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# Immunomodulator FTY720 Induces eNOS-Dependent Arterial Vasodilatation via the Lysophospholipid Receptor $S1P_3$

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***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 and effect dependent of 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 *N′*,*N*-dimethylsphingosine (DMS) prevented activation of eNOS in vitro and inhibited vasodilation in isolated arteries. The endothelial phosphorylation of FTY720 was extremely rapid with almost complete conversion after 10 minutes as determined by mass spectrometry. Finally, we identified the lysophospholipid receptor $S1P_3$ as the S1P receptor responsible for arterial vasodilation by FTY720, as the effect was completely abolished in arteries from $S1P_3$-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;96:913-920.)**

**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 4 of the 5 G protein–coupled receptors for S1P: $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$.[2,5] Recent studies show that the $S1P_1$ receptor and its natural ligand S1P are pivotal to lymphocyte recirculation: mice with a specific deletion of $S1P_1$ in hematopoietic cells showed that thymocytes selectively require $S1P_1$ 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 processes[11] and can be found in significant amounts in serum as part of lipoproteins.[3] The respective receptors $S1P_1$, $S1P_2$, and $S1P_3$ are widely expressed, whereas $S1P_4$ is restricted to lymphoid tissue and $S1P_5$ is present in spleen and white-matter tracts of the central nervous system.[8,12–14] In endothelial cells, we and others have demonstrated that S1P activates Akt and eNOS resulting in vasodilation.[15–17]

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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 $S1P_3$ 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.[18,19]

## 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 BRLy), 50 μg/mL heparin, and antibiotics as described previously.[20]

### 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/J6 mice (Charles River Laboratories, Wilmington, Mass) and eNOS-null male mice and wild-type (WT) controls (Charles River Laboratories, Wilmington, Mass), as well as $S1P_3$-null mice and WT controls.[21,22] The wall tension of the vasculature was measured in mice aortae using established methodology.[23,24] Arterial contraction studies were performed and presented as equilibration dose-response curves for phenylephrine (PE), FTY720, FTY720-P, AAL149 (chiral analogue of FTY720, R-form), AAL151 (chiral analogue form of FTY720, S-form), and AFD298 (phosphorylated form of AAL151, AAL151-P) in thoracic aortae from all mouse strains and deendothelialized aortae from WT-mice, respectively. In arterial relaxation studies, after equilibration and submaximal precontraction with PE (1 μmol/L), relaxation to 10 μmol/L acetylcholine was tested to confirm the integrity of the endothelium. After washing, rings were again contracted with PE and the direct effects of FTY720, FTY720-P, or its analogues were assessed. At the end of the experiment, the relaxation response to acetylcholine was confirmed. Selected studies were performed in rings treated with nitro-L-arginine methylester (L-NAME; 50 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.[25] 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.

### Western Blotting and eNOS Activity Assays

HUVECs were cultured in a 1:10 dilution of regular culture medium for 4 hours before experiment and processed for Western blot analysis as described previously.[24] Preincubation with 20 μmol/L DMS was performed for the last 20 minutes. All other substances were added to the media at the indicated times. The following antibodies were used: eNOS (Pharmingen, San Diego), phospho-Akt and phospho-eNOS ($Ser^{1177}$) (New England Biolabs Inc), and phospho-eNOS ($Thr^{495}$) (Upstate, Biomol). Signals were visualized by ECL according to the manufacturer's instructions (Amersham). Enzymatic eNOS activity was determined in HUVECs as previously described by Davda and coworkers.[26] The assay is based on the stoichiometric production of NO and L-[$^3$H]-citrulline from L-[$^3$H]-arginine by NOS.

### Fluorescence Microscopy and Spectrofluophotometric Measurement of NO Release

For detection of intracellular NO generation, the NO-sensitive fluorescence dye DAF-2DA (Merck Biosciences) was applied as described previously.[27] Briefly, HUVECs ($2\times10^5$ 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 spectrofluorphotometrically as described by Rathel and coworkers[28] in a high-sensitivity spectrofluorphotometer (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 FTY 720-P exhibited any vasoactive effect. However, when FTY720 and FTY720-P were added to aortic rings precontracted with phenylephrine (PE) (maximal vasoconstriction 8.1±0.8 mN, n=16), both substances exhibited a marked vasodilatory effect in a dose-dependent manner ($EC_{50}$[−log mol/L]: 7.1±0.1 for FTY720 and 7.0±0.1 for FTY720-P) (Figure 1B through 1D). Both substances were able to attenuate the vasoconstrictive effect of PE by ≈70% (Figure 1D). To test for involvement of eNOS in the FTY720-P– and

