Immunomodulator FTY720 Induces eNOS-Dependent Arterial Vasodilatation via the Lysophospholipid Receptor S1P3


Abstract—The novel immunomodulator FTY720 is effective in experimental models of transplantation and autoimmunity, and is currently undergoing Phase III clinical trials for prevention of kidney graft rejection. FTY720 is a structural analogue of sphingosine-1-phosphate (S1P) and activates several of the S1P receptors. We show that FTY720 induces endothelium-dependent arterial vasodilation in phenylephrine precontracted mouse aortae. Vasodilation did not occur in thoracic aortic rings from eNOS-deficient mice, implicating an effect dependent on activation of the eNOS/NO pathway. Accordingly, FTY720 induced NO release, Akt-dependent eNOS phosphorylation and activation in human endothelial cells. For biological efficacy, FTY720 required endogenous phosphorylation, since addition of the sphingosine kinase antagonist \( \text{N}_{\text{H}}1032, \text{N}_{\text{H}} \)-dimethylsphingosine (DMS) prevented activation of eNOS in vitro and inhibited vasodilation in isolated arteries. The endothelial phosphorylation of FTY720 was extremely rapid with also most complete conversion after 10 minutes as determined by mass spectrometry. Finally, we identified the lysophospholipid receptor S1P3 as the S1P receptor responsible for arterial vasodilation by FTY720, as the effect was completely abolished in arteries from S1P3-deficient mice. In summary, we have identified FTY720 as the first immunomodulator for prevention of organ graft rejection in clinical development that, in addition, positively affects the endothelium by stimulating NO production, and thus potentially displaying beneficial effects on transplant survival beyond classical T cell immunosuppression. (Circ Res. 2005;96:000-000.)

Key Words: FTY720 ■ eNOS ■ S1P receptor

The novel immunomodulator FTY720 is currently undergoing Phase III clinical trials for prevention of kidney graft rejection.1 FTY720 shares striking structural homology to sphingosine 1-phosphate (S1P),2 a natural lysophospholipid that is present at high nanomolar (nmol/L) concentrations in serum.3 Recent data show that FTY720 is phosphorylated in vivo by sphingosine-kinase-2 (SphK2),4 and that the FTY720-phosphate metabolite (FTY720-P) is a potent agonist of four of the five G protein–coupled receptors for S1P: S1P1, S1P3, S1P4, and S1P5.2,5 Recent studies show that the S1P1 receptor and its natural ligand S1P are pivotal to lymphocyte recirculation: mice with a specific deletion of S1P1 in hematopoietic cells showed that thymocytes selectively require S1P1 for egress from thymus, whereas both T and B cells require this receptor for egress from peripheral lymphoid organs.6 Thus, it was suggested that the efficacy of FTY720 in transplantation and autoimmunity may relate primarily to an inhibition of effector T cell recirculation from lymphoid organs to peripheral sites of inflammation.

S1P receptor agonists mediate a variety of physiological processes and stimulate multiple signaling pathways resulting in calcium mobilization from intracellular stores, polymerization of actin, chemotaxis/migration, and escape from apoptosis.7–10 S1P is released by platelets during inflammatory processes11 and can be found in significant amounts in serum as part of lipoproteins.3 The respective receptors S1P1, S1P2, and S1P3 are widely expressed, whereas S1P4 is restricted to lymphoid tissue and S1P5 is present in spleen and white-matter tracts of the central nervous system.8,12–14 In endothel...
lial cells, we and others have demonstrated that S1P activates Akt and eNOS resulting in vasodilation.

In this study, we show that the immunomodulator and S1P receptor agonist FTY720 displays direct effects on the vascular endothelium. FTY720 potently induced vasodilation in mouse aortae by activating the Akt/eNOS/NO pathway through the S1P receptor. These findings suggest that, unlike conventional immunosuppressive drugs, FTY720 may preserve vascular structure and function and help prevent cardiovascular morbidity and mortality that often occurs in transplant recipients.

