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Inhibition of atherosclerosis by the serine palmitoyl transferase inhibitor myriocin is associated with reduced plasma glycosphingolipid concentration

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ABSTRACT

Glycosphingolipids (GSL) have been implicated as potential atherogenic lipids. Inhibition of hepatic serine palmitoyl transferase (SPT) reduces plasma sphingomyelin (SM) levels in the absence of changes in cholesterol or triglyceride (TG) concentration and this leads to a reduction of atherosclerosis in apolipoprotein-E gene knockout (apoE$^{-/-}$) mice. The possibility that the reduced atherosclerosis resulting from SPT inhibition is associated with decreases in plasma GSL concentration has not been examined and was the primary aim of this investigation. We show that intraperitoneal delivery of the SPT inhibitor myriocin for 9 weeks inhibits atherosclerosis in apoE$^{-/-}$ mice fed a high fat diet. Lesion inhibition was most pronounced at the aortic arch and distal sites of the thoracic and abdominal aorta. There was also a trend towards a reduction in lesion area at the aortic root. Myriocin treatment resulted in significant reductions in both plasma SM and GSL concentration of 42% and 25%, as assessed by enzymatic and HPLC methods, respectively. Moreover, SM and GSL concentrations were significantly correlated, indicating that SPT inhibition suppresses the synthesis of both these sphingolipids concomitantly. The inhibition of atherosclerosis induced by myriocin was not associated with changes in plasma cholesterol or TG concentrations or lipoprotein profiles as determined by FPLC. These data indicate that therapeutic reduction of plasma SM and/or GSL concentrations may offer a novel treatment for atherosclerosis.

1. Introduction

Atherosclerosis is a major cause of cardiovascular disease (CVD) and accounts for ~50% of all deaths in westernised countries [1]. Atherosclerosis develops as a consequence of multiple pathways that involve inflammation, oxidative stress, dysregulated cellular proliferation and lipid accumulation [2–4]. The accumulation of lipids, including cholesterol, sphingomyelin (SM) and glycosphingolipids (GSL), in atherosclerotic lesions is well known [5–7]. Plasma cholesterol levels

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are widely used as a predictor of CVD risk and plasma SM and GSL concentrations are also associated with atherosclerosis risk [8–10]. In the case of GSL, potential pro-atherogenic properties have been proposed. For example, lactosylceramide (LacCer) promotes cholesterol accumulation in macrophage foam-cells [11], inhibits cellular cholesterol removal via the ABCA1/apoA-I pathway [12], induces monocyte adhesion to endothelial cells [13] and stimulates vascular smooth muscle cell proliferation [14]. Other studies have reported that ganglioside GM3 accelerates low-density lipoprotein (LDL) uptake by macrophages which results in the generation of lipid-laden foam cells [7]. Furthermore, GM3 and GD3 promote the adhesion of platelets to sites of atherosclerotic lesion formation and GD3 stimulates production of reactive oxygen species, regulates smooth muscle cell phenotype and inhibits metalloproteinase-9 expression; all events potentially contributing to plaque instability and atherosclerosis [15,16]. In more general terms, vascular accumulation of GSL could impact on atherogenesis via regulation of cellular signalling, activation, recognition, differentiation, fibrinolytic activity, nitric oxide production, and response to growth factors (see [10,17]).

We have begun to assess whether suppression of GSL synthesis in vitro has any impact on cellular pathways that could be considered as anti-atherogenic. One potential pathway involves the removal of cholesterol from macrophages via ATP-binding cassette transporter A1 (ABCA1) in a process known as reverse cholesterol transport [18,19]. Macrophages in atherosclerotic lesions are derived from circulating monocytes that infiltrate the endothelium and become lodged in the intima where they endocytose modified low density lipoproteins (mLDLs, e.g. modified by proteoglycans, enzymes or oxidants) and become cholesterol-loaded foam cells [1]. We have shown that inhibition of macrophage foam cell GSL synthesis using the glucosylceramide synthase inhibitor D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) stimulated cholesterol efflux [12]. Although this work suggests that GSL synthesis inhibition may afford protection in the context of atherosclerosis development, the key question as to whether modulation of GSL levels in vivo has an impact on atherosclerosis remains unanswered.

