FEBS Letters 581 (2007) 3063–3068

# FTY720-phosphate is dephosphorylated by lipid phosphate phosphatase 3

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Received 23 February 2007; revised 4 May 2007; accepted 21 May 2007

Available online 4 June 2007

Edited by Giulio Superti-Furga

**Abstract FTY720 is a novel immunomodulatory drug efficacious in the treatment of multiple sclerosis. The drug is converted in vivo to the monophosphate, FTY720-P, by sphingosine kinase 2. This conversion is incomplete, suggesting opposing actions of kinase and phosphatase activities. To address which of the known lipid phosphatases might dephosphorylate FTY720-P, we overexpressed the broad specificity lipid phosphatases LPP1–3, and the specific S1P phosphatases (SPP1 and 2) in HEK293 cells, and performed in vitro assays using lysates of transfected cells. Among LPPs, only LPP3 was able to dephosphorylate FTY720-P; among SPPs, only SPP1 showed activity against FTY720-P. On intact cells, LPP3 acted as an *ecto*-phosphatase or FTY720-P, thus representing the major phosphatase involved in the equilibrium between FTY720 and FTY720-P observed in vivo.**


*Keywords:* FTY720; Sphingosine-1-phosphate; Phosphatase

## 1. Introduction

FTY720 is an immunomodulatory agent with a novel mode of action, sequestering lymphocytes from blood and spleen into secondary lymphoid organs, thereby preventing their migration to sites of inflammation [1]. In addition to the effects on lymphocyte recirculation, the drug preserves vascular integrity by enhancing endothelial barrier function [2]. FTY720 is currently in clinical phase 3 trials for the treatment of multiple sclerosis.

Given its structural analogy to sphingosine (Sph), FTY720 is phosphorylated in vivo by sphingosine kinases (SPHKs), in particular the type 2 enzyme [3,4]. The phosphate FTY720-P binds to four G protein-coupled sphingosine 1-phosphate (S1P) receptors [5,6]. Most importantly, it acts as a high-affinity agonist at S1P1 on thymocytes and lymphocytes, thereby inducing aberrant internalization of the receptor. This renders the cells unresponsive to the serum lipid sphingosine 1-phosphate (S1P), depriving them from an obligatory signal to egress from lymphoid organs [1].

When FTY720 is applied to animals or to man, rapid phosphorylation to the monophosphate occurs [5,6]. Conversion to FTY720-P is not complete, a steady state between phosphorylated and non-phosphorylated drug being reached; e.g., in the rat a ratio of FTY720/FT720-P of 1:4 is reached within 1 h, lasting for at least 6 h [6]. This is suggestive of opposing actions of kinase(s) and phosphatase(s) that produce and cleave FTY720-P, respectively. While the responsible kinase (SPHK2) has been identified [3,4], the nature of dephosphorylating activities acting on FTY720-P was unknown so far. Interestingly, Mandala et al. [6] noted that FTY720-P is not cleaved to FTY720 by rat blood ex vivo, indicating dephosphorylation outside blood.

S1P is known to be a substrate for various lipid phosphatases including the S1P-specific phosphohydrolases SPP1 and SPP2 [7,8], and the broad specificity lipid phosphohydrolases (LPP1–3) [9]; in addition, S1P lyase is responsible for the irreversible cleavage of S1P to ethanolamine phosphate and hexadecenal [10]. In contrast to S1P, FTY720-P was shown to be stable upon incubation with active SPL, suggesting that SPL is not responsible for metabolism of the drug [11].

In this study, we determined whether FTY720-P might function as a substrate for lipid phosphatases. We show that among the panel of known lipid phosphatases, the *ecto*-phosphatase LPP3 is able to dephosphorylate FTY720-P. Among SPPs, only SPP1 showed moderate activity against FTY720-P as a substrate, but due to its intracellular location is unlikely to contribute to the balance between FTY720 and FTY720-P in the extracellular space.

## 2. Materials and methods

### 2.1. Cell culture and materials

Human umbilical vein endothelial cells (HUVEC) were cultured at 37 °C and 5% $CO_2$ in EGM™-2 medium (Clonetics). HEK293 cells were cultured in DMEM H21 containing 10% fetal calf serum and non-essential amino acids (Gibco).

Nitrobenzo-2-oxa-1,3-diazole (NBD) labeled derivatives were prepared as described [12,13]. [3-$^3$H] S1P (20 Ci/mmol), [4,5-$^3$H] dihydro-S1P (40 Ci/mmol), and [3-$^3$H] sphingosine (20 Ci/mmol) were from American Radiolabeled Chemicals. Tritium labeled lysophosphatidic acid (1-oleoyl,[oleoyl-9,10-$^3$H(N)] LPA, 47 Ci/mmol) was from Perkin–Elmer. [3-$^3$H] FTY720 (102 Ci/mmol) and [3-$^3$H] FTY720 phosphate (54 Ci/mmol) were synthesized at Novartis.