**Materials and Methods**

Detailed methods are described in the expanded Materials and Methods in the online data supplement available at http://circres.ahajournals.org.

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and cultured in RPMI 1640 supplemented with 15% calf serum, 0.4% bovine pituitary brain extract (GIBCO BRL), 50 μg/mL heparin, and antibiotics as described previously.

**Arterial Tension Studies**

The direct effects of FTY720 or FTY720-P on arterial relaxation and contraction were evaluated in 2-mm rings of thoracic aortae from 3-month-old female C57BL/6j mice (Charles River Laboratories, Bar Harbor, Maine) and eNOS-null male mice and wild-type (WT) controls (Charles River Laboratories, Bar Harbor, Maine), as well as S1P2-null mice and WT controls. The wall tension of the vasculature was measured in mice aortae using established methodology.

**Western Blotting and eNOS Activity Assays**

HUVECs were cultured in a 1:10 dilution of regular culture medium. HUVECs and the aortic rings up to a final concentration of 10 μmol/L were stimulated with FTY720 or FTY720-P for 10 minutes in the presence of indomethacin (10 μmol/L), N,N-dimethylsphingosine (DMS; 10 μmol/L), and indomethacin (Indo, 10 μmol/L). In some experiments, triton X-100 (5 seconds) was used to remove the endothelium, as described. The maintenance of functional smooth muscle cell integrity after manipulation was confirmed by evaluation of endothelium-independent relaxation to sodium nitroprusside (SNP, 1 μmol/L). All animal experiments were approved by the Landesamt für Gesundheit, Ernährung und technische Sicherheit Berlin ethics committee.

**Fluorescence Microscopy and Spectrofluorophotometric Measurement of NO Release**

For detection of intracellular NO generation, the NO-sensitive fluorescence dye DAF-2DA (Merk Biosciences) was applied as described previously. Briefly, HUVECs (2 × 10³ cells) were plated on gelatin-coated coverslips (diameter 12 mm) and incubated for 120 minutes in RPMI containing 1% FCS (vol/vol). DAF-2DA was added for the final 30 minutes of incubation. Cells were washed and stimulated with FTY720 or FTY720-P for 10 minutes in the presence or absence of L-NAME (50 μmol/L). Reactions were stopped by fixing the cells in 2% paraformaldehyde (vol/vol) for 5 minutes at 4°C. Coverslips were examined with a fluorescence microscope equipped with an excitation filter (470 to 490 nm), a dichroic mirror (505 nm), and an emission filter (515 nm).

To quantify relative differences in NO production, DAF-dependent fluorescence in supernatants of stimulated HUVECs was measured spectrofluorophotometrically as described by Rathel and coworkers in a high-sensitivity spectrofluorophotometer (Varian Cary Eclipse; Em, 515 nm; Ex, 495 nm; slit width, 2.5 nm). The validity of the method was confirmed by measurement of different concentrations of saline stock solutions of authentic NO (kindly provided by Dr P. Kleinbongard, Institute of Physiology, University of Düsseldorf, Germany).

**Quantification of Phosphorylation of FTY720 by Reverse-Phase Chromatography With Triethylammonium Acetate and Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)**

To quantify conversion of FTY720 to FTY720-P, endothelial cells or aortic rings were stimulated with FTY720 (1 μmol/L) for different times (0, 2, 5, and 10 minutes). Triethylammonium acetate (TEAA) was added to the supernatants of HUVECs and the aortic rings up to a final concentration of 40 mmol/L. Supernatants were concentrated on a monolithic reversed phase column (Chromolith SpeedROD, Merck). The retained substances were eluted with a stepwise gradient. The eluates of the reversed phase chromatography were lyophilized and analyzed by mass spectrometry. For calibration of the mass spectra, FTY720 and FTY720-P were used as external standard. The mass accuracy was in the range of 0.05%.

**Statistics**

All data expressed as mean±SEM. Comparisons between the groups were performed using nonparametric Wilcoxon-Mann-Whitney-Test. Two-sided probability values less than 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism version 3.02 for Windows (GraphPad Software). If error bars do not appear on figure, the error was within the symbol size.

