End-organ damage in diabetes
Hamidi Shishavan, Mahdi

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Differential Effects of Long Term FTY720 Treatment on Endothelial versus Smooth Muscle Cell Signaling to S1P in Rat Mesenteric Arteries

Mahdi Hamidi Shishavan, Arash Bidadkosh, Saleh Yazdani, Jacob van den Born, Hendrik Buikema, Robert H Henning, Leo E Deelman

(Submitted)
Abstract

The immunosuppressant drug and sphingosine 1-phosphate (S1P) analog FTY720 (Fingolimod) exerts pleiotropic effects on the vasculature and the heart. In patients with multiple sclerosis, FTY720 leads to changes in blood pressure and transient bradycardia. The vascular effects of FTY720 have also been studied acutely in experimental studies but its long term effects on resistance artery function remain unknown. Here we investigated myogenic constriction and S1P induced contraction in small mesenteric arteries of rats chronically treated with FTY720.

Healthy Wistar rats received FTY720 1mg/kg/daily for six weeks. At termination, blood pressure was recorded and small mesenteric arteries collected for vascular studies in a perfusion set up.

Myogenic constriction to increased intraluminal pressure was low, but the addition of S1P profoundly augmented vascular constriction in arteries of both controls and animals chronically treated with FTY720. Interestingly, endothelial denudation completely blocked the response to S1P in arteries of FTY720 treated animals, but not in those of control rats. In acute experiments, presence of FTY720 significantly augmented the contractile response to S1P, an effect that was partially abolished after the inhibition of cyclooxygenase (COX-) derived prostaglandins. In experiments with cultured human endothelial cells, prolonged incubation with FTY720 abolished the expression of the S1P1 receptor subtype while leaving S1P2 receptor expression unaffected.

In conclusion, long term treatment with FTY720 leads to a down-regulation of smooth muscle cell reactivity to S1P, which, however, is compensated by the up-regulation of endothelial cell reactivity to release a contractile factor(s). The latter may include S1P-induced release of contractile prostaglandins via mechanism in which chronic FTY720 treatment down-regulates endothelial S1P1 receptor expression while preserving S1P2 receptor expression.
Introduction

FTY720 (fingolimod) is an analog of sphingosine 1-phosphate (S1P) that acts as an immunosuppressive agent by inhibiting the egress of lymphocytes from secondary lymphoid organs to peripheral blood [1,2] and treatment with FTY720 has been demonstrated effective in patients with multiple sclerosis (MS), autoimmune disease and organ transplantation [3]. The effects of S1P are mediated through specific G protein-coupled S1P receptors of which five different receptor subtypes have been identified: S1P(1-5). Several agonists to these receptors have been developed and FTY720 has been identified as a relatively non-selective agonist which binds to S1P(1-5) [4]. However, the phosphorylated form of FTY720 has no affinity to S1P2 [5].

Apart from its immunomodulatory role, FTY720 has profound effects on the cardiovascular system and administration of S1P and FTY720 is known to induce bradycardia both in human and in rodent animal studies [6]. FTY is known to act directly on the heart and the effects of FTY720 on heart rate are thought to be mediated mainly through S1P3, as selective S1P1 agonists did not induce bradycardia in mice and genetic deletion of the S1P3 subtype resulted in the abrogation of S1P induced bradycardia [7].

In the vascular endothelium, S1P1 is the most predominant subtype of S1P receptor and stimulation of endothelial S1P1 causes vasodilation in arteries through an increase in the production of NO [8]. In addition, S1P3 may also stimulate endothelial NO production, as S1P still elicits NO production following knock down of S1P1 [8,10]. In addition to S1P1 and S1P3 receptors, the endothelium expresses S1P2, of which expression is increased in atherosclerosis and diabetes [11,12]. The effects of S1P2 activation include the induction of pro-inflammatory responses in endothelial cells, regulation of microvascular permeability and stimulation of angiogenesis [12]. However, S1P1 and S1P2 show opposing effects on vascular permeability, indicating that the homeostasis of microvascular permeability may be regulated by their balance [13].

