Evidence for a role of sphingosine-1 phosphate in cardiovascular remodelling in Fabry disease

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Abstract

AIMS: A hallmark of Fabry disease is the concomitant development of left-ventricular hypertrophy and arterial intima-media thickening, the pathogenesis of which is thought to be related to the presence of a plasmatic circulating growth-promoting factor. We therefore characterized the plasma of patients with Fabry disease in order to identify this factor. METHODS AND RESULTS: Using a classical biochemical strategy, we isolated and identified sphingosine-1 phosphate (S1P) as a proliferative factor present in the plasma of patients with Fabry disease. Plasma S1P levels were significantly higher in 17 patients with Fabry disease compared with 17 healthy controls (225 +/- 40 vs. 164 +/- 17 ng/mL; P = 0.005). There was a positive correlation between plasma S1P levels and both common carotid artery intima-media thickness and left-ventricular mass index (r(2) = 0.47; P = 0.006 and r(2) = 0.53; P = 0.0007, respectively). In an experimental model, mice treated with S1P developed cardiovascular remodelling similar to that observed in patients with Fabry disease. CONCLUSION: Sphingosine-1 phosphate participates in cardiovascular remodelling in Fabry disease. Our findings have implications for the treatment of cardiovascular involvement in Fabry disease.
Evidence for a role of sphingosine-1 phosphate in cardiovascular remodelling in Fabry disease

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Introduction

Fabry disease is an X-linked lysosomal storage disorder caused by deficiency of the hydroxylase a-galactosidase A. The disease is characterized by progressive accumulation of neutral glycosphingolipids, mainly globotriaosylceramide (Gb3), in the plasma and within many cell types, including endothelial cells, vascular smooth muscle cells (VSMCs), and cardiomyocytes.1 During childhood, early signs and symptoms include neuropathic pain, angiokeratomas, hypohidrosis, and gastrointestinal dysfunction. In adulthood, involvement of the microvasculature of the kidney, brain, and heart leads to renal failure, ischaemic stroke, and cardiovascular dysfunction.2 Although the condition is X-linked, heterozygous females are also affected, and may even exhibit the full range of disease manifestations, but typically with a later onset than in males.2,3

Cardiovascular remodelling, independent of changes in blood pressure, is a hallmark of Fabry disease and occurs to a similar degree in male and female patients.4 The main cardiac finding is concentric left-ventricular hypertrophy (LVH), which usually develops during or after the third decade of life.5 Affected patients may ultimately develop progressive heart failure, mostly due to diastolic dysfunction.6 Patients may also present with a marked and accelerated increase in intima–media thickness (IMT) of the radial, brachial, and common carotid arteries (CCA), as well as of the aorta.4,7,8

Left-ventricular hypertrophy and increased CCA IMT occur concomitantly in patients with Fabry disease suggesting a common pathogenesis.9 As the amount of Gb3 deposited represents only a fraction of the abnormal left ventricle and arterial wall thickening (around 1–3%), a mechanism(s) other than lipid deposition must be considered in the pathogenesis of the

Methods and results

Using a classical biochemical strategy, we isolated and identified sphingosine-1 phosphate (S1P) as a proliferative factor present in the plasma of patients with Fabry disease. Plasma S1P levels were significantly higher in 17 patients with Fabry disease compared with 17 healthy controls (225 ± 40 vs. 164 ± 17 ng/mL; P = 0.005). There was a positive correlation between plasma S1P levels and both common carotid artery intima–media thickness and left-ventricular mass index (r² = 0.47; P = 0.006 and r² = 0.53; P = 0.0007, respectively). In an experimental model, mice treated with S1P developed cardiovascular remodelling similar to that observed in patients with Fabry disease.

Conclusion

Sphingosine-1 phosphate participates in cardiovascular remodelling in Fabry disease. Our findings have implications for the treatment of cardiovascular involvement in Fabry disease.

Keywords

Fabry disease • Left ventricular hypertrophy • Remodelling • Sphingosine-1 phosphate • Intima–media thickness
cardiovascular remodelling associated with this condition.\textsuperscript{9,10} Consistent with this hypothesis, we found previously that plasma from patients with Fabry disease contains a growth-promoting factor that induces VSMC and cardiomyocyte proliferation in vitro.\textsuperscript{4}

In this report, we have further characterized the plasma of male and female patients with Fabry disease and identified sphingosine-1 phosphate (S1P) as a potent growth-promoting factor that participates in both the cardiac and arterial remodelling associated with the disease.