For metabolic conversion experiments, HUVEC at 80% confluence in 6-well plates were incubated for 1 or 3 h at 37 °C in 1 ml medium per well containing 2 μCi of either [$^3$H] Sph (100 nM), [$^3$H]FTY720 (20 nM), [$^3$H] S1P (100 nM), or [$^3$H] FTY720-P (36 nM). Alternatively,

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*Abbreviations:* Cer, ceramide; DHS1P, dihydro-sphingosine-1-phosphate; LPA, lyso-phosphatidic acid; LPP, lipid phosphate phosphohydrolase; S1P, sphingosine-1-phosphate; SM, sphingomyelin; SPHK, sphingosine kinase; Sph, sphingosine; SPP, sphingosine-1-phosphate phosphatase

0014-5793/$32.00 
doi:10.1016/j.febslet.2007.05.069

the fluorescently labeled compounds, NBD-Sph, NBD-FTY720 or their corresponding NBD-labeled phosphates, were added to the cells at 2 μM.

### 2.2. Transient transfection of HUVEC and HEK293

N-terminally Flag-tagged LPP1, LPP2, and LPP3 were amplified by PCR from pooled human kidney and lung cDNA. The PCR fragments obtained were subcloned into pcDNA3.1 (Invitrogen). For EGFP-tagged expression, non-tagged ORFs were transferred to pEGFP-c2 (Clontech). SPP1 and SPP2 ORFs were cloned as previously described [14]. For EGFP-tagged expression, ORFs in pcDNA6.2-DEST (Invitrogen) were subcloned into pEGFP-c2. Full-length cDNAs encoding human LPP1 and 3, and mouse LPP3, SPP1 and 2 in pCMV-SPORT6 were obtained from Invitrogen.

HUVEC were seeded overnight in 6-well plates to reach 60% confluence. Transient transfection of HUVEC with plasmids encoding LPPs and SPPs was done with LipofectAMINE and PLUS™ reagent (Invitrogen). Cells were incubated with transfection mixtures containing 0.5 μg of DNA, 6 μl of PLUS reagent, and 4 μl of LipofectAMINE in a total volume of 1 ml of medium 199 per well for 105 min. Cells were washed with the complete medium and cultured for 20 h before proceeding with further investigations. For immunofluorescence studies, HUVEC were grown on Lab-Tek glass chamber slides (Nunc) and transfected with plasmids at suboptimal amounts (0.25 μg per 1 ml of transfection mixture). HEK293 cells were transfected using the calcium phosphate method.

To estimate efficiency of transfection, subcellular localization of EGFP-tagged phosphatases or uptake of NBD-labeled lipids, living cells were analyzed with an inverted microscope equipped with a high resolution microscopy camera (Zeiss) as well as oil DIC objectives (Plan-Neofluar 40×/1.30 and Plan-Apochromat 63×/1.40). Excitation/emission filter settings were 450–490/515–565 nm.

### 2.3. In vitro phosphatase assays

Lipid phosphatase activity was estimated by the degree of dephosphorylation of radiolabeled or NBD-labeled substrates. Transfected HEK293 cells were scraped into PBS, collected by centrifugation and resuspended in lysis buffer (50 mM HEPES, pH 7.3, containing 150 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM DTT and protease inhibitor mix from Roche), followed by freeze–thaw cycles. HEK293 lysates (5 μg of total protein) were mixed with substrates: (i) [$^3$H] S1P, 0.25 μM, 0.5 μCi; (ii) [$^3$H] dihydro-S1P (0.08 μM, 0.5 μCi); each plus cold S1P at 1 μM, prepared in 0.1% fatty acid-free BSA (Sigma) as described [7]; (iii) [$^3$H] LPA (0.1 μM, 0.5 μCi), balanced with 2 μM cold lyso-phosphatidic acid (LPA), prepared with 0.1% Triton-X100; (iv) [$^3$H]FTY720-P (0.3 μM, and 1.7 μCi) prepared with bovine serum albumin. For NBD-labeled substrates, 50 μg of total protein were mixed with 5 μM NBD-S1P or NBD-FTY720-P in 0.1% fatty acid-free BSA. Incubations were done in total reaction volumes of 100 μl at 37 °C for 15 min.