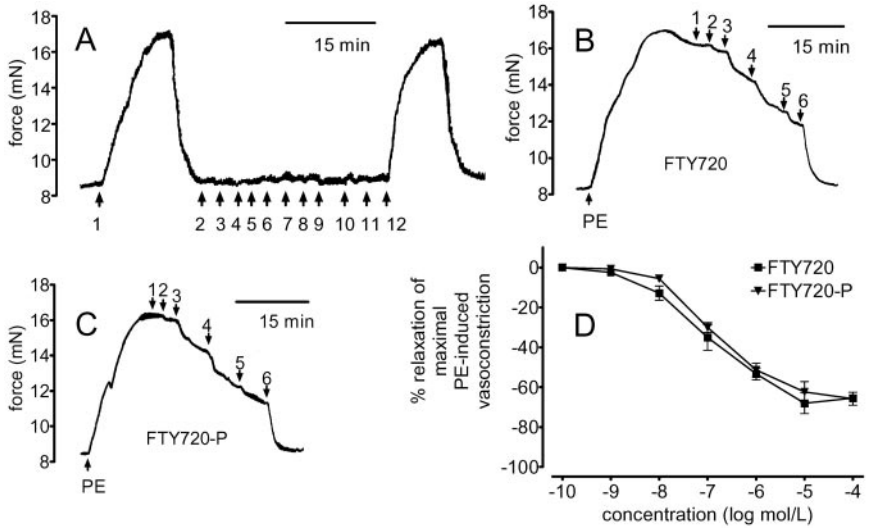


**Figure 1.** FTY720 and FTY720-P induce vasodilation in isolated mouse aortae. A, Thoracic aortic rings at basal tone were stimulated with phenylephrine (PE, 1 μmol/L, 1 and 2), cumulative doses of FTY720 (2=1 nmol/L, 3=10 nmol/L, 4=100 nmol/L, 5=1 μmol/L, and 6=10 μmol/L), and cumulative doses of FTY720-P (7=1 nmol/L, 8=10 nmol/L, 9=100 nmol/L, 10=1 μmol/L, and 11=10 μmol/L). Shown are representative tracings from 1 experiment of 7. 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=10μmol/L) were evaluated. Shown are representative tracings from 1 experiment of 9. D, Dose-response curves of the 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-induced vasodilatory effect, we pretreated the aortae with the eNOS antagonist L-NAME (50 μmol/L). This completely abolished the vasodilation induced by FTY720 and FTY720-P (Figure 2A and 2C). In addition, neither substance had any vasodilatory effect in eNOS-deficient mice compared with their wild-type controls (Figure 2B and 2C), suggesting that the vasodilatory actions of FTY720 and FTY720-P are completely mediated by eNOS. There was no vasodilation induced by either FTY720 or FTY720-P (Figure 2C) after endothelial denudation. In contrast, the vasodilatory effect of neither FTY720-P nor FTY720 was affected by indomethacin (10 μmol/L) (Figure 2C).

## 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 com-

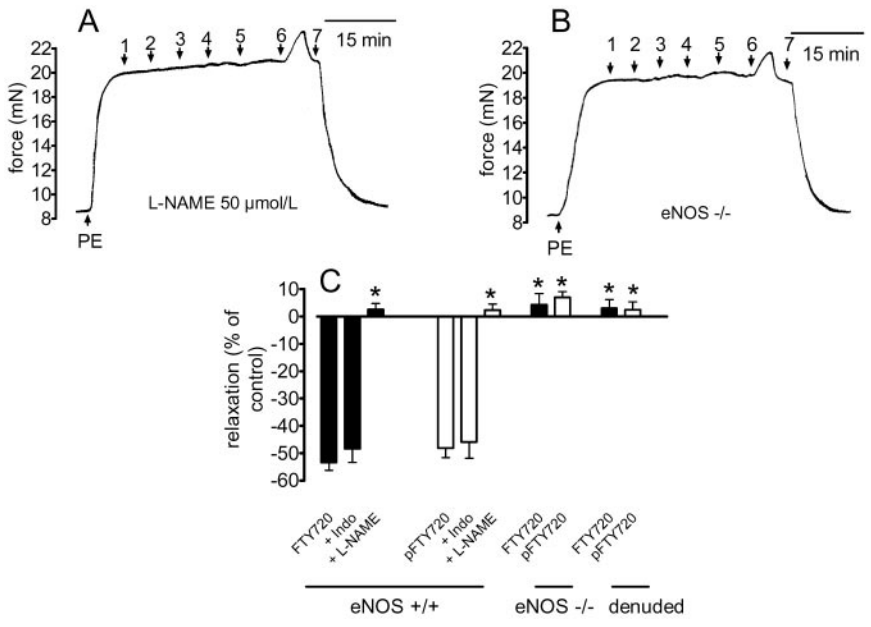


**Figure 2.** FTY720 and FTY720-P activate eNOS. A, Thoracic aortic rings from mice were precontracted with PE (10 μmol/L, arrow) in the presence of L-NAME (50 μmol/L), and direct relaxation responses to cumulative doses of FTY720-P (1=1 nmol/L, 2=10 nmol/L, 3=100 nmol/L, 4=1 μmol/L, and 5=10 μmol/L), acetylcholine (ACH, 6=10 μmol/L), and sodium nitroprusside (SNP, 7=10 μmol/L) were evaluated. Shown is a representative tracing from 1 experiment out of 5. 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 1 experiment out of 5. 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.