As is the case in humans, plasma and aortic GSL levels were found to be increased with atherosclerotic lesion development in apoE$^{-/-}$ mice, thereby identifying an appropriate in vivo model to study the role of GSL in atherogenesis [10]. Subsequent research showed that increasing vascular GSL burden in apoE$^{-/-}$ mice by crossing with α-galactosidase deficient mice (the latter mimic the GSL storage disorder known as Fabry disease) resulted in accelerated atherosclerosis [20]. This finding therefore provided further support to the view that GSL may be pro-atherogenic. Other recent work from two independent groups has shown that inhibition of serine palmitoyl transferase (SPT), the enzyme catalysing the first step in the sphingolipid biosynthetic pathway, dramatically reduced atherosclerotic lesion formation in apoE$^{-/-}$ mice [21,22]. The aim of these studies was in fact to assess the potential impact of SM depletion on atherogenesis and the rationale was based on (as noted above) the observation that plasma SM levels are positively correlated with atherosclerosis risk [9]. In light of the original studies by Miyake et al. reporting that inhibition of SPT with myriocin suppresses GSL synthesis in vitro [23], we speculated that the inhibition of atherosclerosis observed in myriocin-treated apoE$^{-/-}$ mice may not be exclusively due to inhibition of SM synthesis but also to reduced GSL synthesis [24]. The aim of the present study was therefore to examine whether myriocin-mediated inhibition of atherosclerosis in apoE$^{-/-}$ mice was associated with a reduced concentration of plasma GSL.

2. Materials and methods

2.1. Materials

All organic solvents were analytical grade and purchased from Merck (Darmsdadt, Germany). Purified leech (*Macrobdella decora*) ceramide glycanase (E.C.3.2.1.123) was from V-Labs (Covington, LA) and myriocin, (2*S*,3*R*,4*R*,6*E*)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxo-6-eicosenoic acid, from Sigma (Castle Hill, NSW, Australia). All other reagents were of the highest purity available and purchased through standard commercial suppliers.

2.2. Animals and diet

Male apoE$^{-/-}$ mice were supplied by the Animal Resources Centre (Canning Vale, WA, Australia). Mice were fed standard chow until 10 weeks of age then changed to a high fat diet containing 22% (w/w) fat and 0.15% (w/w) cholesterol (Diet No. SF00-219, Specialty Feeds, Glen Forest, WA, Australia). At the same time the mice were changed to a high fat diet, myriocin or vehicle control administration commenced as described previously [22]. In brief, mice were injected intra-peritoneally (i.p.) with filter sterilized (0.2 μm) myriocin dissolved in phosphate-buffered saline (PBS) or with PBS for controls ($n = 7$ in each group). Injection volume for all mice was 100 μl and myriocin dose was of 0.3 mg/kg every 48 h.

2.3. Assessment of atherosclerotic lesions

After 9 weeks on the high fat diet ± myriocin treatment, mice were fasted overnight, euthanased, plasma collected and perfusion fixed hearts and aortae dissected and the root, arch, and descending thoracic (at the branch point of third intercostal pair) and abdominal (at the coeliac branch point) aortic sections prepared for assessment of lesion area based on previous methods [25,26] as described in detail [27]. Morphometric data was collected for all four sites after sections were subjected to Verhoeff staining.

2.4. Plasma lipid analysis

Plasma cholesterol, TG and SM analysis was by enzymatic methods [10,28]. Plasma GSL analysis was by normal phase HPLC [10,29] with minor modifications. In the present study, 0.1 U ceramide glycanase was used to hydrolyse total neutral and charged GSL in 40 μl plasma and the resultant GSL-derived glycans fluorescently labelled with 2-aminobenzamide (2-AB) and analysed as a single sample (rather than analysing neutral and charged glycans separately).

