Sphingosine-1-Phosphate Receptor Agonist, FTY720, Restores Coronary Flow Reserve in Diabetic Rats

Hongzeng Xu, PhD; Yuanzhe Jin, PhD; Haifeng Ni, PhD; Shengda Hu, PhD; Qin Zhang, MD

**Background:** Impairment of coronary flow reserve (CFR) has been generally demonstrated in diabetic patients and animals with microvascular complications but without obvious obstructive coronary atherosclerosis. There have been few studies investigating CFR in cases of relatively well-controlled therapy. The purpose of this study is to evaluate the effect of treatment with a Sphingosine-1-phosphate (S1P) receptor potent agonist, FTY720, on early diabetic rats in terms of CFR.

**Methods and Results:** Male Sprague-Dawley (SD) rats were divided into 3 groups: (1) streptozotocin-uninjected rats (control rats); (2) streptozotocin-injected hyperglycemic rats (diabetic group); and (3) FTY720-fed and streptozotocin-injected hyperglycemic rats. FTY720 (1.25 mg/kg per day orally) was administrated for 9 weeks in SD rats (from 6 weeks old to 15 weeks old). CFR was evaluated by \(^{13}\)N-ammonia positron emission tomography. No obvious pathological changes of macrovascular atherosclerosis were observed in each group. Diabetic rats had impaired CFR compared with the control group (1.39±0.26 vs. 1.94±0.24, P<0.05). Treatment with FTY720 for 9 weeks attenuated the heart histological changes and improved CFR in 32% of diabetic rats (1.84±0.36 vs. 1.39±0.26, P<0.05).

**Conclusions:** In summary, long-term therapy with the Sphingosine-1-phosphate receptor agonist, FTY720, improved CFR by attenuating the heart histological changes, and it might have a beneficial effect on coronary microvascular function in diabetic rats. (Circ J 2014; 78: 2979–2986)

**Key Words:** Coronary flow reserve; Diabetes; FTY720; Microcirculation; Sphingosine-1-phosphate
Methods

Materials

All reagents, unless otherwise specified, were of analytical grade and commercially available, and were used as received without further purification. FTY720 and streptozotocin were purchased from Sigma-Aldrich Inc (St. Louis, MO, USA). A MicroPET system (Inveon, Siemense Inc, Erlangen, Germany) supplied by Jiangsu Institute of Nuclear Medicine was used for in vivo imaging.

All of the animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Jiangsu Institute of Nuclear Medicine. Experiments were performed in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines on animal research. All efforts were made to minimize the number of animals used and reduce their suffering.

Animals

Male Sprague-Dawley rats (Shanghai Super-B&K Laboratory Animal Corp Ltd, Shanghai, China), weighing 160–180 g (5 weeks old) at the beginning of the study, received a single intraperitoneal injection of streptozotocin (60 mg/kg, dissolved in 0.1 mol/L sodium citrate buffer, pH=4.5) (Sigma) to induce high glucose. Control animals received only the citrate buffer. Only rats that developed sustained hyperglycemia with a serum glucose level of 300 mg/dl were included in the study. After confirmation of diabetes mellitus (DM), rats were divided randomly into 3 groups: (1) streptozotocin-uninjected rats (control group, n=8); (2) streptozotocin-injected hyperglycemic rats (diabetic group, n=8); and (3) FTY720-fed and streptozotocin-injected hyperglycemic rats (FTY720-treated group, n=8). To examine the effect of FTY720 on heart microvessels, we fed diabetic rats with or without oral FTY720 administration (with an effective dose of 1.25 mg · kg⁻¹ · day⁻¹) for a duration of 9 weeks.

The animals were maintained in a room controlled at a temperature range of 20–26˚C and relative humidity of 40–70%. The weight and serum glucose level of all groups were tested every 2 weeks during the experiment.

Positron Emission Computed Tomography and Data Analysis

After treated with FTY720 for 9 weeks, all animals underwent the Positron Emission Tomography study at rest and under dipyridamole vasodilatation conditions. First, the rats received an injection of ¹³N-ammonia (3.5–5.0 mCi) in the tail vein. Dynamic serial transaxial images were acquired for 10 min (Figure 1A). The proportion of ¹³N radioactivity in plasma was measured in rats with a catheter inserted in a femoral artery. Blood samples were collected (150 μl) at 30, 60, 120, 180 and 300 s from the femoral artery catheter. After deproteinization and centrifugation, the supernatant was transferred to new tubes. Triplicates of the counting tubes containing the supernatant were counted individually using a gamma counter for 1 min. Sixty minutes after the first ¹³N-ammonia injection, 150 μg · kg⁻¹ · min⁻¹ dipyridamole (Beijing Yongkang Co) was infused intravenously over 4 min. ¹³N-ammonia (3.5–5.0 mCi) was injected at the end of the third minute after dipyridamole infusion and serial images of the heart were recorded 3 min (Figure 1B). The whole PET study took an average time of 20 min.