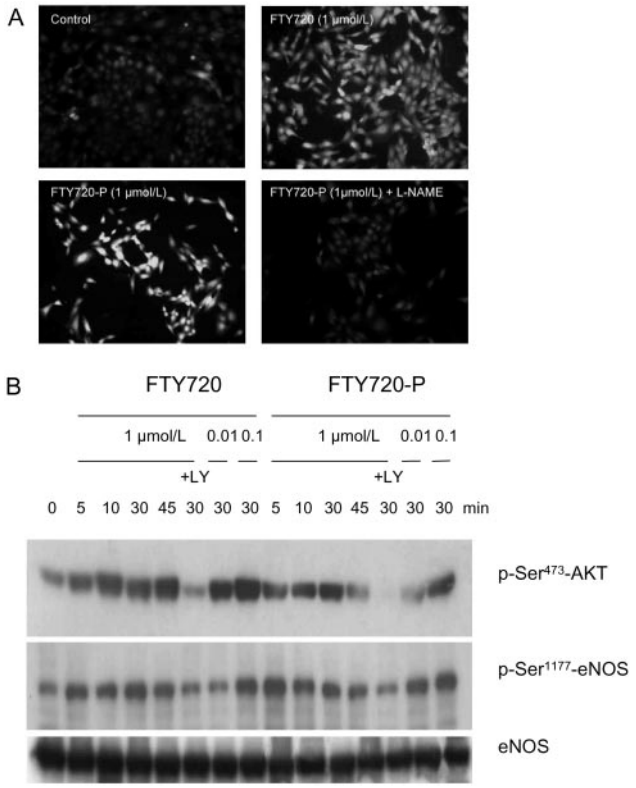


**Figure 3.** FTY720 and FTY720-P activates eNOS. A, HUVECs loaded with DAF-2DA and preincubated with L-NAME (50 μmol/L) were stimulated for 30 minutes with FTY720 (10 μmol/L) or FTY720-P (10 μmol/L) and NO-generation detected with a fluorescence microscope. Shown are representative results for 1 experiment of 3. B, HUVECs were stimulated with FTY720 and FTY720-P for 5, 10, 20, 30, and 45 minutes with 1 μmol/L FTY720 and FTY720-P, respectively. To demonstrate dose-dependent effects, HUVECs were stimulated with 0.01 μmol/L, 0.1 μmol/L, and 1 μmol/L FTY720 and FTY720-P, respectively, for 30 minutes. Effect of PI3-Kinase inhibition on Akt and eNOS-phosphorylation at $Ser^{1177}$ by FTY720 and FTY720-P was tested by preincubation with 10 μmol/L LY294002. Equal loading was confirmed by Western blot analysis of total eNOS protein. All results are representative of 1 experiment of 4.

pletely 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 spectrofluophotometrically as described.[28] The stimulation leads to a 3.13±0.56-fold increase in NO production, which was abolished by pretreatment with L-NAME (data not shown). In addition, [$^3$H]arginine/citrulline-based eNOS acitivity assays revealed a ≈3-fold induction of eNOS activity by FTY720-P (control: 68±13 fmol/well; FTY720-P, 1 μmol/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 $Ser^{1177}$ in endothelial cells.[24] 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 μmol/L) (Figure 3B). This was closely associated with an increase in eNOS phosphorylation at $Ser^{1177}$ 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 μmol/L) was completely reduced to control levels in the [$^3$H]arginine/citrulline assay (control: 68±13fmol/well; FTY720-P, 1 μmol/L: 212±23 fmol/well; FTY720-P+LY294002: 42±18fmol/well; n=3). eNOS-phosphorylation status at $Thr^{495}$ was not affected by FTY720-P (data not shown).

## 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.[29] In vivo, this phosphorylation is initiated by sphingosine-1-phosphate kinases.[4] 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′*,*N*-dimethylsphingosine (DMS, 10 μmol/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 [$^3$H]arginine/citrulline assay after 30 minutes of incubation with FTY720 (1 μmol/L) (control: 68±13fmol/well; FTY720, 1 μmol/L: 193±18 fmol/well; FTY720+DMS 58±18fmol/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

