac

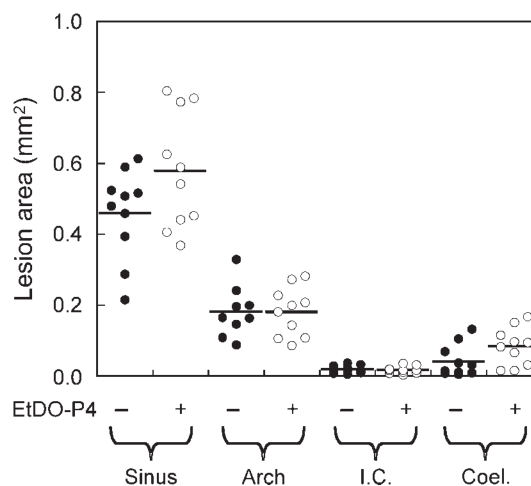

Fig. 3. Morphometric analysis of atherosclerotic lesions. Two groups of 10 male apoE$^{-/-}$ mice were maintained on a high-fat diet for 94 days. One group (+) received daily intraperitoneal injections of EtDO-P4 (10 mg/kg) incorporated into PLVs as a delivery vehicle; the other group (−) received the vehicle alone. Lesions were assessed at the aortic sinus (Sinus), arch (Arch), third intercostal branch (I.C.) and celiac branch (Coel.). Data are derived from 10 mice in each group, except for the vehicle-treated (−) Arch samples, where n = 9 due to sample loss during tissue sectioning. Mean values are indicated by the horizontal bars. Significance was assessed by Mann-Whitney U test.

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branch. Atherosclerotic lesions were detected at all four sites. However, no significant difference in lesion size was observed between the control and EtDO-P4-treated groups (**Fig. 3**). A trend for increased lesion area was detected at the aortic sinus and at the celiac branch in the EtDO-P4-treated group, but this was not statistically significant (Fig. 3).

DISCUSSION

We have previously shown that the SPT inhibitor myriocin potently inhibits atherosclerosis in apo$E^{-/-}$ mice and that this is associated with a 20% to 25% reduction in plasma GSL concentration (12, 13). In the present study, we were able to achieve approximately twice the level of GSL synthesis inhibition (~50% reduction), and this did not significantly impact on lesion area. It therefore appears that the anti-atherogenic actions of myriocin are probably not due to GSL synthesis inhibition.

Although we have focused on plasma GSL levels in this study, previous work has shown that EtDO-P4 administered as a PLV complex (10 mg/kg intraperitoneally every 12 h for 8 weeks) also reduced tissue GSL levels in murine liver, kidney, heart, and brain by 34%, 49%, 40%, and 16%, respectively (15). In other previous studies, vascular GSL levels were reported to be regulated by a combination of in situ synthesis and influx from the plasma compartment, predominantly in association with lipoproteins (19). Based on the known inhibition of GSL synthesis induced by EtDO-P4 in multiple murine organs and the evidence indicating that vascular GSL levels are at least partially regulated by plasma GSL levels, it seems reasonable to predict that GSL levels in the vasculature will also be reduced under our current experimental conditions.

Previous studies demonstrating reduction in atherosclerotic lesion size with myriocin treatment of apo$E^{-/-}$ mice fed a high-fat diet have reported reductions in plasma SM concentrations of 64% (10), 59% (11), and 42% (12). Interestingly, we detected a 24% reduction in plasma SM concentration in EtDO-P4-treated mice. Although the reasons for this are presently not clear, the data do suggest that SM levels may need to be reduced below a certain threshold in order for atherosclerosis to be inhibited. Alternatively, the major anti-atherogenic mechanism for myriocin may be related to additional pathways, for example, modulation of the signaling sphingolipids sphingosine-1-phosphate and ceramide-1-phosphate or additional actions related to regulation of hepatic apoA-I and HMG-CoA reductase gene expression, which have an athero-protective impact on plasma lipoprotein profile (11, 14, 20).

The physiological mechanisms resulting in an increased weight gain in the EtDO-P4-treated mice in the present study remain unknown. On the basis of the evidence that a fraction of plasma EtDO-P4 may cross the blood-brain barrier (15), we can speculate that EtDO-P4 may centrally regulate appetite or satiety. Interestingly, a previous study has shown that depletion of plasma membrane GSLs induced by 1-phenyl-2-decanoylamino-3-morpholino-1-propanol treatment significantly reduced the binding of serotonin to 5-$HT_{7(a)}$ receptors (21). If EtDO-P4 was able to reduce GSL synthesis in hypothalamic neurons expressing 5-$HT_{7(a)}$ receptors in vivo, serotonin signaling would be predicted to be impaired, thus resulting in decreased sensation of satiety, increased appetite, and increased weight gain.

In conclusion, despite the previously published positive correlations between plasma and aortic GSL concentrations and the development of atherosclerosis, and the in vitro studies indicating that GSLs may be pro-atherogenic, our current data indicate for the first time that inhibition of GSL synthesis does not inhibit atherosclerosis in vivo.

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