For imaging studies, animals were under isoflurane anesthesia that was delivered through a nose cone at a concentration of 2.0% volume and 2L/min oxygen flow. Animals were positioned and immobilized supine, with their medial axis parallel to the axial axis of the scanner, with their thorax region in the center of field of view. Imaging experiments were performed on the Siemens Inveon Dedicated Small Animal PET Scanner, with an in-plane resolution of 0.48 mm and an energy
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Table 1. General Characteristics for the Control, the Streptozotocin-Induced Diabetic Group and the FTY720 Treatment Group

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n=8)</th>
<th>DM (n=6)</th>
<th>DM+FTY720 (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>6</td>
<td>170±13.8</td>
<td>168±12.6</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>355±32.5</td>
<td>242±12.5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>487±98.2</td>
<td>277±14.6</td>
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<tr>
<td></td>
<td>15</td>
<td>525±102.9</td>
<td>298±21.8</td>
</tr>
<tr>
<td>Urine glucose</td>
<td>6,9,12,15</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>(qualitative)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose</td>
<td>6</td>
<td>6.8±0.6</td>
<td>19.8±1.4</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td>9</td>
<td>7.1±0.4</td>
<td>22.12±2.2</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>7.3±0.6</td>
<td>25.3±3.10</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD, *P<0.01 vs. Control, **P<0.05 vs. DM. DM, diabetes mellitus; FTY720, Sphingosine-1-phosphate (S1P) receptor potent agonist.

Table 2. CFR and Perfusion Data at Baseline and During Hyperemia in the Controls, Diabetic and FTY720 Treatment Groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n=6)</th>
<th>DM (n=6)</th>
<th>DM+FTY720 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>10.2±1.8</td>
<td>27.5±2.7*</td>
<td>15.3±2.49*</td>
</tr>
<tr>
<td>MBFStress (ml·min⁻¹·g⁻¹)</td>
<td>6.13±1.23</td>
<td>5.96±1.03</td>
<td>6.04±1.37</td>
</tr>
<tr>
<td>MBFrest (ml·min⁻¹·g⁻¹)</td>
<td>11.62±1.58</td>
<td>8.35±1.58*</td>
<td>11.14±2.12*</td>
</tr>
<tr>
<td>CFR</td>
<td>1.94±0.24</td>
<td>1.39±0.26*</td>
<td>1.84±0.36*</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD, *P<0.05 vs. Control, **P<0.05 vs. DM. CFR, coronary flow reserve; MBF, myocardial blood flow. Other abbreviations as in Table 1.

Histopathological Evaluation and Immunohistochemistry

After imaging experiments, the rats were sacrificed and their left ventricles were dissected and myocardial sections were removed immediately for histological processing. The tissue samples were fixed in paraformaldehyde solution (4%) and embedded in paraffin. From the paraffin blocks, 4-μm-thick serial sections were examined for detailed morphological analysis. The heart tissue was estimated by using hematoxylin and eosin (H&E) staining.

All sections of formalin-fixed, paraffin-embedded heart samples for immunohistochemical analysis were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase in methanol for 30 min at room temperature. The sections were microwaved to retrieve the antigen by using an antigen unmasking solution for 5 min. After incubation with 5% skim milk for 1 h at room temperature, the sections were incubated with the primary antibodies (rabbit anti-rat CD31 (1:100), anti-transforming growth factor (TGF)-β (1:100), anti-collagen type I (1:100) overnight at 4°C). Then, the tissues were washed several times in phosphate-buffered saline (PBS) and incubated with the secondary antibody for 45 min. Subsequently, the tissues were visualized with a 3, 3′-diaminobenzidine kit and examined under a microscope and photographed.

The collagen distribution in myocardial tissue was observed by Masson’s trichrome staining and collagen immunohistochemistry staining. In order to observe the changes of the capillaries in the myocardium, endothelial cells were stained with anti-CD31. To determine capillary density, the number of CD31 brown positive-staining cells was counted in a double-blind fashion from 10 different fields of each section (n=5) at a ×200 magnification. The average number of the vessels in one section was used for the assessment of microvascular density. All images were reviewed and analysed using Image Pro Plus 6.0.