In contrast to the endothelium, the role of S1P receptors is less well understood in the vascular smooth muscle layer. Smooth muscle cells obtained from rat aorta predominantly express S1P2 and S1P3 and stimulation of these receptors activates several intracellular messengers including Rho kinase, intracellular calcium and MAPK [14]. Selective S1P3 antagonism induced relaxation of dog cerebral arteries that were pre-contracted with S1P and decreased S1P induced calcium signaling in cultured human coronary artery smooth
muscle cells, while selective S1P2 antagonists were without effects [15]. However, S1P2 antagonism has also been reported to inhibit S1P induced contractions of cultured coronary artery smooth muscle cells [16]. A further role for S1P2 receptors in vascular contraction has been demonstrated in mice genetically deleted of S1P2, resulting in impaired contractions to phenylephrine and KCl in mesenteric and renal resistance vessels [17]. In addition, a lack of S1P2 receptor induces decreases myogenic response, which is governed by TNFα in proximal cerebral arteries [18]. As FTY720/fingolimod is entering the clinic, a proper understanding of its vascular action upon chronic treatment seems warranted. Moreover, characterization of the effects of chronic treatment with fingolimod likely enhances our understanding of the diverse roles of S1P receptors in the vascular system, which may lead to improved and more specific therapies in cardiovascular disease. As FTY720 is known to affect all S1P receptors but S1P2, we hypothesized that long term FTY720 treatment alters the balance of vascular S1P signaling in the microvasculature, leading to altered vascular function. We, therefore, investigated vascular function of mesenteric arteries of rats to pressure and response to S1P after that were treated with FTY720 for six weeks.

Materials and methods

Animals and Study Design

The experiments were performed according to the NIH Guideline for the use of laboratory animals and were approved by the research ethics committee at the University of Groningen. Twelve male *Wistar* rats from Harlan weighing between 180-200 gr were randomly assigned to control or FTY720 treatment. Rats in the active treatment group received daily 1 mg/kg body weight FTY720 (Novartis, Switzerland) via drinking water for 6 weeks. A week prior to treatment, animals were housed under standard conditions for acclimation at the animal facilities of the University of Groningen. During the study, *ad libitum* food and tap water were provided. After six weeks of treatment, rats were anesthetized with 2.5% isoflurane in oxygen and data of hemodynamic function recorded by a pressure transducer catheter, which was inserted into the carotid artery (Datex-Ohmeda, Cardiocap/5, USA). Afterwards, mesenteric arteries were isolated and transferred in normal physiologic saline for further studies.
Vascular reactivity of mesenteric arteries

A third order branch of the superior mesenteric artery with a diameter of 249±3 µm, was dissected from surrounding fat tissue and mounted in a perfusion set up for pressurized vessels (Living System Instrumentation, Burlington, VT, USA). To evaluate the luminal diameter of the vessels, an inverted light microscope attached to a video camera and video dimension analyzer was integrated in the setup. The vessel chamber in the setup was filled and continuously recirculated with warmed (37°C) and oxygenated (5% CO₂ in O₂) Krebs solution with a pH of 7.4. Intraluminal pressure was set at 60 mmHg and arteries were allowed to equilibrate for 30 minutes as described previously [19]. Measurements were initiated by adding a single dose of phenylephrine (PE, 10⁻⁶ mol/L) to the vessel chamber to check contractile smooth muscle viability in both endothelium-intact or denuded vessels. Hence, the endothelium was removed by back and forth rubbing the vessel lumen with a hair. Successful removal of the endothelium was confirmed by the absence of a dilative response to subsequent addition of acetylcholine (ACh; 3*10⁻⁵ mol/L).