2.5. *Lipoprotein sub-fractionation*

Fresh plasma (200 μl, stored at 4 °C < 48 h) from 2 mice (1 control and 1 myriocin-treated) was mixed with 200 μl 20 mM sodium phosphate buffer, pH 7.8, and this diluted plasma subjected to FPLC (Pharmacia AKTAexplorer 100, Uppsala, Sweden) using a Superose-6 and Superose-12 column (10 mm × 370 mm each) in series as described previously [30]. The columns were eluted at a flow rate of 0.25 ml/min with 20 mM sodium phosphate buffer, pH 7.8, and the eluant was monitored at 280 nm.

2.6. *Statistical analysis*

All data are presented as means ± S.E. 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. Difference were considered significant where $P < 0.05$.

3. Results

In order to confirm that myriocin inhibits atherosclerosis in apoE$^{-/-}$ mice fed a high fat diet we used i.p. administration at a dose of 0.3 mg/kg every 48 h for 9 weeks and assessed lesion area in the proximal and distal aorta. In control animals, injected with PBS, extensive lesions developed at all four sites examined. Lesions were most advanced at the aortic root with complex lesions containing necrotic regions and cholesterol crystals clearly detected (Fig. 1). Similarly, significant lesions were present at the inner curvature of the aortic arch and at the proximal branch points of the arch as well as in the thoracic and abdominal aortic segments at the third intercostal and coeliac