**Results**

FTY720 and FTY720-P Induce NO-Mediated Arterial Vasodilation

To examine the effects of FTY720 and FTY720-P on vascular tone, both substances were added to rings of mouse thoracic aortae at basal tone (Figure 1A). Neither FTY720 nor FTY720-P exhibited any vasoactive effect. However, when FTY720 and FTY720-P were added to aortic rings precontracted with phenylephrine (PE) (maximal vasoconstriction (EC₅₀ = log
vasodilatory effects of FTY720 and FTY720-P in PE (1 μmol/L) precontracted aortic rings from mice (n=9). Values are shown as mean±SEM.

FTY720-P and FTY720 Induce NO Release and Stimulate eNOS Phosphorylation via Akt

In agreement with the organ studies, FTY720 (10 μmol/L) and FTY720-P (10 μmol/L) potently enhanced NO generation in human umbilical vein endothelial cells (HUVECs) in vitro, as measured by DAF-2DA–dependent fluorescence (Figure 3A). Pretreatment with 10 μmol/L L-NAME completely abolished NO release by FTY720-P. To quantify relative differences in NO production, we stimulated HUVECs with FTY720-P (1 μmol/L) for 30 minutes and measured DAF-dependent fluorescence of the supernatant spectrofluorophotometrically as described. The stimulation leads to a 2.4±0.5-fold increase in NO production, which was abolished by pretreatment with L-NAME (data not shown). In addition, [1H]arginine/citrulline-based eNOS activity assays revealed a 4.3-fold induction of eNOS activity.

Figure 1. FTY720 and FTY720-P induce vasodilation in isolated mouse aortae. A, Thoracic aortic rings at basal tone were stimulated with phenylephrine (PE, 1 μmol/L), and cumulative doses of FTY720 (2=1 nmol/L, 3=10 nmol/L, 4=100 nmol/L, 5=1 μmol/L, and 6=10 μmol/L), and cumulative doses of FTY720-P (7=1 nmol/L, 8=10 nmol/L, 9=100 nmol/L, 10=1 μmol/L, and 11=10 μmol/L) were evaluated. Shown are representative tracings from one experiment of seven. B and C, Thoracic aortic rings from mice were precontracted with PE (10 μmol/L, arrow), and direct relaxation responses to cumulative doses of FTY720 (B) or FTY720-P (C) (1=1 nmol/L, 2=10 nmol/L, 3=100 nmol/L, 4=1 μmol/L, and 5=10 μmol/L) and sodium nitroprusside (SNP, 6=10 μmol/L) were evaluated. Shown are representative tracings from one experiment of nine. D, Dose-response curves of the

Figure 2. FTY720 and FTY720-P activate eNOS. A, Thoracic aortic rings from mice were precontracted with PE (10 μmol/L, arrow) in the presence of L-NAME (50 μmol/L), and direct relaxation responses to cumulative doses of FTY720-P (1=1 nmol/L, 2=10 nmol/L, 3=100 nmol/L, 4=1 μmol/L, and 5=10 μmol/L), acetylcholine (ACH, 6=10 μmol/L), and sodium nitroprusside (SNP, 7=10 μmol/L) were evaluated. Shown is a representative tracing from one experiment out of five. B, Thoracic aortic rings from eNOS-knockout mice were precontracted with PE (10 μmol/L, arrow) and direct relaxation responses to cumulative doses of FTY720-P (1=1 nmol/L, 2=10 nmol/L, 3=100 nmol/L, 4=1 μmol/L, and 5=10 μmol/L), acetylcholine (ACH, 6=10 μmol/L), and sodium nitroprusside (SNP, 7=10 μmol/L) were evaluated. Shown is a representative tracing from one experiment out of five. C, Vasodilative properties of FTY720 (10 μmol/L, filled column) and FTY720-P (10 μmol/L, open column) in wild-type mice (eNOS+/+) and eNOS knockout-mice (eNOS−/−) in the presence or absence (control) of indomethacin (Indo, 10 μmol/L), L-NAME (50 μmol/L), or after endothelium removal with triton X-100. *P<0.05 significant changes vs control, n=6 experiments.
Phosphorylation of FTY720 Is Required for eNOS Activation