Intraluminal myogenic constriction was studied by obtaining active pressure-diameter curves over a pressure range of 60-140 mmHg in steps of 40 mmHg. Each pressure step was maintained for 3 minutes to reach a stable response. Subsequently the pressure was set back to 60 mmHg and the vessel segments were incubated with 1*10⁻⁵ mol/L S1P for 20 min after which the active pressure-diameter curve was obtained again. Finally, to obtain the passive pressure-diameter curves, S1P and calcium containing Krebs solution was washed out and replaced by calcium-free Krebs solution supplemented with ethyleneglycol-bis-(b-aminoethylether) tetra acetic acid (EGTA, 2 mmol/L), and the pressure-step measurements repeated.

To also obtain vascular reactivity to PE, ACh and SNP, additional arteries of the aforementioned mesenteric were isolated, and the endothelium kept intact or removed (i.e. as in the above) before relaxation responses were assessed. For that matter, vessels were first pre-constricted with 3*10⁻⁷ mol/L PE. Subsequently, endothelium-dependent dilation curves to ACh (10⁻⁹ - 10⁻⁵ mol/L) were assessed in the intact vessels and endothelium-independent dilation curves to SNP (10⁻⁹ - 10⁻⁵ mol/L) in the denuded vessels. The latter measurements were all performed at 60 mmHg.

In order to measure the acute effects of FTY720 on contraction mediated by S1P receptors, additional mesenteric arteries of control rats were cut into vascular rings (1-2 mm in width).
Arterial rings were mounted on a small wire in individual bath chambers and equilibrated for 30 min in Krebs bicarbonate solution bubbled with 95% O₂ and 5% CO₂ at 37°C. Then, the calculated length of the vessel at 100 mmHg was determined by stepwise increasing the distance between two stainless steel wires in steps of 10 mm until the calculated transmural pressure exceeded 120 mmHg [20]. Vessels were held at each length for 1 min and the generated force and internal circumference were used to calculate the wall tension. The internal circumference and corresponding wall tension for each point could thus be fitted on an exponential curve for determination of L100 (i.e. calculated length of the vessel at 100 mmHg). Arteries were allowed to equilibrate for 30 min in a standard Krebs solution at an internal circumference of 0.9 L100 before S1P (10⁻⁹ - 10⁻⁵ mol/L) -induced contraction was studied. Hence, contractions to S1P were studied both in the absence and presence of FTY720 (10⁻³ mol/L) and/or indomethacin (10⁻⁵ mol/L as to block the involvement of cyclooxygenase (COX-)derived prostaglandins).

**Cell culture**

HUVECs were obtained from the Endothelial Cell Facility of the University Medical Center Groningen/University of Groningen and grown to confluence in six-well plates and treated with FTY720 (10⁻⁷ mol/L) or vehicle for 24 hours before being washed once with 0.5 ml PBS. Subsequently, S1P receptor protein levels were analyzed by Western blotting. For the Western blot analysis, cells were homogenized in radio-immunoprecipitation assay buffer, and protein concentration was determined according to the DC protein assay (Biorad, Veenendaal, Netherlands) using bovine albumin as a standard. Denatured protein (20 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4%–20% precise protein gels (Pierce, Rockford, IL, USA), transferred to nitrocellulose membranes and incubated with primary antibodies against S1P1 (EDG-1 ab11424 Abcam, Cambridge, UK) and S1P2 (EDG-5 Antibody (H-64), Santa Cruz, Heidelberg, Germany), Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG, Santa Cruz, Heidelberg, Germany), Heerhugowaard, Netherlands) was used as secondary antibody. Signals were detected by the West Pico Chemiluminescent Substrate method (Life technologies, Bleiswijk, Netherlands) and quantified by densitometry.
Chemicals and compounds

Vascular studies were performed using daily-prepared Krebs bicarbonate solution with the following composition \( \text{NaCl}, 120.4; \text{KCl}, 5.9; \text{CaCl}_2, 2.5; \text{MgCl}_2, 1.2; \text{NaH}_2\text{PO}_4, 1.2; \text{glucose}, 11.5; \text{NaHCO}_3, 25.0 \) in (mmol/L). S1P was purchased from Sigma Aldrich (Netherlands). FTY720 was generously provided by Novartis and was dissolved in demi water. Other chemicals and compounds were purchased from Merck (Merck, The Netherlands).