**Methods**

This study was approved by the Institutional Review Board of the local ethic committees and complies with the Declaration of Helsinki. All human subjects provided written informed consent. All animal experiments were performed in accordance with the Swiss federal animal regulations and approved by the veterinary office of Vaud.

**Study participants**

Seventeen adults with Fabry disease (9 men: age range, 25–56 years; 8 women: age range, 24–61 years) from the cohort of patients followed at the Centre Hospitalier Universitaire Vaudois in Lausanne were enrolled in the study. No patient was receiving enzyme replacement therapy (ERT) at baseline. Both groups are part of populations that have been previously described.\textsuperscript{4} All hemizygous males had the classic form of the disease, based on clinical manifestations and the detection of \( \alpha \)-galactosidase A levels <1.5 mmol/h per mL plasma. Diagnosis of heterozygous females was confirmed by mutation analysis.

To determine the normal plasma level of S1P, 17 healthy individuals, matched for sex and age, were studied. Blood pressure, IMT measurements, and laboratory studies were performed in all patients and healthy controls, and echocardiography in all patients. Left-ventricular mass (LVM) index was measured in the patients and compared with normal reference values according to guidelines of the European society of cardiology.\textsuperscript{11}

**Reagents**

Sphingosine-1 phosphate was purchased from Avantis Polar Lipids, anti-\( \alpha \)-actinin antibody from Novocastra, and anti-atrial natriuretic peptide (ANP) antibody from Biomol International.

**Cell culture and measurement of vascular smooth muscle cell proliferation**

Vascular smooth muscle cells from Wistar rat aorta were isolated and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and antibiotics, as previously described.\textsuperscript{4} Vascular smooth muscle cells were seeded on 96-well plates and incubated for 12 h. Cells were subsequently washed and incubated with various concentrations of S1P. Cell proliferation was quantified using the cell titre 96 Aqueous non-radioactive cell proliferation assay (promega) following the manufacturer’s instructions.

**Plasma lipid fraction extraction and fractionation**

To identify the circulating growth-promoting factor(s), we isolated the lipid fraction in pooled plasma samples from the 17 patients with Fabry disease and 17 matched healthy individuals. Vascular smooth muscle cells were exposed to extracted plasma and proliferation was measured using the soluble tetrazolium salt (MTS) assay. Fresh heparinized human blood was centrifuged at 3000g for 5 min at 4°C. Cell-free plasma was removed and centrifuged at 14 000g for 5 min at 4°C. Each plasma sample was extracted by rocking with 20 volumes of chloroform:methanol (2:1) for 15 min at 37°C and then centrifuged at 14 000g for 5 min at 4°C. The supernatants were transferred to fresh tubes, partitioned against one-fifth volume of distilled water at 4°C, and stored overnight prior to two-phase separation. The upper phase was removed following centrifugation and dried under nitrogen. The dry residue was resuspended in 400 \( \mu \)L of serum-free medium and 100 \( \mu \)L applied to cultured VSMCs seeded on a 96-well plate. Cells were cultured for 18 h and proliferation was quantified as described above.

A sample of 5 mL heparinized plasma from five patients was distributed in 2 mL ependorf tubes in 50 \( \mu \)L aliquots then extracted as described earlier. Each of 20 tubes (1 mL of plasma) of dried residues were resuspended in 1 mL water trifluoroacetic acid 1% and applied on C18 Sep-Pak cartridge, which was pre-equilibrated with water trifluoroacetic acid 1%. The bound material was eluted with different concentrations of acetonitrile (0, 20, 40, 60, and 80%). An aliquot of 25 \( \mu \)L from each fraction was dried and resuspended in DMEM then applied to VSMCs; this step was replicated three times.

The highly active fractions from extracted Fabry plasma between 0 and 20% were mixed, dried and resuspended in 40% acetonitrile/60% trifluoroacetic acid 1% in water. The obtained material was fractionated by gel filtration HPLC using two columns (two protein-pak 60 in tandem, Macrophere GPC 60 A 7u Alltech) at a flow rate of 0.5 mL/min. An aliquot of 25 \( \mu \)L from each fraction was dried and resuspended in DMEM then applied to VSMC, this was carried out in triplicate. The tandem column was calibrated with somatostatin-28 (3000 Da) and somatostatin-14 (1500 Da).

The highly active fractions from plasma of patients with Fabry disease were mixed, dried, and resuspended in chloroform/methanol (70/30%). The obtained material was fractionated by HPLC on a Vydac C18 column. Elution was performed in isocratic chloroform/methanol (70/30%) for 15 min followed with a linear gradient of methanol at a flow rate of 1 mL/min from 30 to 60% in 5 min.