In our experiments, FTY720 and FTY720-P had comparable effects although several studies have demonstrated that FTY720 becomes biologically active only after phosphorylation. In vivo, this phosphorylation is initiated by sphingosine-1-phosphate kinases. To test whether the effect of FTY720 we observed is due to phosphorylation by endogenous SPK, we performed the vasodilatation studies with FTY720 and FTY720-P, respectively, in the presence of the SPK inhibitor N\textsuperscript{\textregistered}D,N-dimethylsphingosine (DMS, 10 \textmu mol/L). DMS completely abolished the vasodilatory effect of FTY720, whereas the potent vasodilatory effect of FTY720-P was preserved (Figure 4A and 4B). We then tested the effect of SPK inhibition on Akt and eNOS phosphorylation by FTY720 in HUVECs in vitro. DMS potently inhibited both Akt and eNOS phosphorylation (Figure 4B) by FTY720, suggesting that endogenous phosphorylation of FTY720 by the endothelial SPK is necessary for its activatory effect on Akt and eNOS.

Inhibition of phosphorylation of FTY720 by DMS prevented production of NO as measured by the \[^{[3]}\text{H} \text{arginine/citrulline} \text{assay} after 30 minutes of incubation with FTY720 (1 \textmu mol/L) (control: 68±13 fmol/well; FTY720, 1 \textmu mol/L: 193±18 fmol/well; FTY720+DMS 58±18 fmol/well).

Conversion of FTY720 to FTY720-P as Measured by MALDI-MS

To determine the kinetics and extent of endogenous FTY720 phosphorylation and conversion to active FTY720-P, we analyzed the FTY720 and FTY720-P content in supernatants of HUVECs and whole aortae preparations using mass spectrometry (Figure 5A and 5B). Two minutes after incubation of HUVECs or aortae with FTY720, more than 70% of FTY720 was phosphorylated (Figure 5A and 5B). Ten minutes after incubation, more than 90% was phosphorylated. These data indicate an extremely rapid and efficient conversion of FTY720 to its active metabolite. Time course experiments using maximal doses of FTY720 and FTY720-P allowed the detection of a slightly earlier onset of the vasodilative effect of FTY720-P in comparison to FTY720 (Figure 5C).

AAL 151 and AFD298 but not AAL151 Can Activate eNOS

To investigate the specificity of FTY720-P to activate eNOS, we performed experiments with AAL151 (chiral analogue of FTY720, R-form, which can be phosphorylated), AFD298 (phosphorylated AAL151), and AAL149 (chiral analogue of FTY720, S-form, which cannot be phosphorylated) in rings of mouse thoracic aortae precontracted with phenylephrine (PE). AAL151 and AFD298 exhibited a marked vasodilator effect in a dose-dependent manner (EC\textsubscript{50} [log mol/L]: 6.9±0.3 for AAL151 and 7.3±0.3 for AFD298) (Figure 6A). In eNOS-deficient mice, AAL151 and AFD298 had no

by FTY720-P (control: 68±13 fmol/well; FTY720-P, 1 \textmu mol/L: 212±23 fmol/well).