For analysis, 400 μl PBS was added to the reaction mixtures and lipids were extracted by adding of 707 μl of chloroform/methanol/HCl/5 M NaCl (300:300:7:100 v/v) and mixing. After sonication, phases were separated by centrifugation, and the organic phase was recovered, dried, and dissolved in chloroform/methanol (19:1, v/v). The labeled lipids were resolved by thin-layer chromatography (TLC) on SilicaGel 60 TLC plates (Merck) with 1-butanol/acetic acid/water (3:1:1, v/v) or $CHCl_3$/MeOH/$H_2O$/$NH_4OH$ (28% w/w) 200:150:29:1 as previously described [13]. In these systems, NBD-labeled FTY720 derivative and [3-$^3$H]FTY720 as well as their corresponding phosphates co-migrate. Radiolabeled S1P and FTY720-P were identified by co-migration with unlabeled standards, visualized using vanillin/sulfuric acid [15]. TLC plates were exposed to Kodak BioMax MR Film at −80 °C; for NBD-labeled lipids, plates were photographed and bands quantified by using AlphaImager 2200 (Alpha Innotech). S1P phosphatase activity was expressed as nmol of sphingosine formed per minute per milligram of total protein. Protein concentrations in cell lysates were determined using Bradford reagent (BioRad).

## 3. Results and discussion

### 3.1. Metabolic conversion of FTY720 in cell cultures

Primary endothelial cells (HUVEC) in culture were incubated with [$^3$H]FTY720, and for comparison with [$^3$H]Sph for 1 and 3 h, followed by lipid extraction and separation of products by TLC (Fig. 1A). While Sph was converted by the cells to S1P, ceramide (Cer), and sphingomyelin (SM) as products expected from sphingolipid metabolism [13,16], FTY720 was converted to FTY720-P as the only detectable product (Fig. 1A) even after 24 h of incubation (data not shown). The low rate of [$^3$H] FTY720 phosphorylation in HUVEC was strongly increased in SPHK2-overexpressing cells in comparison to the vector-transfected cells (data not shown). A similar conversion pattern was seen when using as substrates a fluorescently labeled FTY720 derivative [12] and for comparison a fluorescently labeled Sph derivative [13]; while the NBD-Sph derivative was rapidly metabolized to its phosphate and to corresponding Cer and SM species, the NBD-FTY720 derivative was phosphorylated, only (Fig. 1B). Similar metabolic

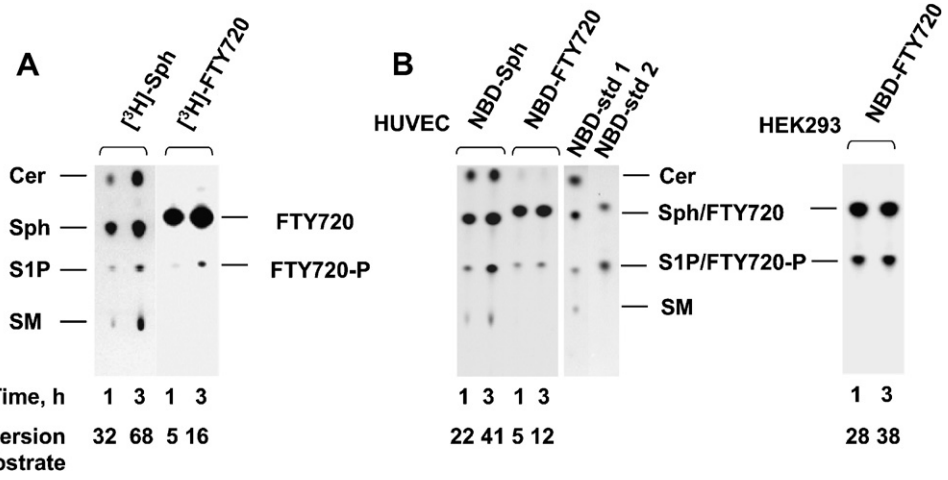


Fig. 1. Metabolic conversion of FTY720 by cells in culture. (A) HUVEC were incubated with [$^3$H] Sph or [$^3$H] FTY720 (2 μCi; 100 and 20 nM, respectively) followed by lipid extraction and separation of products by TLC. (B) Metabolic conversion of NBD-labeled FTY720 (2 μM) derivative in HUVEC and HEK293 cells. Cells were exposed to NBD-labeled derivatives for 1 and 3 h. NBD-std1: NBD-labeled standards as NBD-Cer, NBD-Sph, NBD-S1P, NBD-SM; NBD-std2: NBD-labeled FTY720 and FTY720-P derivatives. The percentage of substrate conversion is indicated.