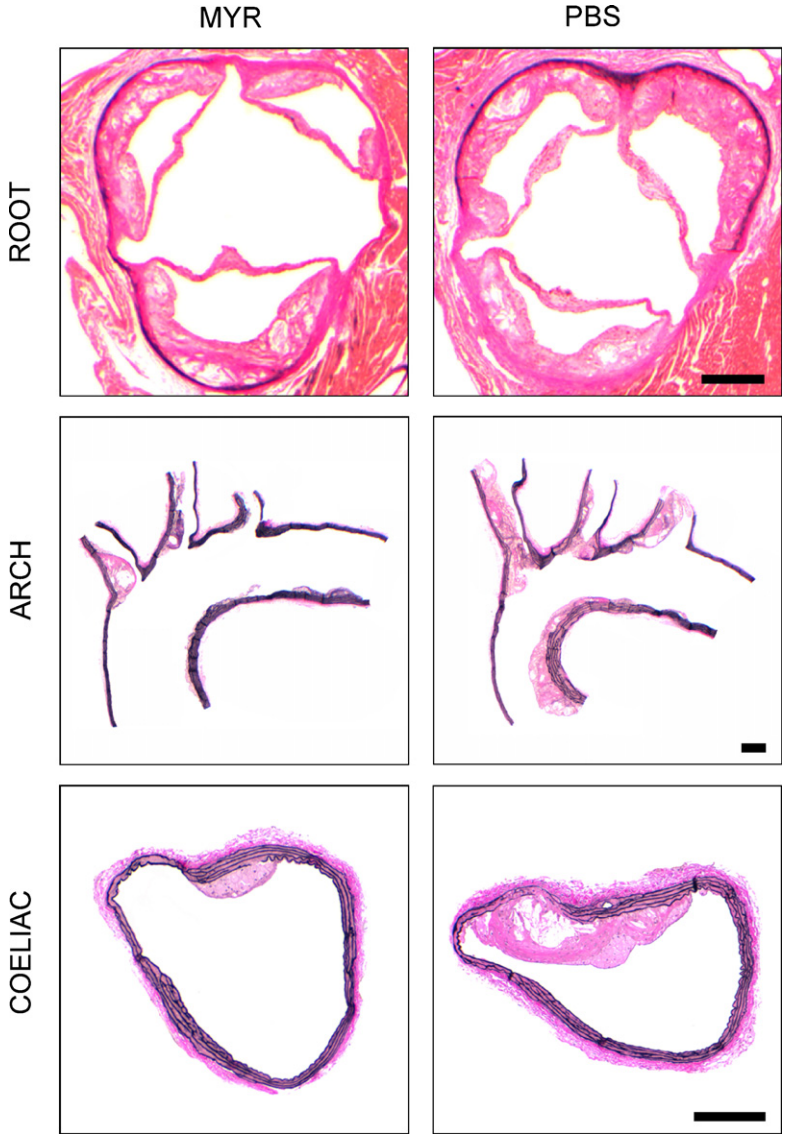

Fig. 1 – Impact of myriocin on atherosclerosis development at the aortic root, arch and coeliac branch point. ApoE$^{-/-}$ mice were fed a high fat diet for 63 days and concurrently treated i.p. with myriocin (MYR) at 0.3 mg/kg/48 h or phosphate buffered saline (PBS) as vehicle control. Lesions were assessed at the aortic root (ROOT), arch (ARCH) and coeliac branch point (COELIAC). Representative sections are shown after Verhoeff staining. Scale bar = 250 μm.