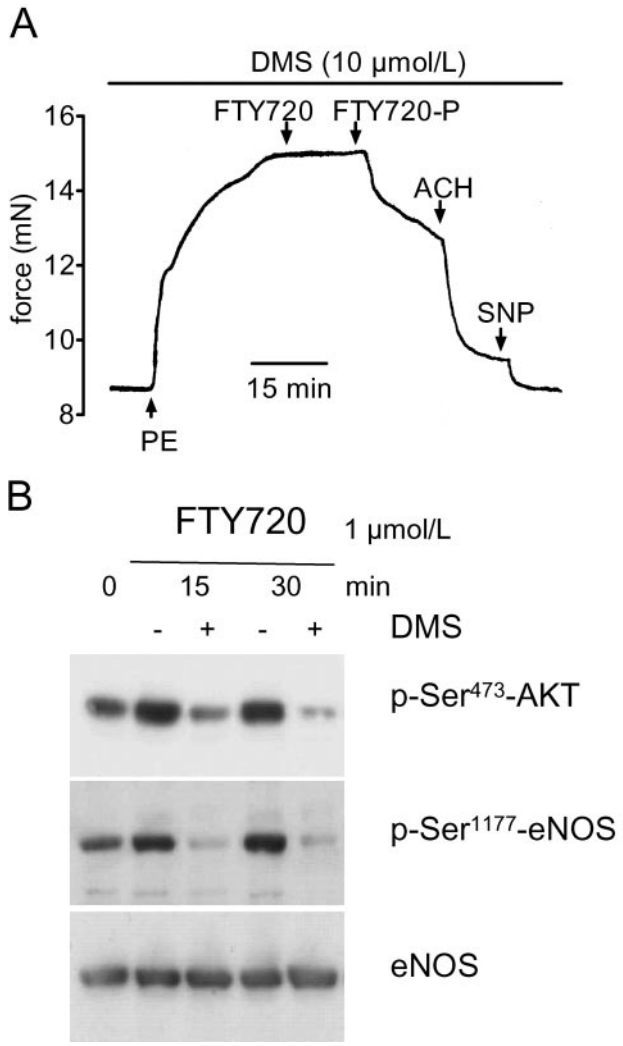


**Figure 4.** Dimethylsphingosine inhibits phosphorylation of eNOS and Akt by FTY720 in vitro and prevents vasodilation by FTY720 but not FTY720-P in isolated arteries. A, Thoracic aortic rings from mice were precontracted with phenylephrine (PE, 10 µmol/L, arrow) in the presence of *N,N*-dimethylsphingosine (DMS, 10 µmol/L), and direct relaxation responses to FTY720 (10 µmol/L), FTY720-P (10 µmol/L), acetylcholine (ACH, 10 µmol/L), and sodium nitroprusside (SNP, 10 µmol/L) were evaluated. Shown is a representative tracing from 1 experiment out of 3. B, HUVECs were stimulated with 1 µmol/L FTY720 in the presence or absence of 10 µmol/L DMS for 15 and 30 minutes. Cell lysates were analyzed for Akt and eNOS phosphorylation at $Ser^{1177}$ by Western blotting. All results are representative of 1 experiment of 3.

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 vasodilatory effect in a dose-dependent manner ($EC_{50}$[−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 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 $Ser^{1177}$ (Figure 7C). Again, AAL149 did not show any effect on Akt or eNOS phosphorylation (Figure 6C).

### FTY720-P Mediates Vasodilation Through Activation of the $S1P_3$ Receptor

We have previously shown that S1P activates eNOS via the $S1P_3$ receptor in vitro as well as in isolated arteries.[24] Acetylcholine- and SNP-induced vasodilation in PE-precontracted mice from $S1P_3$ receptor knockout mice was not significantly different from the vasodilation in littermates (Figure 7A and 7B). To test the role of $S1P_3$ in mediating the vasodilatory effects of FTY720 and FTY720-P, we made use of mice deficient for the $S1P_3$ 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 $S1P_3$-deficient mice (Figure 7C and 7D), suggesting a crucial role for $S1P_3$ 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 $S1P_1$ and/or $S1P_3$ 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 in 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 $S1P_3$-deficient mice, demonstrating a critical role of the eNOS/NO pathway and an involvement of the $S1P_3$ receptor. Phosphorylation of eNOS by FTY720 occurred at $Ser^{1177}$ and coincided with activation of Akt. Phosphorylation of eNOS at $Thr^{495}$ 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 $Ca^{2+}$ mobilization[24,39] in vitro, and both S1P and FTY720 induce vasodilation via the $S1P_3$ receptor in isolated arteries. However, there are also substantial differences between S1P and FTY720 in respect to their vasoactive functions: whereas S1P has a vasoconstrictor effect on basal arterial tone in isolated arteries[24,33,40] and decreases myocar-