Data analysis and calculations

Data are expressed as mean ± SEM; n indicates the number of the rats or the number of investigated arteries. To characterize myogenic response, the following parameters were calculated from the pressure-diameter curve of each individual artery. Myogenic tone, describing myogenic behavior of an artery at a given pressure, was expressed as percent decrease in active diameter from the maximally dilated (passive) diameter determined at the same pressure in calcium-free/EGTA solution, i.e., myogenic constriction (%) = \( 100 \left\{ (D_{\text{Ca-free}} - D_{\text{Ca}})/D_{\text{Ca-free}} \right\} \), where \( D \) is the diameter in calcium free (\( D_{\text{Ca-free}} \)) or calcium-containing (\( D_{\text{Ca}} \)) Krebs. The Area Under the Curve (AUC) for myogenic constriction (MC) were calculated from pressure curves and expressed as arbitrary units (AUs) using graph pad prism(5.0) Statistical differences were determined by t-test using SPSS. Differences were considered significant at \( p<0.05 \) (two-tailed).

Results

Body weight and hemodynamic effects of FTY720 treatment

Animal body weight and hemodynamics are presented in Table 1. Body weights were similar in control treated and FTY720 treated animals. FTY720 treatment significantly reduced heart rate, although no effects of FTY720 were observed on blood pressure after six weeks of treatment.
Table 1. Animal body weight and hemodynamic effects of FTY720.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 6)</th>
<th>FTY720 (n = 6)</th>
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<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>472±12</td>
<td>449±13</td>
</tr>
<tr>
<td><strong>Heart Rate (bpm)</strong></td>
<td>408±7</td>
<td>361±7 *</td>
</tr>
<tr>
<td><strong>Systolic Blood Pressure (mmHg)</strong></td>
<td>137±7</td>
<td>135±7</td>
</tr>
<tr>
<td><strong>Diastolic Blood Pressure (mmHg)</strong></td>
<td>83±7</td>
<td>83 ±4</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM, * p<0.01 vs control.

**Long term of FTY720 treatment effects on mesenteric artery reactivity**

To investigate the effects of long term FTY720 treatment on the microvasculature, we examined vascular myogenic function and response to S1P of isolated mesenteric arteries in a perfusion setup. To determine the role of the endothelium in vascular function, measurements were performed in intact and denuded blood vessels and presented as a pressure range of 60-140 mmHg and calculated as area under the curve (AUC) respectively (figure 1A, 1B).
Figure 1. Responses of endothelium-intact and -denuded mesenteric arteries of rats chronically treated with or without FTY720. (A) Pressure induced myogenic constriction. (B) Area under the curve of pressure induced myogenic constriction. (C) S1P induced constriction. (D) Area under the curve of S1P induced constriction. Data are expressed as mean ± SEM. * p<0.05 vs control.

Next, myogenic constriction of mesenteric vessels was assessed after pretreatment with S1P (10 µM). Addition of S1P to mesenteric arteries resulted in a strong constriction at 60 mmHg in both intact and denuded control and FTY720 treated animals (figure 1C). Subsequent increases in intraluminal pressure resulted in an increase of myogenic constriction in vessels from control and intact vessels from FTY720 treated animals. In contrast, in FTY720 treated animals addition of S1P to endothelium denuded vessels did not result in an increase of myogenic constriction (figures 1C and 1D). As a result no...
differences were observed in constriction in endothelium-intact vessels compared with endothelium-denuded vessels of untreated animals. In FTY720 treated animals, however, responses to S1P were completely blocked in denuded arteries. The latter suggests that the endothelium is essential for maintaining S1P mediated constriction after long term treatment with FTY720.