**Mass spectrometry analysis**

The lipid fraction of plasma from patients with Fabry disease were mixed, dried, resuspended in acetonitrile/acetic acid and analysed by electrospray ionization mass spectrometry (ESI-MS). Electrospray ionization mass spectrometry analysis was performed with a linear ion trap mass spectrometer (Thermo-Fisher), operating in positive ion mode, with data collected from 50 to 1000 amu.

**Circulating plasma sphingosine-1 phosphate measurement**

Sphingosine-1 phosphate was first measured using solid-phase extraction followed by derivatization with o-phthalaldehyde and fluorescence detection, as described by Butter et al.\textsuperscript{12}

**Blood pressure measurements, tissue analysis, and immunohistochemical staining in a Fabry mouse model**

One hundred and twenty-nine strain mice were obtained from the Jackson Laboratory. Ten-week-old mice were injected intraperitoneally with S1P 0.4 mg/kg/day (\( n = 8 \)) or with vehicle (phosphate buffered saline; \( n = 8 \)) as a control for 4 weeks. Following 4 weeks of S1P treatment, mean arterial blood pressure was measured in control and S1P-treated mice, as described previously.\textsuperscript{13} Subsequently, mice were killed by intravenous injection of pentobarbital 100 mg/kg. Hearts and aorta were collected. Cardiac weight index was determined. Tissue
samples were immersion-fixed in 4% paraformaldehyde, embedded in paraffin, and serial sections were cut for haematoxylin–eosin, ANP, and α-actin staining. Serial sections collected every 4 μm were analysed. Aortic IMT was evaluated by computer-assisted morphometric analysis. All measurements were performed in the same 16 mice.

**Statistics**
Results are reported as the mean ± SD unless otherwise indicated. GraphPad Prism Software (San Diego, USA) was used for statistical analysis. Groups were compared using the Kruskal–Wallis non-parametric one-way analysis of variance and Mann–Whitney U tests. Differences were considered significant where P-value was < 0.05. Correlations between circulating levels of S1P and CCA IMT and LVM index were assessed by regression analysis (Pearson product-moment correlation coefficient).

**Results**

**Study participants**
The demographic and baseline clinical characteristics of the patients and controls are shown in Table 1. The male Fabry group differed significantly from the male control group in terms of CCA IMT (725.8 ± 117.0 vs. 594.0 ± 62.0 μm; P = 0.045). There was no significant difference between the female Fabry group and the female control group. An increase in LVM index was observed in male and female patients with Fabry disease.

**Plasma of patients with Fabry disease induces vascular smooth muscle cell proliferation**
Extracted plasma from patients with Fabry disease significantly increased VSMC proliferation compared with plasma from healthy subjects (Figure 1A, n = 17 P = 0.0078). The most abundant component present in the fractioned plasma of patients with Fabry disease peaked at 380.6 Da, corresponding to the mass of S1P (Figure 1B).

**Plasma sphingosine-1 phosphate levels are significantly increased in Fabry patients compared with healthy subjects**
Circulating S1P levels were significantly increased in patients with Fabry disease compared with healthy controls (225 ± 40 vs. 164 ± 17 ng/mL; P = 0.005) (Figure 2A). Males with Fabry disease had significantly increased plasma S1P levels compared with healthy males (213 ± 31 vs. 161 ± 19 ng/mL; P = 0.008) (Figure 2B). Sphingosine-1 phosphate concentrations were not significantly different between heterozygous females and healthy females (198.0 ± 47.0 vs. 167.5 ± 15.0 ng/mL; P = 0.18); however, some heterozygous females had S1P levels as high as hemizygous males (Figure 2C).

**Sphingosine-1 phosphate increases vascular smooth muscle cell proliferation in vitro in a dose-dependent manner**
Sphingosine-1 phosphate significantly increased the proliferation of VSMCs in a dose-dependent manner as evaluated with optic density at 490 nm. Cell proliferation increased from 0.436 ± 0.0015 in the absence of S1P to 0.432 ± 0.06 (P = 0.7), 0.498 ± 0.035 (P = 0.02), and 0.577 ± 0.002 (P = 0.007) with S1P concentrations of 100 nM, 1 μM, and 10 μM, respectively (Figure 3A). This proliferative effect was present at S1P concentrations similar to those measured in the plasma of the patients with Fabry disease. At lower S1P concentrations (100 nM), the planar cell surface area (PCSA) of VSMCs was increased (Figure 3B). This suggests a hypertrophic mechanism at the origin of cardiac remodelling. In contrast, at higher S1P concentrations, while VSMC PCSA was