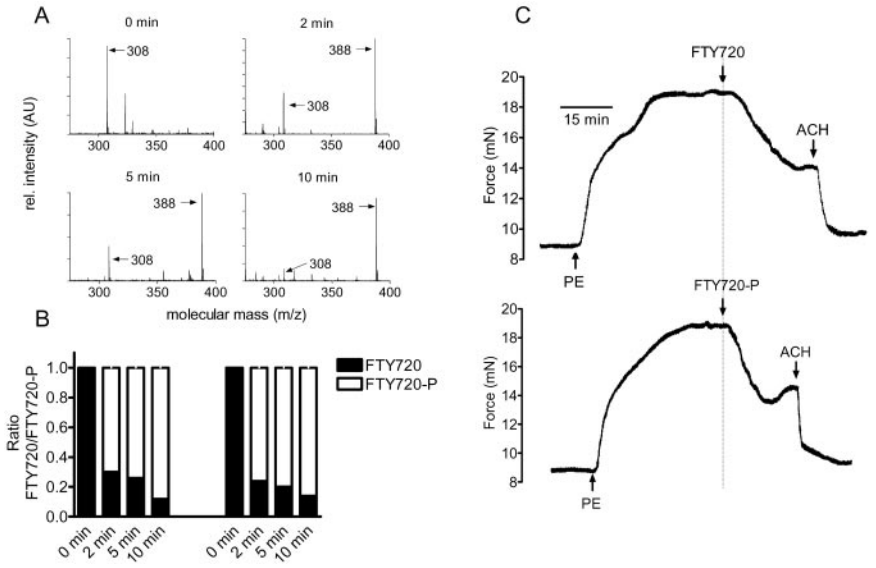


**Figure 5.** FTY720 is rapidly converted to FTY720-P in HUVECs and whole aorta. A, MALDI mass spectra of desalted thoracic aortic rings supernatants after incubation with FTY720 in dependence of the incubation time (0, 2, 5, and 10 minutes). An aliquot (1/10) of the desalted supernatant was cocrystallised with 1 $\mu$L of the matrix solution of 2,5-dihydroxybenzoic acid (DHB; 20mmol/L in water/acetonitril 50/50-vol-%) on the MALDI target. Mass signal at 308 Da is caused by FTY720, and the mass signal at 388 Da is caused by FTY720-P. Quantification of the FTY720 / FTY720-P ratio is given in Figure 6C. B, Time-dependent conversion of FTY720 to FTY720-P in HUVECs and whole mice aorta. Shown is the ratio of FTY720/FTY720-P as measured by mass spectrometry. C, Thoracic aortic rings from mice were precontracted with PE (10 $\mu$mol/L, arrow) and direct time-dependent relaxation responses to FTY720 (1 $\mu$mol/L, top panel) and FTY720-P (10 $\mu$mol/L, bottom panel) were evaluated. Vertical line indicates the time of FTY720 and FTY720-P application and allows comparison of the beginning of the arterial vasodilation. Shown is a representative tracing from 1 experiment out of 3.

dial 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 $S1P_3$ in isolated arteries[24] and appears to depend on $S1P_2$[42] in human coronary smooth muscle cells in vitro. Interestingly, FTY720 has no affinity to $S1P_2$, 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[2]: 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.[2] A pertinent review of the literature has shown that FTY720 is assumed to be phosphorylated by sphingosine-kinase type 1 and 2,[4,29,43] with sphingosine-kinase type 2 being favored.[44] However, we cannot differentiate between the actions of these enzymes in our system. Neither can we exclude that other enzymes are 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 knowl-

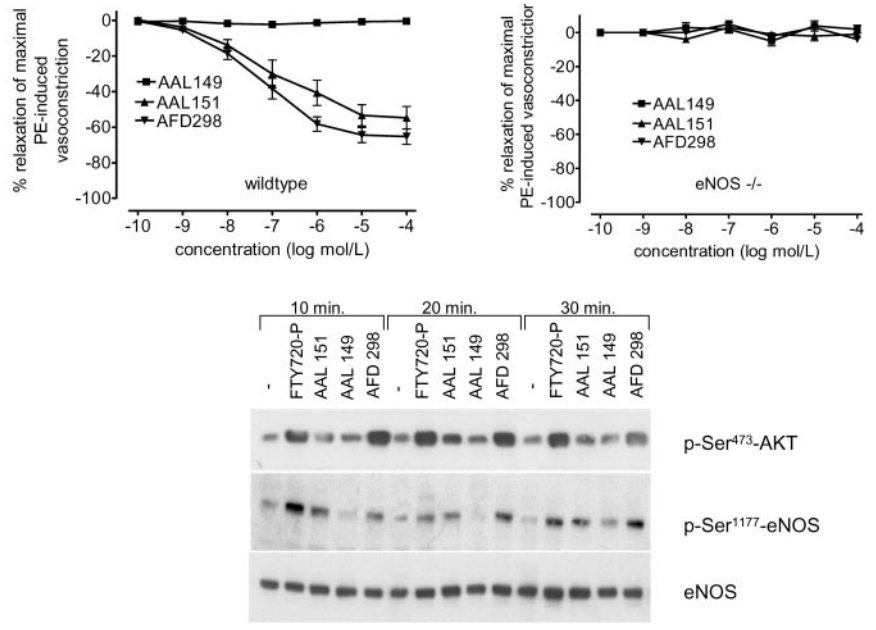


**Figure 6.** FTY720 stereoisomers AAL151 and AFD298 activate eNOS and induce vasodilation, whereas nonphosphorylable chiral analogue AAL149 had not effect. A, Dose-response curves of the vasodilatory effects of AAL151, AFD298, and AAL149 in PE (1 $\mu$mol/L)-precontracted aortic rings from wild-type mice (n=6). Values are shown as mean±SEM. B, Dose-response curves of the vasodilatory effects of AAL151, AFD298, and AAL149 in PE (1 $\mu$mol/L)-precontracted aortic rings from $eNOS^{-/-}$ mice (n=4). Values are shown as mean±SEM. C, HUVECs were stimulated with 1 $\mu$mol/L AAL151, AFD298, and AAL149 for 10, 20, and 30 minutes. Cell lysates were analyzed for Akt and eNOS phosphorylation by Western blotting. All results are representative of 1 experiment of 3.