Moreover, the treatment effects of FTY720 appear specific for S1P-mediated responses since contraction to PE was not significantly different between treated and untreated animals, neither in endothelium-intact nor in endothelium-denuded artery segments (figure 2A; response to PE: intact 46±6%, FTY720 intact 60±8%, denuded 70 ±10%, FTY720 denuded 48±9 % of KCl). Also, the similar responses to ACh and SNP in arteries of untreated and FTY720 treated animals (figure 2B and 2C) further rules out that the above described differences in the response to S1P were due to some general (d-)effect of FTY720 treatment on endothelial and/or vascular smooth muscle cell function.
Acute effects of FTY720 treatment on mesenteric artery contraction

To further investigate how FTY720 treatment may have affected S1P mediated contraction of endothelium intact mesenteric arteries, we also studied the acute effects of FTY720 on S1P induced contractions in isolated mesenteric artery ring preparations. Incubation with FTY720 caused a significant leftward shift of the dose-response curve to S1P (figure 3, Table 2). Addition of indomethacin, a nonselective inhibitor of cyclooxygenase (COX) 1 and 2, partially reversed the effects of FTY720. These results suggest that FTY720 acutely enhances S1P contraction through a mechanism that at least in part involves COX-derived
prostaglandins. Furthermore, it seems that this acute effect of FTY720 was specific for S1P-induced contraction since responses to KCl remained unaffected in presence of FTY720 (data not shown).

Table 2. Concentration-response parameters for S1P induced contractions of mesenteric arteries.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Log EC50</th>
<th>Emax</th>
</tr>
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<tbody>
<tr>
<td>CON (n=6)</td>
<td>-6.30 ±0.10</td>
<td>36.2 ±4.6</td>
</tr>
<tr>
<td>CON + INDO (n=6)</td>
<td>-6.27 ±0.06</td>
<td>34.0 ±4.5</td>
</tr>
<tr>
<td>FTY720 (n=6)</td>
<td>-6.65 ±0.09*</td>
<td>43.7 ±4.7</td>
</tr>
<tr>
<td>FTY720 + INDO (n=6)</td>
<td>-6.52 ±0.13</td>
<td>37.8 ±4.7</td>
</tr>
</tbody>
</table>

Values are means ± SEM; n = no. of arteries; Emax= maximal contraction (% of KCL). * p < 0.05 vs control (CON)

Figure 3. Acute effects of FTY720 on S1P mediated contraction. Pre-treatment with FTY720 caused a leftward shift of the CR curve to S1P; this could be in part reversed by addition of indomethacin (*p<0.05 EC50 FTY720 vs control).
Effect of FTY720 on endothelial S1P receptor subtype expression

Since the presence of endothelium was essential for maintaining S1P mediated constriction after long term treatment with FTY720, we investigated the effects of FTY720 on the expression of S1P receptor subtypes in cultured human endothelial cells (HUVEC). FTY720 treatment significantly reduced S1P1 protein expression while S1P2 expression was unaffected (figure 4), indicating that FTY720 treatment shifts the relative expression of S1P receptors towards S1P2.

Figure 4. Effect of FTY720 on the expression of S1P1 and S1P2 in cultured human endothelial cells (HUVEC). The expression of S1P1 was significantly reduced by FTY720. Data are presented as mean ± SEM. * p < 0.05 vs control.

Discussion
Chapter 5

This is the first functional study to characterize the chronic effects of FTY720 treatment on contractile responses to S1P in small resistance arteries of Wistar rats. The main finding of this study is that the endothelium of mesenteric arteries of healthy animals is able to compensate for a complete loss of responsiveness of the smooth muscle layer to S1P after FTY720 treatment through a mechanism which enhances production of contractile prostaglandins by the endothelium.