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**Table 1** Baseline clinical characteristics of the study populations

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Males (n = 9)</th>
<th>Controls (n = 9)</th>
<th>P-value</th>
<th>Females (n = 8)</th>
<th>Controls (n = 8)</th>
<th>P-value</th>
</tr>
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<tr>
<td>Age (years)</td>
<td>42.8 ± 8.8</td>
<td>50.0 ± 7.0</td>
<td>NS</td>
<td>40.1 ± 13.6</td>
<td>49.0 ± 7.5</td>
<td>NS</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>126.1 ± 12.3</td>
<td>118.0 ± 6.7</td>
<td>NS</td>
<td>122.7 ± 16.4</td>
<td>118.0 ± 9.8</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>75.2 ± 8.1</td>
<td>73.7 ± 3.2</td>
<td>NS</td>
<td>74.5 ± 5.9</td>
<td>69.4 ± 4.9</td>
<td>NS</td>
</tr>
<tr>
<td>Serum total cholesterol (mmol/L)</td>
<td>5.20 ± 0.99</td>
<td>4.81 ± 0.72</td>
<td>NS</td>
<td>5.33 ± 0.72</td>
<td>5.10 ± 0.49</td>
<td>NS</td>
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<tr>
<td>Serum HDL-cholesterol (mmol/L)</td>
<td>1.48 ± 0.29</td>
<td>1.50 ± 0.44</td>
<td>NS</td>
<td>1.51 ± 0.43</td>
<td>1.55 ± 0.32</td>
<td>NS</td>
</tr>
<tr>
<td>Total/HDL-cholesterol ratio</td>
<td>3.59 ± 0.25</td>
<td>2.90 ± 0.65</td>
<td>NS</td>
<td>3.59 ± 0.43</td>
<td>3.62 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Serum LDL-cholesterol (mmol/L)</td>
<td>3.13 ± 0.95</td>
<td>3.0 ± 0.54</td>
<td>NS</td>
<td>3.20 ± 0.96</td>
<td>2.93 ± 0.36</td>
<td>NS</td>
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<tr>
<td>Serum triglycerides (mmol/L)</td>
<td>1.27 ± 0.56</td>
<td>1.10 ± 0.43</td>
<td>NS</td>
<td>1.25 ± 0.39</td>
<td>0.81 ± 0.31</td>
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<tr>
<td>Diabetes (n)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Smoking (%)</td>
<td>22.0</td>
<td>20.0</td>
<td>12.5</td>
<td>25.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Left-ventricular mass index (g/m²)</td>
<td>149.6 ± 64.3  (normal ≤ 115)</td>
<td>–</td>
<td>104.9 ± 39.3 (normal ≤ 96)</td>
<td>–</td>
<td>104.9 ± 39.3 (normal ≤ 96)</td>
<td>–</td>
</tr>
<tr>
<td>Common carotid artery IMT (μm)</td>
<td>725.8 ± 117.3</td>
<td>594.6 ± 62.4</td>
<td>0.045</td>
<td>709.4 ± 193.7</td>
<td>586.7 ± 81.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

HDL, high-density lipoprotein; IMT, intima–media thickness; LDL, low-density lipoprotein.
Figure 1  (A) Vascular smooth muscle cell proliferation is significantly increased in the presence of extracted lipid fraction from the plasma of $n = 17$ patients with Fabry disease compared with that from $n = 17$ healthy subjects. Results are expressed in arbitrary units and represent the mean of six experiments of absorbance at 490 nm, normalized with absorbance obtained from vascular smooth muscle cells incubated with Dulbecco’s modified Eagle’s medium alone ($P = 0.0078$). (B) Electrospray ionization mass spectrometry showed that the most abundant plasma lipid compound peaked at 380.6 Da, corresponding to the mass of sphingosine-1 phosphate.
Figure 2. (A) Plasma sphingosine-1 phosphate levels of $n = 17$ healthy subjects vs. $n = 17$ patients with Fabry disease ($P = 0.005$). (B) Plasma sphingosine-1 phosphate levels of $n = 9$ healthy males vs. $n = 9$ males with Fabry disease ($P = 0.008$). (C) Plasma sphingosine-1 phosphate levels of $n = 8$ healthy females vs. $n = 8$ females with Fabry disease.