Western Blotting

We used Western blot analysis according to the manufacturer’s instructions to investigate vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and interleukin (IL)-6 protein expression. Proteins from the heart tissue extracts (100 μg per sample) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Non-specific antibody binding was blocked by a pre-incubation of the membrane in 1×Tris-buffered saline containing 5% skim milk for 1 h at room temperature. The membranes were incubated with anti-VCAM-1 antibody (1:500; Sigma), anti-IL-6 antibody (1:500; Sigma) and anti-ICAM-1 antibody (1:500; Sigma) at 4°C overnight with constant shaking. This was followed by incu-
observed an increase of CFR by 31% compared in the diabetic group (1.84 ± 0.36 vs. 1.39 ± 0.26, P<0.05), indicating that FTY720 preserved the coronary artery diastolic ability.

Effect of FTY720 on Histological Changes

Conventional histology with H&E and Masson staining showed moderate myodegeneration and sporadic vacuolation in the left ventricular myocardium of the diabetic control rats, without signs of severe tissue damage, and no obvious pathological changes of large vessel atherosclerosis.

After H&E staining, neat myocardial cells that had a clear structure, were compact, with less extracellular matrix, and a small amount of fibroblasts, could be seen in the normal rats. While in the diabetic group, disorderly arranged myocardial cells, distortion, and enlarged cell gaps were shown. Cardiomyocyte size significantly increased in the diabetic group compared to the control group, while in the FTY720 treatment group, a trend towards diminished cardiomyocyte hypertrophy, but no statistically significant effects, were found in comparison with the control group. The state was somewhat in between, and compared with the model group, narrowed cell gaps and reduced interstitial and perivascular extracellular matrix were shown (Figure 2A).

After Masson staining, the distribution of collagen tissue was almost equal in the non-diabetic control group; the collagen fiber network among adjacent cells was intact with less collagen fiber content. In the diabetic group, the broken and disorderly arranged collagen fiber network around myocardial cells increased interstitial and perivascular extracellular matrix, and increased the number of fibroblasts with inflammatory cells infiltrated, as shown in Figure 2B. Compared with the control group, the relative myocardial collagen content in the diabetic group (1.84±0.36 vs. 1.39±0.26, P<0.05), indicating that FTY720 preserved the coronary artery diastolic ability.

Statistical Analysis

Data are expressed as mean±standard deviation (SD) and analyzed by using SPSS 17.0 software. For comparisons between 2 variables, the unpaired Student’s t-test was used. A two-tailed P<0.05 was considered statistically significant.

Results

Characteristics of Diabetic Animals

Three rats died during the experimental period; 2 in the vehicle-treated diabetic group and 1 in the FTY720-treated group. Serum levels of glucose were increased in diabetic rats, which were higher than 18 mmol/L in 6 weeks old. These results indicate that hyperglycemia was successfully induced. Interestingly, the blood glucose level in the FTY720-treated group became lower than that in the diabetic group after being treated for 4 weeks, and this lasted until the end of our experiment.

Diabetic rats showed lower body weight, significantly increased urine glucose levels and daily water intake. These parameters were not significantly altered by FTY720 treatment (Table 1). The changes in weight and blood glucose level for each group are shown in Table 1.

Chronic FTY720 Treatment Restores CFR in Diabetic Animals

Eighteen rats were studied following 15N-ammonia injection at baseline and under pharmacologic intervention conditions (Table 2). Although there was no obvious different average MBF value between the 3 groups, under dipyridamole stimulation conditions, diabetic rats had significantly decreased CFR compared with the non-diabetic control group (1.39±0.26 vs. 1.94±0.24, P<0.05). After given FTY720 for 9 weeks, we observed an increase of CFR by 31% compared in the diabetic group (1.84±0.36 vs. 1.39±0.26, P<0.05), indicating that FTY720 preserved the coronary artery diastolic ability.

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Compared with the control group, the relative myocardial collagen content in the diabetic group increased significantly (P<0.01), while in the CFR group, the arrangement of collagen tissue and the structure of the fiber network were better (Figure 2B). Compared with the diabetic group, collagen content in the FTY720 treatment group decreased significantly (P<0.05) suggesting that FTY720 can remarkably inhibit the proliferation of collagen fibers.
Effects of FTY720 on the Expression of Collagen and the Pro-Fibrotic Molecule TGF-β