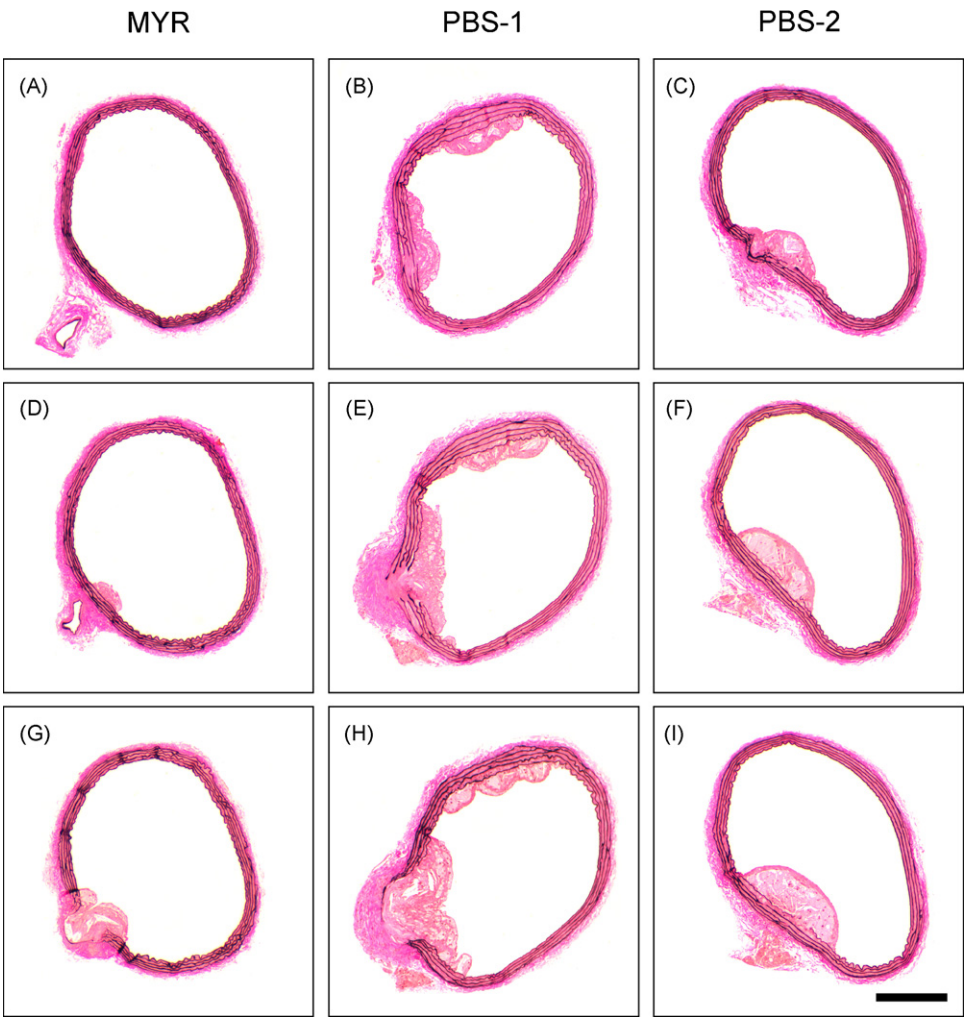

Fig. 2 – Impact of myriocin on atherosclerosis development at the third intercostal branch point of the thoracic aorta. ApoE$^{-/-}$ mice were fed a high fat diet for 63 days and concurrently treated i.p. with myriocin (MYR) at 0.3 mg/kg/48 h i.p. or phosphate buffered saline (PBS-1 and PBS-2) as vehicle control. Six lesions sections were cut at 100 μm steps both above and below the branch point. For "MYR" and "PBS-1", sections are shown at 200 μM (A and B) and 100 μM (D and E) above the branch and at the branch (G and H). For "PBS-2", sections are shown just below the branch (C) and at 100 μM (F) and 200 μM (I) below the branch point. Representative sections are shown after Verhoeff staining. Scale bar = 250 μm.

branches, respectively (Figs. 1 and 2). There was a trend towards reduced lesion size at the root in the myriocin treated mice, however, this did not reach statistical significance (Fig. 3). At all three of the remaining sites, myriocin significantly reduced lesion development and this was particularly noticeable at the third intercostal and coeliac branch points where average lesion area was reduced by 69% and 64%, respectively (Fig. 3).

Lesion morphology at the aortic root, arch and coeliac branch points was uniform in most of the control animals (data not shown), whereas variability was noted at the third intercostal branch point (e.g. Fig. 3). This variation is demonstrated by lesion morphology that ranged from highly focused lipid-rich lesions with a well defined fibrous cap that extended well into the arterial lumen (Fig. 2, PBS-2) to a broader, thinner lesion that extend around the inner lumenal surface (Fig. 2, PBS-1). It was clear that despite the differences in lesion morphology observed at this site, cross-sectional areas were similar in the control animals and myriocin treatment significantly inhibited lesion development (Fig. 3).

Previous studies have shown that oral administration of myriocin (0.3 mg/kg/day) reduced apoE$^{-/-}$ mouse plasma cholesterol and TG concentrations by 74% and 47%, respectively, and this was associated with major reductions in both very low density lipoprotein (VLDL) and low density lipoprotein (LDL) lipoprotein fractions and a doubling of high density lipoprotein (HDL) level [31]. In contrast, i.p. administration of myriocin (using the same approach as we have here) reduced liver SPT activity by 50% and reportedly had no significant impact on either plasma cholesterol or TG concentration or on lipoprotein profile [22]. We also assessed these parameters and found that myriocin did not significantly reduce plasma cholesterol or TG concentrations (Table 1) or appear to modify lipoprotein profile (Fig. 4); in general agreement with Hojjati et al. [22]. When we analysed plasma SM concentrations, we