Figure 3. (A) Proliferation of vascular smooth muscle cells exposed to different concentrations of sphingosine-1 phosphate. Results are expressed in arbitrary units and represent the mean of six experiments of absorbance at 490 nm ($P = 0.02$ and $P = 0.007$ vs. untreated vascular smooth muscle cells). (B) Progressive variation of planar cell surface area (PCSA) of vascular smooth muscle cells exposed to increasing concentrations of sphingosine-1 phosphate. PCSA was quantified by computer-assisted morphometry. Values are mean ($\pm$ SD) PCSA of 150 cells. (C) Number of live vascular smooth muscle cells exposed to increasing concentrations of sphingosine-1 phosphate as assessed by fluorescence-activated cell sorting (FACS).
decreased, the number of cells increased as indicated by FACS analysis (Figure 3C), suggesting hyperplasia.

**Treatment of mice with sphingosine-1 phosphate increases aortic intima–media thickness**

Mean aortic IMT was significantly greater in S1P-treated mice than controls (74.1 ± 21.0 vs. 37.5 ± 5.1 μm; P = 0.0007) (Figure 4A). Haematoxylin–eosin staining of the aortic wall revealed a normal appearance and composition of the intima–media (Figure 4B). The predominant cellular component of the intima–media was VSMCs, as revealed by immunostaining for α-actin (Figure 4B).

**Treatment of mice with sphingosine-1 phosphate induces cardiac hypertrophy**

The cardiac weight index of S1P-treated mice was 27% greater than that of control mice (P = 0.03) (Figure 5A). The development of cardiac hypertrophy was independent of blood pressure as S1P-treated mice had a lower mean arterial blood pressure than controls (Figure 5B). Furthermore, haematoxylin–eosin staining showed a hypertrophic response of cardiomyocytes to S1P. This was confirmed by the presence of diffuse ANP staining in samples from S1P-treated mice compared with patchy staining in control samples (Figure 5B).

**Plasma sphingosine-1 phosphate levels correlate with common carotid artery intima–media thickness and left-ventricular mass index in Fabry patients**

There was a positive correlation between circulating S1P levels and CCA IMT in patients with Fabry disease (r² = 0.47; P = 0.007, Figure 6A). Plasma S1P levels also correlated positively with LVM index in Fabry patients (r² = 0.53; P = 0.0007, Figure 6B). Figure 6C shows the correlation between S1P levels and CCA IMT (r² = 0.55; P = 0.0001) in males and females in the Fabry and control populations.
Males with Fabry disease had a greater IMT and higher S1P-values compared with male controls. The ranges of IMT and S1P-values were greater in heterozygous females than in female controls.

Discussion

In the present study, we identified S1P as a biologically active growth-promoting factor involved in cardiovascular remodelling in both males and females with Fabry disease. Male patients had significantly higher plasma S1P levels compared with healthy controls. Moreover, there was a strong correlation between plasma S1P levels and LVM index, and increased CCA IMT in patients with Fabry disease. In addition, S1P-treated mice developed comparable cardiovascular remodelling to that observed in patients with Fabry disease.

We showed previously that vascular remodelling occurs to a similar degree in male and female patients with Fabry disease. Patients with Fabry disease presented with a strong correlation between LVM index and CCA IMT, in the absence of arterial hypertension, suggesting a common pathogenesis to their development. Using an in vitro assay, we demonstrated that plasma from both males and females with Fabry disease induced proliferation of rat VSMCs and neonatal mice cardiomyocytes. These results suggested the existence of a circulating growth-promoting factor in Fabry disease. In a recent paper, Aerts et al. reported that globotriaosylsphingosine (lyso-Gb3) was markedly elevated in the plasma of male Fabry patients but not in female patients. Interestingly, exposure of VSMCs to lyso-Gb3 promoted cell proliferation. However, the signalling pathway and cardiovascular expression of lyso-Gb3 receptors remain elusive.