Transforming growth factor (TGF)-β plays an important role in the pathogenesis of the fibrotic effects in diabetic complications. Immunohistochemical staining for TGF-β demonstrated a similar pattern to that observed with respect to collagen content. As shown in Figure 3C, diabetic rats had significantly increased TGF-β immunostaining compared with the control rats (5.66±0.92% vs. 3.22±0.56%, P<0.05). TGF-β immunostaining in the FTY720-treated diabetic group was significantly lower than that in the vehicle-treated diabetic rats (4.42±0.29% vs. 5.66±0.92%, P<0.05), but significantly higher compared with the control rats (P<0.05). To assess the extent of myocardial interstitial fibrosis, we measured the collagen content by collagen staining the sections of the left ventricle from experimental animals. As shown in Figure 3B, quantitative analysis of the fibrotic region of the left ventricle myocardium indicated significantly increased interstitial fibrosis in DM animals vs. control (11.7±0.87% vs. 6.21±0.67%, P<0.05). FTY720 treatment significantly reduced the extent of myocardial interstitial fibrosis compared with the diabetic rats (7.96±0.80% vs. 11.7±0.87%, P<0.05), but it was significantly higher compared with the control rats (P<0.01).

Effects of FTY720 on Ventricle Capillary Density

The myocardial microvessel density difference was quantitative assessed by using CD31-positive staining endothelial cells. Endothelial cells in coronary microvasculature were regularly arranged, and the capillary walls were thin and smooth in most control rats (Figure 4A). However, there was a significant decrease of cardiac capillary density in the diabetic group compared to the control group (11.4±0.79% vs. 22.54±3.59%, P<0.01). FTY720 treatment for 9 weeks significantly alleviated the damage of the microvasculature and a trend towards increased capillary density was found compared to the control group (17.26±1.40% vs. 11.4±0.79%, P<0.05).

Effects of FTY720 on the Expression of Pro-Inflammatory and Endothelial Adhesion Molecule Expression

As shown in Figure 5, Western blot analysis results show that the diabetic group had a significant increase of IL-6 in cardiac tissues, while in the FTY720 treatment group, the level of IL-6
was lower than that of the DM group. Compared to controls, diabetic rats had significantly increased VCAM-1 and ICAM-1 protein relative levels (VCAM-1 protein: 0.29±0.04 vs. 0.12±0.03; ICAM-1 protein: 0.78±0.02 vs. 0.39±0.05, P<0.01). However, FTY720 treatment of diabetic rats resulted in significantly decreased VCAM-1 and ICAM-1 protein levels compared with the untreated diabetic rats (VCAM-1 protein: 0.18±0.04 vs. 0.29±0.04; ICAM-1 protein: 0.59±0.07 vs. 0.78±1.02, P<0.05).

Discussion

In the present study, we successfully constructed the diabetic rat model and studied the effect of FTY720 on CFR with 13N-ammonia microPET. Our experimental results showed that there was a lower flow coronary vasodilator response in the majority of diabetic rats. After oral administration of FTY720, at a dose of 1.25 mg · kg–1 · day –1 for 9 weeks, the CFR of diabetic rats increased. It indicated that FTY720 improves the vasodilator response of myocardial microvessels. To the best of our knowledge, this is the first report on the administration of FTY720, which prevented the progression of coronary microcirculatory disturbance.

Evidence from clinical and experimental studies showed that CFR is reduced in diabetic mellitus because of functional and structural abnormalities of the coronary microvascular circulation such as endothelial dysfunction, hyperglycemia, perivascular fibrosis, endothelial adherence molecules and collagen disposition. An abnormal extracellular component of coronary resistance, like smooth muscle cell proliferation, might also play an important role. A previous study has shown that inward hypertrophic remodeling of coronary arterioles isolated from diabetic mice is associated with a decrease in vascular wall stiffness and reduced MBF and CFR. In this study, our findings were consistent with the current literature and showed that FTY720 treatment lowered the expression of TGF-β and collagen, and reduced the extent of myocardial interstitial fibrosis in diabetic rat heart tissues.

Sphingosine-1-phosphate receptor agonists have a wide effect on the proliferation of microvessels and inhibited the apoptosis of vessel endothelial cells. Some studies have shown that S1P could control the vascular tone; however, there are few studies that examined the effect of the S1P receptor agonist on the impact of myocardial perfusion. As one type of selective S1P receptor agonist drug, FTY720 has been proven to be of low toxicity. This study revealed that after 3 weeks of FTY720 treatment, there was no impact on kidney and liver hemodynamics. Our 13N-NH3 PET animal myocardial perfusion experiment indicated that the oral administration of FTY720 for 9 weeks could restore the CFR of diabetes rats. And pathology experiment showed that long-term administration of FTY720 improves myocardial microangiopathy.