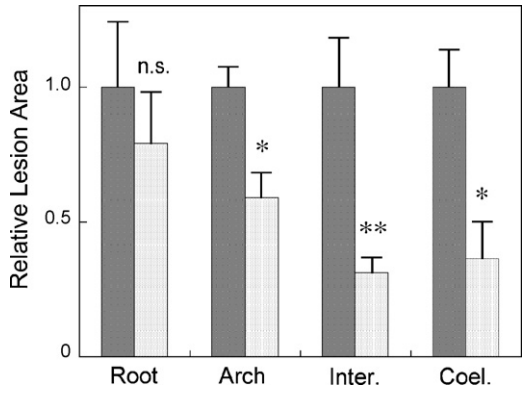

Fig. 3 – Morphometric analysis of atherosclerotic lesions. ApoE$^{-/-}$ mice were fed a high fat diet for 63 days and concurrently treated i.p. with myriocin at 0.3 mg/kg/48 h i.p. or phosphate buffered saline as vehicle control. Lesion areas were measured in myriocin (dark bars) and PBS (light bars) treated mice ($n = 7$ in each group) at the aortic root (Root), arch (Arch) third intercostal branch (Inter.) and coeliac branch (Coel.). All PBS control conditions were assigned a value of 1.0 and relative changes in lesion area induced by myriocin are shown. Data are means ± S.E. $^{*}P < 0.05$, $^{**}P < 0.01$ assessed by Mann–Whitney U test.

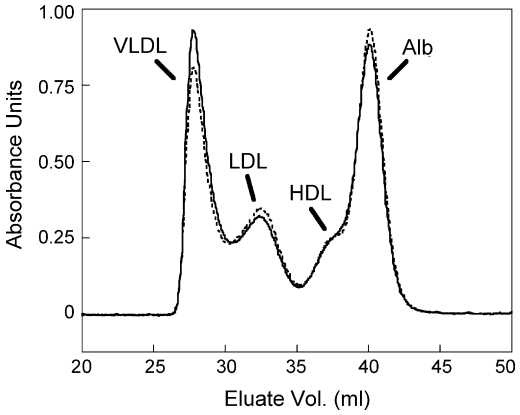

Fig. 4 – Analysis of plasma lipoprotein profile by FPLC. ApoE$^{-/-}$ mice were fed a high fat diet for 63 days and concurrently treated i.p. with myriocin (solid line) at 0.3 mg/kg/48 h i.p. or phosphate buffered saline (broken line) as vehicle control. Plasma samples were analysed by FPLC and the elution positions of the major lipoprotein fractions and albumin assessed by monitoring absorbance at 280 nm. VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; Alb, albumin.

found that myriocin treatment resulted in a significant 42% decrease overall which was not as profound as the 59% reduction in plasma SM previously reported [22]. Nonetheless, this result does confirm that highly significant reductions in plasma SM concentration are achievable in the absence of overt changes to other potentially atherogenic lipids (cholesterol and TG) or lipoprotein profile.

The primary aim of the present study was to examine potential changes in plasma GSL concentration that may be induced with myriocin treatment. In order to examine this we analysed GSL glycans by HPLC; a method which we have previously shown is a quantitative and sensitive enough for analysis of individual mouse plasma samples [10,29]. In agreement with our previous work, LacCer, GalNAcβ1-1Galβ1-4Glc-Cer (GA2) and *N*-glycolyl GalNAcβ1-4[NeuNGcα2-3]Galβ1-4Glc-Cer (gGM2) represented the three major plasma GSL identified (Fig. 5A). In the mice treated with myriocin, a significant 25% reduction in total GSL concentration (LacCer + GA2 + gGM2) was observed (Table 1). Furthermore, plasma SM and GSL concentrations were significantly correlated (Fig. 5B). This data therefore suggests that the inhibition of atherosclerosis induced by myriocin may be due to reductions in both plasma SM and GSL concentrations.

Table 1 – Impact of SPT inhibition on plasma lipid concentrations

TC (mM)	TG (mM)	SM (mM)	GSL (μM)
PBS			
25.5 (2.0)	1.24 (0.30)	1.94 (0.14)	68.9 (3.8)
MYR			
27.9 (3.4)	1.81 (0.35)	1.13 (0.08)	51.7 (4.4)
n.s.	*n.s.*	*0.0004*	*0.0143*

ApoE$^{-/-}$ mice fed a high fat diet were injected i.p. every 48 h with 100 μl phosphate buffered saline (PBS) or PBS containing myriocin 0.3 mg/kg (MYR) for 63 days. Plasma lipids were then determined as described in Section 2 after an over-night fast. Values are means ($n = 7$) with S.E. in parentheses and level of significance (Student's *t*-test, *P*-value) in italics. n.s., not significant.