Chronic treatment of FTY720 leads to bradycardia without reduction of blood pressure in Wistar rats. This balance in maintenance of blood pressure indicates the compensatory mechanism of vascular system to adjust the blood pressure tone, however in the current study various vascular function such as vascular tone and vascular response to PE remained unchanged in control mesenteric arteries compare with FTY720. This finding is partially concordant with previously findings of Spijkers et al., who found FTY720 not to affect vascular contraction, per se, in non-hypertensive rats [21].

In this study, our findings are in line with several previous studies demonstrating that FTY720 causes sustained internalization and degradation of S1P receptors [22]. Addition of S1P did not affect the vascular contraction of intact vessels in chronically treated animals compared with control. In contrast, in denuded arteries, long term FTY720 treatment caused a complete inhibition of S1P mediated constrictions, indicating that the smooth muscle layer of FTY720 treated animals had completely lost responsiveness to S1P. As smooth muscle cells are known to express S1P1 and S1P3, our findings most likely reflect the functional consequences of down-regulation of these receptor subtypes in the smooth muscle layer of FTY720 treated animals. Moreover, our findings indicate that the endothelium compensates for the loss of S1P signaling of the smooth muscle layer, as S1P responsiveness was maintained in intact vessels of FTY720 treated animals. As S1P mediated augmentation of myogenic constriction arteries was completely absent in denuded mesenteric, it is unlikely that the endothelium of intact arteries compensates through impaired productions of relaxing factors. Indeed, relaxation responses to acetylcholine of mesenteric arteries did not differ between vehicle and FTY720 treated animals. Rather, the endothelium most likely produces endothelium-derived contractile factors (EDCFs), which maintained S1P mediated constrictions in intact mesenteric arteries of FTY720 treated
animals. A well-known source of EDCFs consists of cyclooxygenase (COX)-derived contractile prostaglandins.

Our findings are in line with those of Spijkers et al., who reported correlation between S1P and hypertension in spontaneous hypertensive rats (SHR) [23]. Previously, a link between FTY720 and endothelial COX has been described in a study showing that FTY720 caused contractions of isolated carotid arteries of spontaneous hypertensive rats (SHR), which could be blocked by denudation, indomethacin and thromboxane synthase inhibitors [21]. However in mast cells, FTY720 inhibits prostaglandin and thromboxane secretion independently of S1P receptors [24]. Our findings on mesenteric arteries elucidate that S1P mediated contractions after FTY720 treatment augments the COX secretion in endothelial cells and enhances sensitivity to S1P; as reflected by a significantly lower log EC50 value. It is known that in smooth muscle cells S1P can regulate COX activities via protein kinase B and mitogen-activated protein kinases pathways [25]. In addition, our data shows that inhibiting COX partly reversed the effects of FTY720, indicating that the effects of FTY720 were at least in part mediated through COX-derived PGs in endothelium. Taken together, our results and previous studies suggest that the endothelium may enhance vascular contraction in FTY720 treated animals by stimulating endothelial COX mediated contractile prostaglandin production.

Relative expression of S1P1 and S1P2 after FTY720 treatment caused a shift of S1P receptors towards S1P2. Although coupling of S1P2 to COX has not yet been demonstrated in aortic vascular endothelial cells, stimulation of S1P2 of endothelial cells from mouse retina enhanced COX expression [26]. Furthermore, S1P induced COX-2 expression and PGE2 formation through S1P2 in renal mesangial cells [27]. Therefore, the enhanced endothelium mediated contraction in FTY720 treated animals may be caused through enhanced contractile prostaglandin production caused by a shift in the relative expression of S1P1 and S1P2.

In conclusion, to the best of our knowledge, this is the first study to demonstrate that the endothelium of mesenteric arteries of healthy animals may be able to compensate for the complete loss of responsiveness of the smooth muscle layer to S1P after long term FTY720 treatment through a mechanism that involves enhanced production of contractile
prostaglandins by the endothelium. Although this mechanism did not cause changes in blood pressure in healthy animals, it may contribute to the development of hypertension in conditions in which the COX system is already activated, such as in spontaneous hypertensive rats (SHR).

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References