Figure 5 (A) Cardiac weight index in mice treated with vehicle (n = 8 control) or sphingosine-1 phosphate (n = 8 sphingosine-1 phosphate), 0.4 mg/kg/day intraperitoneally for 4 weeks (P = 0.03). (B) Arterial blood pressure of mice treated with vehicle or sphingosine-1 phosphate as under (A). Values are the means of eight animals per group. (C) Haematoxylin–eosin and atrial natriuretic peptide staining of heart sections of mice treated with vehicle or sphingosine-1 phosphate as under (A). Photomicrographs show representative sections of myocardium (Scale bar = 20 μm).
In the present work, we isolated and identified S1P in the plasma of patients with Fabry disease at significantly higher concentrations compared with those seen in control plasma. Females showed a wider range of S1P concentrations than female controls, but the difference was not significant. Interestingly, we found no significant difference in plasma S1P levels between male and female Fabry patients who shared a comparable penetrance of cardiovascular remodelling, as previously shown. Moreover, there was a strong correlation between S1P concentration and both CCA IMT and LVM index, in males and females with Fabry disease. This indicates that cardiovascular remodelling and plasma S1P levels are, at least in part, independent of the level of residual circulating α-galactosidase A, and that plasma S1P, unlike plasma Gb3 and lyso-Gb3, may be considered as a marker of the severity of cardiovascular remodelling in Fabry disease.

Ceramide metabolism generates a cascade of bioactive lipids, all of which carry a specific signalling capacity. This sphingolipid signalling network is expressed in the cardiovascular system. In healthy subjects, S1P is present in the plasma at higher levels than in the tissues, and exists largely in forms bound to high-density lipoproteins. S1P is generated by the phosphorylation of sphingosine by sphingosine kinases and is irreversibly degraded by a specific lyase that is exclusively located at the endoplasmic reticulum.

Sphingosine-1 phosphate has been shown to induce in vitro VSMC proliferation, by a variety of signal transduction pathways including ERK and RhoA, and hypertrophy of neonatal rat cardiomyocytes. Our in vitro study showed that S1P promotes marked proliferation of VSMCs in a dose-dependent manner. These data demonstrate that S1P is a potent circulating growth-promoting factor because its effect was observed at concentrations similar to those measured in plasma from patients with Fabry disease (plasma concentrations only 1.3 times greater than in healthy controls). Interestingly, at lower S1P concentrations, the VSMC phenotype was mainly hypertrophic, whereas at higher S1P concentrations the VSMC phenotype was predominantly hyperplasic. These findings suggest that both proliferative mechanisms may be involved in the arterial remodelling of Fabry disease, with the participation of the individual mechanisms depending directly on plasma S1P levels. The mechanism responsible for increased plasma S1P levels in patients with Fabry disease has yet to be established.

Overall results, i.e. the increase of plasma S1P concentrations and the strong correlation between S1P concentrations and both CCA IMT and LVM index in patients with Fabry disease, and the in vitro effect of S1P on VSMCs, suggest that S1P may be a key factor that triggers the development of LVH and arterial intima–media thickening in Fabry disease. We therefore injected S1P into an in vivo model of Fabry disease in mice. Animals developed cardiovascular remodelling that was very similar to that observed in humans with Fabry disease. Image analysis revealed a significant thickening of the aortic IMT relative to control animals. The predominant cellular component of the arterial wall was VSMCs, as revealed by immunostaining for α-actinin. Cardiac weight index was also significantly greater compared with controls. Microscopic analysis revealed a marked hypertrophy of cardiomyocytes at the origin of the increased cardiac mass as assessed by diffuse ANP expression. Significant cardiovascular remodelling developed in S1P-treated mice after only 1 month of daily injections (half-life of S1P, 3–5 min), demonstrating that S1P is also a potent hypertrophic mediator in vivo.

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**Figure 6** (A) Correlation between plasma sphingosine-1 phosphate levels and common carotid artery intima–media thickness (CCA IMT) in the Fabry population. (B) Correlation between plasma sphingosine-1 phosphate levels and left-ventricular mass in the Fabry population. (C) Correlation between plasma sphingosine-1 phosphate levels and CCA IMT in male and female patients with Fabry disease and controls.
animal studies which reported that systemic administration of S1P may reduce blood pressure. Sphingosine-1 phosphate interacts with high-affinity G-protein coupled receptors that can sense very small changes in S1P level, thus small increases in S1P may have functional and morphological consequences. Receptors S1P1–3 are widely expressed in endothelial cells and VSMCs of the artery tree and in cardiomyocytes, and their biological roles have been shown by the generation of knockout mice. The importance of S1P1 in angiogenesis is exemplified by the embryonic lethality of S1P1 knockout. There is evidence that S1P1 and S1P2 receptors promote, whereas S1P2 receptors antagonize, VSMC proliferation in vitro in response to S1P. Cardiomyocyte hypertrophy appears to be mediated by S1P1 receptors as an anti-S1P1 receptor antibody has been found to block S1P-mediated hypertrophy.