4. Discussion

Previous studies showed that myriocin administered either orally (0.3 mg/kg/day) or i.p. (0.3 mg/kg/48 h) significantly reduced atherosclerosis in apoE$^{-/-}$ mice [21,22]. These studies both suggested that reduced plasma SM levels were responsible for the inhibition of atherosclerosis; however, it was not clear if additional atherogenic lipids may be altered with myriocin treatment. Based on the knowledge that myriocin administered via the i.p. route inhibits hepatic SPT activity by 50% [22], that SPT catalyses the first step in the GSL biosynthetic pathway (Fig. 6), and that the vast majority of plasma GSL are derived from the liver [32], we predicted that plasma GSL levels may also be reduced with myriocin treatment. We used an i.p. administration route to avoid the potentially confounding anti-atherogenic reductions in plasma cholesterol and TG concentrations and alterations in plasma lipoprotein profile that result from oral administration [31]. Our data indicate that reductions in both plasma SM and GSL concentrations may play a role in the anti-atherogenic mechanisms associated with myriocin. The development of more selective GSL synthesis inhibitors and methods to quantify both inhibitors and GSLs in distinct lesion sites would obviously help to resolve the specific contributions of GSL to atherosclerosis.

Another novel observation in the present study concerns the site-specific inhibition of lesions in mice treated with myriocin.

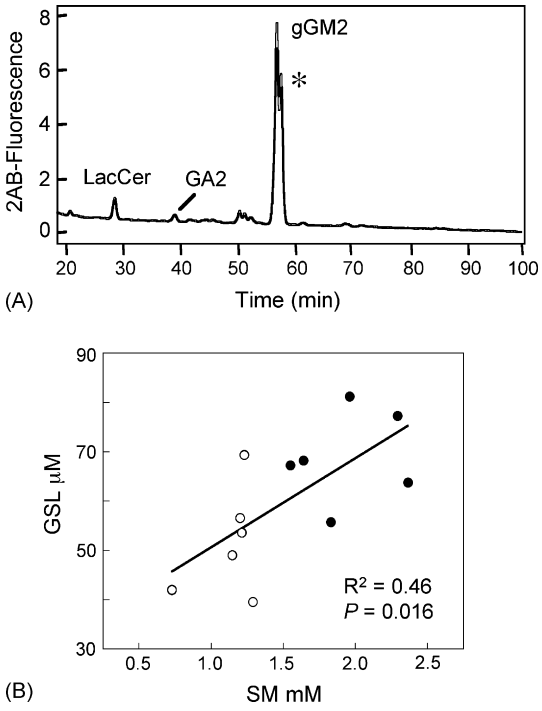

Fig. 5 – Analysis of plasma GSL concentration by HPLC and correlation of GSL and SM concentrations. (A) Plasma GSL from a control PBS treated mouse were subjected to ceramide glycanse digestion and the resulting glycans labelled with 2-aminobenzamide (2-AB) and analysed by normal phase HPLC. The elution positions of lactosyl ceramide (LacCer), GalNAcβ1-1Galβ1-4Glc-Cer (GA2) and N-glycolyl GalNAcβ1-4[NeuNGcα2-3]Galβ1-4Glc-Cer (gGM2) are shown. The asterisk denotes previously identified gGM2 lactone [10]. (B) Correlation of plasma GSL and SM concentrations. Myriocin treated (○), PBS treated (●).