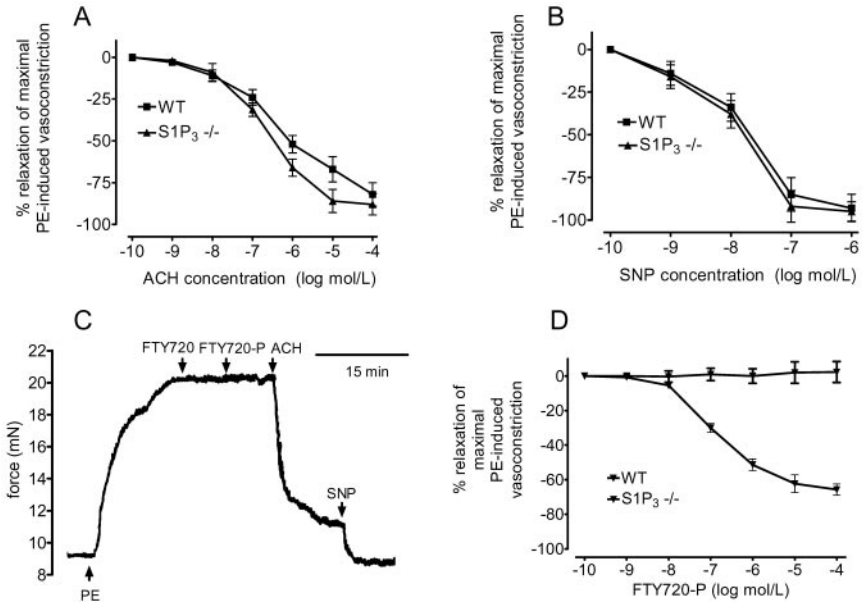


**Figure 7.** Vasodilation by FTY720 and FTY720-P is mediated by activation of the $S1P_3$ receptor. A and B, Thoracic aortic rings from $S1P_3$-knockout mice and littermates were precontracted with PE (10 $\mu$mol/L) and direct relaxation responses to acetylcholine (ACH, A) and sodium nitroprusside (SNP, B) were evaluated; n=5 experiments. C, Thoracic aortic rings from $S1P_3$-knockout mice and wild-type controls were precontracted with PE (10 $\mu$mol/L) and direct relaxation responses FTY720 (10 $\mu$mol/L) and FTY720-P (10 $\mu$mol/L), ACH (10 $\mu$mol/L), and SNP (10 $\mu$mol/L) were evaluated. Shown is a representative tracing from 1 experiment out of 5 D, Thoracic aortic rings from $S1P_3$-knockout mice and wild-type controls were precontracted with PE (10 $\mu$mol/L) and direct relaxation responses to cumulative concentrations of FTY720-P were evaluated; n=5 experiments.

edge, 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.[45] Our data suggest that the beneficial effects of FTY720 on kidney graft rejection for which it is currently undergoing Phase III clinical trials[1] 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.[46] However, caution should be applied when discussing potential benefits of agonism at $S1P_3$ 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 $S1P_3^{-/-}$ mice.[47] 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.[3] FTY720 was reported to be fatal for mice at doses of 10 mg/kg[46], and pFTY720 was toxic at doses above 1.0 mg/kg.[47] 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.[1]

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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## References

1. Tedesco-Silva H, Mourad G, Kahan BD, Boira JG, Weimar W, Mulgaonkar S, Nashan B, Madsen S, Charpentier B, Pellet P, Vanrenterghem Y. Fty720, a novel immunomodulator: efficacy and safety results from the first phase 2a study in de novo renal transplantation. *Transplantation*. 2004;77:1826–1833.
2. Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, Bruns C, Prieschl E, Baumruker T, Hiestand P, Foster CA, Zollinger M, Lynch KR. The immune modulator fty720 targets sphingosine 1-phosphate receptors. *J Biol Chem*. 2002;277:21453–21457.
3. Kimura T, Sato K, Kuwabara A, Tomura H, Ishiwara M, Kobayashi I, Ui M, Okajima F. Sphingosine 1-phosphate may be a major component of plasma lipoproteins responsible for the cytoprotective actions in human umbilical vein endothelial cells. *J Biol Chem*. 2001;276:31780–31785.
4. Paugh SW, Payne SG, Barbour SE, Milstien S, Spiegel S. The immunosuppressant fty720 is phosphorylated by sphingosine kinase type 2. *FEBS Lett*. 2003;554:189–193.
5. Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, Thornton R, Shei GJ, Card D, Keohane C, Rosenbach M, Hale J, Lynch CL, Rupprecht K, Parsons W, Rosen H. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science*. 2002;296:346–349.
6. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on s1p receptor 1. *Nature*. 2004;427:355–360.
7. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol*. 2003;4:397–407.
8. Hla T, Lee MJ, Ancellin N, Paik JH, Kluk MJ. Lysophospholipids: receptor revelations. *Science*. 2001;294:1875–1878.
9. Pyne S, Pyne N. Sphingosine 1-phosphate signalling via the endothelial differentiation gene family of G-protein–coupled receptors. *Pharmacol Ther*. 2000;88:115–131.
10. Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS, Webb B, Lefebvre S, Chun J, Gray N, Rosen H. Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem*. 2004;279:13839–13848.
11. English D, Welch Z, Kovala AT, Harvey K, Volpert OV, Brindley DN, Garcia JG. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. *FASEB J*. 2000;14:2255–2265.
12. Chun J, Goetzl EJ, Hla T, Igarashi Y, Lynch KR, Moolenaar W, Pyne S, Tigyi G. International Union of Pharmacology, XXXIV: lysophospholipid receptor nomenclature. *Pharmacol Rev*. 2002;54:265–269.