Several of the S1P receptors that can be activated by FTY720-P have been reported to induce Akt activation after stimulation with S1P. In addition, we and others have shown that S1P-mediated Akt activation in turn activates eNOS via phosphorylation of Ser\textsuperscript{1177} in endothelial cells. Therefore, we examined the effects of FTY720 and FTY720-P on Akt and eNOS activation in our system. Incubation of cells with FTY720 and FTY720-P induced a marked Akt activation in a dose- and time-dependent manner (0.01 to 1 \textmu mol/L) (Figure 3B). This was closely associated with an increase in eNOS phosphorylation at Ser\textsuperscript{1177} by FTY720 and FTY720-P (Figure 3B). LY294002, as a selective inhibitor of Akt activation by PI-3K, completely abolished Akt and eNOS phosphorylation. In the presence of LY294002, NO formation by FTY720-P (1 \textmu mol/L) was completely reduced to control levels in the \[^{[3]}\text{H} \text{arginine/citrulline} \text{assay} (control: 68±13 fmol/well; FTY720-P, 1 \textmu mol/L: 212±23 fmol/well; FTY720-P+LY294002:

42±18 fmol/well; n=3). eNOS-phosphorylation status at Thr\textsuperscript{695} was not affected by FTY720-P (data not shown).

Preparation of one experiment of three. B, HUVECs were stimulated with FTY720 and FTY720-P for 5, 10, 20, 30, and 45 minutes with 1 \textmu mol/L FTY720 and FTY720-P, respectively. To demonstrate dose-dependent effects, HUVECs were stimulated with 0.01 \textmu mol/L, 0.1 \textmu mol/L, and 1 \textmu mol/L FTY720 and FTY720-P, respectively, for 30 minutes. Effect of PI3-Kinase inhibition on Akt and eNOS-phosphorylation at Ser\textsuperscript{1177} by FTY720 and FTY720-P was tested by preincubation with 10 \textmu mol/L LY294002. Equal loading was confirmed by Western blot analysis of total eNOS protein. All results are representative of one experiment of four.
vasodilatory effect compared with their wild-type controls (Figure 6A and 6B). The nonphosphorylatable FTY720 analogue AAL149 did not show any significant vasodilatory properties (Figure 6A). None of these substances had any effect on basal arterial tone (data not shown). In cultured endothelial cells, AAL151 (1 μmol/L) and AFD298 (1 μmol/L) induced a marked Akt activation in a time-dependent manner (Figure 7C). This was closely associated with an increase in eNOS phosphorylation at Ser1177 (Figure 7C). Again, AAL149 did not show any effect on Akt or eNOS phosphorylation (Figure 6C).

**FTY720-P Mediates Vasodilation Through Activation of the S1P3 Receptor**

We have previously shown that S1P activates eNOS via the S1P3 receptor in vitro as well as in isolated arteries.24 Acetylcholine- and SNP-induced vasodilation in PE-precontracted mice from S1P3 receptor knockout mice was not significantly different from the vasodilation in littermates (Figure 7A and 7B). To test the role of S1P3 in mediating the vasodilatory effects of FTY720 and FTY720-P, we made use of mice deficient for the S1P3 receptor.21 Neither FTY720 (10 μmol/L) nor FTY720-P in a dose-dependent manner were able to induce vasodilation in PE-precontracted aortae from S1P3-deficient mice (Figure 7C and 7D), suggesting a crucial role for S1P3 in FTY720-mediated vasodilation.

**Discussion**

There is increasing evidence that the natural serum lysophospholipid S1P regulates vascular tone and endothelial barrier function,30–33 and that this process involves G protein–coupled receptors from the lysophospholipid receptor family. Earlier studies with S1P receptor–transfected CHO cells and antisense oligonucleotides have suggested a potential involvement of S1P1 and/or S1P3 in these biological functions.34–36 However, both endothelial and smooth muscle cells, which act in concert to regulate vessel tone, express several of the S1P receptors,12,37 and the distinctive role of individual S1P receptor subtypes in the vasculature with respect to the regulation of vasomotion remains elusive.