There has been controversy about the effect of S1P agonist on the expression of vascular inflammation factor and endothelial adhesion molecule. Some studies support the use of the S1P receptor agonist to prevent inflammation factor-mediated monocyte adhesion to aortic endothelium in mice, while another study showed that S1P could induce an increase in E-selectin expression by the way of NF-κB activation. However, the present study results showed that FTY720 could lower the expression of VCAM-1, ICAM-1 and IL-6 in diabetic rat heart tissue. A recent study report that IL-6 and tumor necrosis factor (TNF)-α were independently associated with CFR, and a PET-CFR study showed that IL-6 should be a determinant of CFR. This may be one of the microvascular protection mechanisms of FTY720. Interestingly, the FTY720 treatment group showed a lower glucose level than the diabetic group; one of the mechanisms of FTY720 treatment may be that it could prevent the manifestation of DM by promoting the retention of activated immune cells in the lymph nodes, thereby avoiding islet infiltration and β-cell destruction by proinflammatory cytokines. This protective effect might account for why FTY720 could restore the CFR of diabetic rats and improve vascular resistance.

Diabetes could be a coronary artery disease equivalent. The earlier stage of diabetes impacts the vascular system and causes endothelial dysfunction, such as the increase of reactive oxygen species, the lower release of NO, and the stimulation of endothelial cells to release a vascular inflammation factor and bring about the state of apoptosis. This procedure is also called “endothelial activation”, which reflects an early injury procedure to the vascular wall, and could lead to the disturbance of the microvascular blood stream and the lower myocardial perfusion. The coronary microvascular endothelial dysfunction has an important role in the development of atherosclerosis. Some studies have demonstrated that FTY720
could promote angiogenesis, lower the vascular permeability, stimulate endothelial cells to release NO by the PI3K/AKT pathway and increase the local blood flow. Another study has shown that endothelium-derived hyperpolarizing factor (EDHF) dysregulation is present in various vascular beds of diabetic animal models and humans with vascular dysfunction caused by an impairment of endothelial-dependent vasodilation; EDHF also plays a crucial role in modulating vasomotor tone in microvessels. How FTY720 influences the endothelium-derived relaxing factor and the mechanism that impacts the endothelial function remains unclear.

Overall, there is growing evidence that lower CFR is an independent risk factor of cardiovascular events. Therefore, study drugs to improve coronary artery microcirculation have an important clinical value. Myocardial 13N-NH3 PET imaging provides a relatively precise method for evaluating coronary microcirculation. We used this method to study the effect of FTY720 on the restored CFR of the diabetic rat. This reverse effect might have important implications for coronary microcirculation, but the mechanism is complex and future research is needed. In this study, the animal model was given a large dose of streptozotocin and was not provided with high cholesterol food to feed on; this was the type 1 diabetic model. CFR is affected in the experimental rats not only by microvascular function but also by epicardial coronary stenosis. However, in this study, we did not find obvious pathogenesis of macrovascular complications, including atherosclerosis in diabetic rats. Whether FTY720 could restore the CFR, which deteriorated because of coronary macrovessel stenosis, is still unknown. The next step in our research would be to use a low dose injection of streptozotocin (30 mg/kg) along with a high-fat diet to induce high blood pressure and high cholesterol levels to study the effect of FTY720 on CFR in a diabetic model that has obstructive coronary atherosclerosis.

Study Limitations

The main drawback of this study is the high cost and the limited availability of the imaging equipment in some places throughout the world. However, PET has recently proved itself as a highly precise method for the assessment of CFR. In addition, these results will reinforce PET’s key role in cardiology and help to make it a more accessible method in the future. Other limitations of this study include a low number of rats used and the short length of FTY720 treatment. One of the most important limitations of this study is the fact that we were not able to compare the different dose effects of the drugs separately, thus we cannot conclude whether the changes observed were due mostly to the effectiveness of the drugs. It is important to design a study that includes a larger number of doses given to the subjects, a longer treatment course, and to create groups that could receive each of the drugs independently. In addition, some studies report that FTY720 has side-effects such as transient bradycardia and atrioventricular block. Some studies reported that heart rate and blood pressure can impact CFR. Further research is required to investigate whether FTY720 has an impact on the heart rate of diabetic rats.

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Disclosures

The authors have declared that no competing interests exist.

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**Appendix**

The contributions of the authors to this study were as follows: Conceived and designed the experiments: H.X., H.N., Y.J. Performed the experiments: H.X., H.N., S.H. Analyzed the data: H.N., S.H. Wrote the paper: H.X., H.N. Sponsored the experiments: Y.J.