13. Anliker B, Chun J. Lysophospholipid g protein-coupled receptors. *J Biol Chem.* 2004.
14. Ishii I, Fukushima N, Ye X, Chun J. Lysophospholipid receptors: signaling and biology. *Annu Rev Biochem*. 2004;73:321–354.
15. Igarashi J, Bernier SG, Michel T. Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase: differential regulation of AKT and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. *J Biol Chem*. 2001;276:12420–12426.
16. Tanimoto T, Jin ZG, Berk BC. Transactivation of vascular endothelial growth factor (VEGF) receptor flk-1/kdr is involved in sphingosine 1-phosphate-stimulated phosphorylation of AKT and endothelial nitric-oxide synthase (eNOS). *J Biol Chem*. 2002;277:42997–43001.
17. Morales-Ruiz M, Lee MJ, Zollner S, Gratton JP, Scotland R, Shiojima I, Walsh K, Hla T, Sessa WC. Sphingosine 1-phosphate activates akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. *J Biol Chem*. 2001;276:19672–19677.
18. Joosten SA, van Kooten C, Sijpkens YW, de Fijter JW, Paul LC. The pathobiology of chronic allograft nephropathy: Immune-mediated damage and accelerated aging. *Kidney Int*. 2004;65:1556–1559.
19. Oflaz H, Turkmen A, Kazancioglu R, Kayacan SM, Bunyak B, Genchallac H, Erol B, Mercanoglu F, Umman S, Sever MS. The effect of calcineurin inhibitors on endothelial function in renal transplant recipients. *Clin Transplant*. 2003;17:212–216.
20. Levkau B, Scatena M, Giachelli CM, Ross R, Raines EW. Apoptosis overrides survival signals through a caspase-mediated dominant-negative NF-κB loop. *Nat Cell Biol*. 1999;1:227–233.
21. Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJ, Kingsbury MA, Zhang G, Brown JH, Chun J. Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, lp(b3)/edg-3. *J Biol Chem*. 2001;276:33697–33704.
22. Ishii I, Ye X, Friedman B, Kawamura S, Contos JJ, Kingsbury MA, Yang AH, Zhang G, Brown JH, Chun J. Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate (S1P) receptors, s1p(2)/lp(b2)/edg-5 and s1p(3)/lp(b3)/edg-3. *J Biol Chem*. 2002;277:25152–25159.
23. Tepel M, Jankowski J, Ruess C, Steinmetz M, van der Giet M, Zidek W. Activation of Na+, H+ exchanger produces vasoconstriction of renal resistance vessels. *Am J Hypertens*. 1998;11:1214–1221.
24. Nofer JR, van der Giet M, Tolle M, Wolinska I, von Wnuck Lipinski K, Baba HA, Tietge UJ, Godecke A, Ishii I, Kleuser B, Schafers M, Fobker M, Zidek W, Assmann G, Chun J, Levkau B. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest*. 2004;113:569–581.
25. van der Giet M, Schmidt S, Tolle M, Jankowski J, Schluter H, Zidek W, Tepel M. Effects of dinucleoside polyphosphates on regulation of coronary vascular tone. *Eur J Pharmacol*. 2002;448:207–213.
26. Davda RK, Chandler LJ, Crews FT, Guzman NJ. Ethanol enhances the endothelial nitric oxide synthase response to agonists. *Hypertension*. 1993;21:939–943.
27. Sugimoto K, Fujii S, Takemasa T, Yamashita K. Detection of intracellular nitric oxide using a combination of aldehyde fixatives with 4,5-diaminofluorescein diacetate. *Histochem Cell Biol*. 2000;113:341–347.
28. Rathel TR, Leikert JJ, Vollmar AM, Dirsch VM. Application of 4,5-diaminofluorescein to reliably measure nitric oxide released from endothelial cells in vitro. *Biol Proced Online*. 2003;5:136–142.
29. Sanchez T, Estrada-Hernandez T, Paik JH, Wu MT, Venkataraman K, Brinkmann V, Claffey K, Hla T. Phosphorylation and action of the immunomodulator fty720 inhibits vascular endothelial cell growth factor-induced vascular permeability. *J Biol Chem*. 2003;278:47281–47290.
30. McVerry BJ, Garcia JG. Endothelial cell barrier regulation by sphingosine 1-phosphate. *J Cell Biochem*. 2004;92:1075–1085.