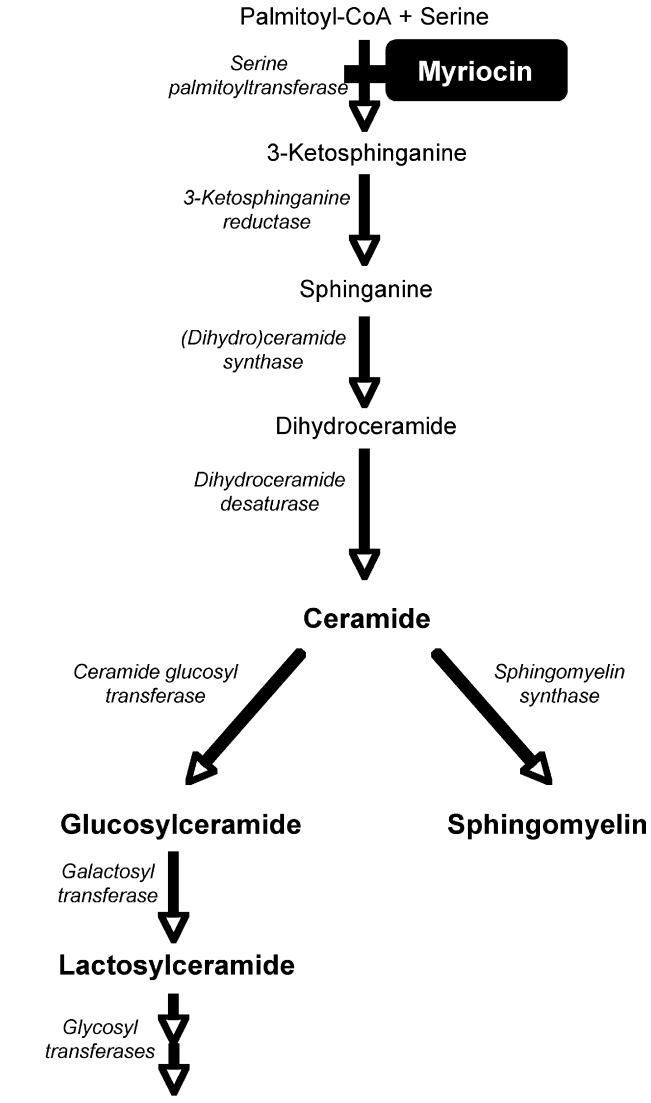

Fig. 6 – Simplified overview of the sphingolipid biosynthetic pathway. Serine palmitoyltransferase catalyses the initial step in both glycosphingolipid (e.g. lactosylceramide, GM1, GM2, globoside) and sphingomyelin biosynthesis and our data indicate that the in vivo inhibition of this enzyme by myriocin results in a reduction of both these sphingolipid classes.

We detected more potent inhibition of atherosclerotic lesions with increasing distance from the heart (Fig. 3). While the mechanisms responsible for this site-specific action are unclear, this may be a general phenomenon associated with pharmacological treatment of apoE$^{-/-}$ mice since the anti-atherogenic actions of vitamin E plus coenzyme Q_{10} [33] as well as probucol [26] are also more potent at the distal lesion sites.

While speculative, it seems reasonable to propose that targeting of SM and/or GSL synthesis could offer a novel pharmacological approach to treat human atherosclerosis that may be complementary to lowering total plasma cholesterol concentrations [24]. This could represent a new therapeutic approach for patients who do not respond well to statin treatment and opens up opportunities for a dual treatment approach (i.e. cholesterol and SM/GSL synthesis inhibition) to treating CVD in the population at large.

In conclusion, we have shown that myriocin inhibits the development of atherosclerosis in apoE$^{-/-}$ mice fed a high fat diet and that the anti-atherogenic effect is more pronounced at distal sites of the aorta. The inhibition of atherosclerosis was not associated with reductions in plasma cholesterol or TG concentration or with altered lipoprotein profile but was associated with significant reductions in plasma SM and GSL concentrations. These data suggest that therapeutic reduction of plasma GSL concentration may contribute to the previously reported anti-atherosclerotic actions associated with SPT inhibition.

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