Some reports have suggested that the biologically active high-density lipoprotein compound S1P is responsible for antiatherogenic actions on vascular endothelial cells of the myocardium. Recently, we showed that patients with Fabry disease are somehow protected from atherosclerotic plaques relative to controls with a similar number of cardiovascular risk factors. Our data suggest that the increased plasma S1P concentration in patients with Fabry disease may be in part responsible for the cardiovascular remodelling and the anti-atherogenic effect observed in Fabry disease. These findings open new perspectives that have implications for the treatment of cardiovascular complications in Fabry disease.

In conclusion, our data provide evidence that S1P is a biactive mediator that participates in the development of cardiovascular remodelling in patients with Fabry disease. Sphingosine-1 phosphate-treated mice developed cardiovascular anomalies similar to those observed in patients with Fabry disease, further supporting the potent cardiovascular effects of S1P. In addition, the strong correlation of plasma S1P levels with LVH and with increased CCA IMT, in the absence of arterial hypertension, provide evidence of the role of S1P as a marker of the severity of cardiovascular remodelling in Fabry disease.

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References


CARDIOVASCULAR FLASHLIGHT

An indirect shot to the heart

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A 49-year-old man presented with breathlessness. Chest X-ray (Panels A–C) showed a foreign body in line with the thoracic vertebra. Further CT imaging confirmed a metallic object embedded in the right ventricle (Panels D–K). He had no history of cardiac or thoracic surgery and no history of penetrating trauma. On further questioning it was discovered that he was shot in the back of the right knee with an air-rifle pellet 27 years ago (Panel L). This had subsequently migrated into the venous system and embolized to the right heart.

Migration and venous or arterial embolization of penetrating foreign bodies are rare, but recognized. If they result in obstruction, then percutaneous or surgical retrieval is indicated. In this patient, this finding was considered to be incidental and unrelated to his symptoms. After joint cardiothoracic/interventional cardiology discussion, no further treatment was recommended. He is due to have CT monitoring in 2 years.

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SUPPLEMENT MATERIAL TO METHODS

VSMC metabolic activity assay

VSMCs from Wistar Kyoto rat aortas were isolated by collagenase digestion. Cells were cultured in DMEM-containing 10% fetal bovine serum (FBS) and antibiotics. VSMCs prepared from these rats were not contaminated with fibroblast or endothelial cells, as evidenced by positive immunostaining of smooth muscle α-actin with fluorescein isothiocyanate-conjugated α-actin antibody. VSMCs with early passage (<15) were seeded on a 96-well plate (2000 cells/well) and incubated for 24 hours. Subsequently, cells were washed three times with phosphate-buffered saline (PBS) and incubated for 16–18 hours with 100 µl DMEM containing increasing percentages of plasma from patients with Fabry disease or healthy subjects, and then assayed for cell proliferation. Only plasma from non-ERT treated patients was used in the present study.

VSMC proliferation was quantified using the Cell Titer 96® AQeuous non-radioactive cell proliferation assay kit (Promega Corporation, Madison, WI, USA). This assay is based on measuring the cellular conversion of the colorimetric reagent MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) into formazan by dehydrogenase enzymes found only in metabolically active, proliferating cells. Absorbance was measured at 490 nm using a 96-well enzyme-linked immunosorbent assay plate reader.

Comparative metabolic activity was assessed using culture media containing 25% of extracted plasma (volume/volume) originating either from patients with Fabry disease or from healthy controls. All assays were carried out in parallel; each individual assay was replicated six times. Arbitrary units represent absorbance at 490 nm of formazan generated from MTS. For each experiment, cells were incubated in parallel with DMEM without plasma and the values obtained were used for normalization.
Circulating plasma S1P measurement

S1P was first measured using solid phase extraction followed by derivatization with o-phthalaldehyde and fluorescence detection, as described by Butter et al. Data were confirmed in our laboratory using N-acylation with $[^3H]$ acetic anhydride. Briefly, S1P was first extracted as described above (section on lipid fraction extraction). Extracted S1P was acylated into N-[${^3H}$]acylated S1P, the reaction mixture was extracted to eliminate sphingosine in lower chloroform phase. Aqueous phase soluble products were separated by reverse phase HPLC on EC NUCLEODUR (250 /4.6 100-5) column (Machrey-Nagel, Oesingen, Switzerland). The mobile phase consisted of isocratic chloroform/methanol (70/30%) for 15 min followed with a linear gradient of methanol containing triethylamine 0.5 %, at a flow rate of 1 ml/min from 15% to 50% in 25 min at flow rate 0.5 ml/min. A volume of 2 ml of Pico-Fluor-40 (Perkin-Elmer, Shelton USA) was added to 0.5 ml collected fraction and the sample counted on 2500 TR liquid scintillation analyzer (Perkin-Elmer).