31. Schaphorst KL, Chiang E, Jacobs KN, Zaiman A, Natarajan V, Wigley F, Garcia JG. Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products. *Am J Physiol Lung Cell Mol Physiol*. 2003;285:L258–L267.
32. Dantas AP, Igarashi J, Michel T. Sphingosine 1-phosphate and control of vascular tone. *Am J Physiol Heart Circ Physiol*. 2003;284:H2045–H2052.
33. Salomone S, Yoshimura S, Reuter U, Foley M, Thomas SS, Moskowitz MA, Waeber C. S1p3 receptors mediate the potent constriction of cerebral arteries by sphingosine-1-phosphate. *Eur J Pharmacol*. 2003;469:125–134.
34. Banno Y, Takuwa Y, Yamada M, Takuwa N, Ohguchi K, Hara A, Nozawa Y. Involvement of phospholipase d in insulin-like growth factor-i-induced activation of extracellular signal-regulated kinase, but not phosphoinositide 3-kinase or AKT, in Chinese hamster ovary cells. *Biochem J*. 2003;369:363–368.
35. Kwon YG, Min JK, Kim KM, Lee DJ, Billiar TR, Kim YM. Sphingosine 1-phosphate protects human umbilical vein endothelial cells from serum-deprived apoptosis by nitric oxide production. *J Biol Chem*. 2001;276:10627–10633.
36. Garcia JG, Liu F, Verin AD, Birukova A, Dechert MA, Gerthoffer WT, Bamberg JR, English D. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by EDG-dependent cytoskeletal rearrangement. *J Clin Invest*. 2001;108:689–701.
37. Lee MJ, Thangada S, Claffey KP, Ancellin N, Liu CH, Kluk M, Volpi M, Sha'afi RI, Hla T. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell*. 1999;99:301–312.
38. Boo YC, Jo H. Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases. *Am J Physiol Cell Physiol*. 2003;285:C499–C508.
39. Butler J, Lana D, Round O, LaMontagne K. Functional characterization of sphingosine 1-phosphate receptor agonist in human endothelial cells. *Prostaglandins Other Lipid Mediat*. 2004;73:29–45.
40. Coussin F, Scott RH, Wise A, Nixon GF. Comparison of sphingosine 1-phosphate-induced intracellular signaling pathways in vascular smooth muscles: differential role in vasoconstriction. *Circ Res*. 2002;91:151–157.
41. Levkau B, Hermann S, Theilmeier G, van der Giet M, Chun J, Schober O, Schäfers M. Hdl stimulates myocardial perfusion in vivo. *Circulation*. 2004;110:3355–3359.
42. Ohmori T, Yatomi Y, Osada M, Kazama F, Takafuta T, Ikeda H, Ozaki Y. Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via S1P2. *Cardiovasc Res*. 2003;58:170–177.
43. Billich A, Bornancin F, Devay P, Mechtcheriakova D, Urtz N, Baumruker T. Phosphorylation of the immunomodulatory drug fty720 by sphingosine kinases. *J Biol Chem*. 2003;278:47408–47415.
44. Allende ML, Sasaki T, Kawai H, Olivera A, Mi Y, van Echten-Deckert G, Hajdu R, Rosenbach M, Keohane CA, Mandala S, Spiegel S, Proia RL. Mice deficient in sphingosine kinase 1 are rendered lymphopenic by fty720. *J Biol Chem*. 2004;279:52487–52492.
45. Vos IH, Joles JA, Rabelink TJ. The role of nitric oxide in renal transplantation. *Semin Nephrol*. 2004;24:379–388.
46. Kiuchi M, Adachi K, Kohara T, Minoguchi M, Hanano T, Aoki Y, Mishina T, Arita M, Nakao N, Ohtsuki M, Hoshino Y, Teshima K, Chiba K, Sasaki S, Fujita T. Synthesis and immunosuppressive activity of 2-substituted 2-aminopropane-1,3-diols and 2-aminoethanols. *J Med Chem*. 2000;43:2946–2961.
47. Forrest M, Sun SY, Hajdu R, Bergstrom J, Card D, Doherty G, Hale J, Keohane C, Meyers C, Milligan J, Mills S, Nomura N, Rosen H, Rosenbach M, Shei GJ, Singer II, Tian M, West S, White V, Xie J, Proia RL, Mandala S. Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. *J Pharmacol Exp Ther*. 2004;309:758–768.