There is recent evidence that, similar to S1P, the immunomodulatory drug FTY720 may affect vascular permeability.29 To our knowledge, the data presented here are the first to show that FTY720 induces endothelium-dependent arterial vasodilation in PE-precontracted isolated arteries. Vasodilation did not occur in thoracic aortic rings from eNOS-deficient or S1P3-deficient mice, demonstrating a critical role of the eNOS/NO pathway and an involvement of the S1P3 receptor. Phosphorylation of eNOS by FTY720 occurred at Ser1177 and coincided with activation of Akt. Phosphorylation of eNOS at Thr495 was not affected by Akt activation, which is in line with earlier observations.38 Moreover, the activation of eNOS was attenuated by the PI3K inhibitor Ly294002, confirming involvement of Akt in eNOS phosphorylation. In this respect, FTY720 closely resembles the vasodilatory action of S1P in PE-precontracted arteries.24 Similar to S1P, FTY720 also activates eNOS by Akt-induced phosphorylation and induces Ca2+ mobilization24,39 in vitro, and both S1P and FTY720 induce vasodilation via the S1P3 receptor in isolated arteries. However, there are also substantial differences between S1P and FTY720 in respect to their vasomotoric functions: whereas S1P has a vasoconstrictor effect on basal arterial tone in isolated arteries24,33,40 and decreases myocardial perfusion in vivo,41 FTY720 has no effect on basal arterial tone in isolated arteries as shown in our study. This vasoconstrictive effect of S1P on basal arterial tone is independent of S1P1, in isolated arteries24 and appears to depend on S1P3,42 in human coronary smooth muscle cells in vitro. Interestingly, FTY720 has no affinity to S1P2, leaving this as a possible explanation for the difference between the effects of S1P and FTY720 on basal arterial tone ex vivo. All
activities of FTY720 required its phosphorylation, because addition of the sphingosine-kinase antagonist DMS prevented activation of eNOS by FTY720 but did not affect vasodilation by synthetic FTY720-P. To exclude receptor-independent effects and confirm the necessity of endogenous phosphorylation of FTY720, we performed experiments with several of its stereoisomers: the phosphorylatable chiral analogue of FTY720, AAL151, and its phosphorylated form, AFD298. Both activated eNOS and induced vasodilation. Accordingly, the nonphosphorylatable chiral analogue of FTY720, AAL149, had no effect. This is in line with the earlier observation that only FTY720-P targets S1P receptors and displays biological activity. A pertinent review of the literature has shown that FTY720 is assumed to be phosphorylated by sphingosine-kinase type 1 and type 2, with sphingosine kinase type 2 being favored. However, we cannot differentiate between the actions of these enzymes in our system. Neither can we exclude that other enzymes involved in the phosphorylation of FTY720. No matter which enzyme is responsible, our MALDI-MS data on the conversion of FTY720 revealed that it must be extremely efficient with 70% conversion already after 2 minutes. To our knowledge, this is the first report to show such rapid kinetics in vitro and in whole artery preparations.

Endothelial integrity, especially the expression of protective vasoactive agents, such as NO, may be a key factor in the sensitivity of transplanted organs such as the allogenic kidney to transplantation-mediated injury. Our data suggest that the beneficial effects of FTY720 on kidney graft rejection for which it is currently undergoing Phase III clinical trials may
depend not only on its immunosuppressive function but also on its vasoactive, NO-generating potential in the endothelium we have characterized in our study. Accordingly, optimal efficacy of FTY720 in models of transplantation required at least 5-fold higher concentrations than those needed for maximal lymphocyte trapping in lymphoid organs. However, caution should be applied when discussion potential benefits of agonism at S1P3 receptors such as S1P itself, which was reported to be rapidly fatal to mice when administered by bolus iv injection in wild-type but not S1P3−/− mice. It appears that not only dose but also modus of application of S1P may be important: whereas IV bolus administration of high doses of 1 mg/kg in mice are fatal, slow continuous infusion has no cardiac side effects. FTY720 was reported to be fatal for mice at doses of 10 mg/kg, and pFTY720 was toxic at doses above 1.0 mg/kg. It is important to be aware of these experimental data, especially with FTY720 currently undergoing Phase III clinical trials and being close to clinical approval for kidney graft rejection.

In summary, we have identified FTY720 as the first immunomodulator for prevention of organ graft rejection in clinical development that, in addition, positively affects the endothelium by affecting NO production and thus possibly displaying beneficial effects in patients beyond classical T cell immunosuppression.

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