VSMC fluorescence-activated cell sorting (FACS) analysis

Cells were seeded in 12-well plates (50 000 cells/well) in DMEM 10% fetal bovine serum (FBS) and incubated for 24 h. Subsequently cells were washed three time with PBS and incubated with DMEM 0.4% lipid-free BSA containing increasing concentrations of S1P (0, 1 nM, 10 nM, 100 nM, 1 µM and 10 µM) for 48 h. Then, cells were washed with PBS and trypsinized. Cells were resuspended in 1 ml DMEM 10% FBS and propidium iodide (PI) was added at final concentration of 1 µg/ml. Flow cytometry was performed with a cytofluorometer (Becton Dickinson, Basel, Switzerland), using CellQuest software from the manufacturer. Cells were gated by forward- and side-scatter signals (FSC and SSC). A total of 9000 cells were analyzed in each experiment. PI was used to discriminate live and dead cells. Mean fluorescence intensity (MFI) was the parameter that reflected VSMC size.
**Planar cell surface area (PCSA)**

VSMC was seeded on cover slips and incubated for 24 h. Subsequently, cells were washed three times with PBS and incubated with DMEM 0.4% lipid-free bovine serum albumin (BSA) containing various concentrations of S1P (0, 1 nM, 10 nM, 100 nM, 1 µM and 10 µM) for 48 h. Cells were fixed in 4% paraformaldehyde/PBS overnight at 4 °C, washed with PBS and permeabilized in 0.1% triton X-100/PBS for 10 minutes. Cells were blocked with PBS 2% BSA for 1 h at room temperature. Cell F-actin was labelled with Phalloidin-Fluoroprobe (Fluoroprobe Interchim, France) 0.16 nmol/ml final concentration in PBS for 1 h at room temperature. The samples were mounted in 50% glycerol/PBS with 100 mg/ml 1,4-diaminobicyclo[2.2.2]octane. The slides were examined with a Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany). Pictures were acquired using a colour digital camera connected to the microscope. PCSA was quantified by computer-assisted morphometry. Randomly identified images were transmitted to a computer (HP Workstation xw4000) connected to the microscope (Leica), and then analyzed with appropriate software.

Measurements were performed by readers who were blind to the treatment groups associated with the samples. At least six images and 150 cells were counted for each S1P concentration assessed.

**Mice**

Mice strain 129 (B6; 129 genetic background) were obtained from the Jackson Laboratory. Control and S1P-treated mice used in our experiments were wild-type littermates of Fabry mice. Mice were fed regular rodent chow and water *ad libitum* throughout the study. Only males were used in the study and each of the two groups. From 10 weeks of age the S1P-treated mice received intraperitoneal injections of S1P 0.4 mg/kg/day for 4 weeks. All of the experiments were approved by the local institutional animal committee (Service de la consommation et des affaires vétérinaires).
Blood pressure measurement

Mean arterial blood pressure was measured in the three groups of mice (control, S1P-treated and Fabry) as described previously. Briefly, mice were weighed, sedated and their left carotid artery was catheterized. Following full recovery from anaesthesia, the arterial line was connected to a pressure transducer, and 30 minutes after, mean blood pressure was recorded.

Histological analysis

After blood pressure measurement, mice were euthanized by intravenous injection of pentobarbital 100 mg/kg. Hearts and aorta were rapidly and carefully excised and the heart was weighed. Organs were immersion-fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and serial sections were cut for analysis by hematoxylin-eosin staining. Serial sections collected every 4 µm were used for analysis.

Immunohistochemistry

Sections of 3 µm thickness of heart were cut and deparaffinized in xylene. The sections were then incubated for 30 min in 0.3% hydrogen peroxide solution in water to block endogenous peroxidase activity. The sections were washed three times in water and digested with trypsin 10 min at 37°C then rinsed with water and incubated in the blocking reagent (Dako containing 0.5% of BSA) for 1 h. The samples were later incubated with polyclonal antibodies against ANP (1:3000 dilutions) for 24 h at 4°C. After washing in TBS, the specimens were incubated in anti-rabbit peroxidase ready to use Dako for 40 min and treated with 3,3-diaminobenzidine tetra-hydrochloride to reveal sites of immunoreactivity. The slides were counter-stained with hematoxylin for 30 s, dehydrated in ascending grades of ethanol, cleared in xylene, and mounted with permanent mounting medium.
References
