

Fingolimod Reduces Hemorrhagic Transformation Associated With Delayed Tissue Plasminogen Activator Treatment in a Mouse Thromboembolic Model

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Background and Purpose—The sphingosine 1-phosphate receptor agonist fingolimod reduces infarct size in rodent models of stroke and enhances blood–brain barrier integrity. Based on these observations, we hypothesized that combination of fingolimod with tissue plasminogen activator (tPA) would reduce the risk of hemorrhagic transformation associated with delayed administration of tPA.

Methods—We evaluated the effects of fingolimod in a mouse model of thromboembolic stroke, in which both the beneficial effect of reperfusion associated with early tPA treatment and hemorrhagic transformation associated with delayed administration mimic clinical observations in humans.

Results—Our results demonstrate that fingolimod treatment attenuates the neurological deficit and reduces infarct volume after in situ thromboembolic occlusion of the middle cerebral artery. Combination of fingolimod and tPA improves the neurological outcome of the thrombolytic therapy and reduces the risk of hemorrhagic transformation associated with delayed administration of tPA.

Conclusion—This study confirms the protective efficacy of fingolimod as a treatment against ischemic stroke in another rodent model of stroke (thromboembolic occlusion), and suggests that fingolimod could potentially be used in combination with tPA to reduce the risk of brain hemorrhage. (*Stroke*. 2013;44:505-511.)

Key Words: fingolimod ■ hemorrhage ■ stroke ■ tPA ■ thromboembolic model

Acute cerebral ischemia is a major cause of mortality and disability worldwide, but no successful pharmacotherapy has been established that can benefit patients beyond the time window of thrombolysis with recombinant tissue-type plasminogen activator (tPA).¹ Because of risks related to hemorrhage, this window is within 3 to 4.5 hours after the onset of symptoms.² The tPA has also shown neurotoxicity in experimental models of cerebral ischemia.³ Therefore, a combination treatment that would reduce the deleterious effects of tPA, while maintaining the benefits of recanalization, might extend the usability and efficacy of tPA.

Fingolimod (FTY720, Gilenya) is a sphingosine analog that, when phosphorylated, acts on sphingosine 1-phosphate receptors, regulating cellular and physiological mechanisms, including proliferation, apoptosis, adhesion, migration, differentiation/morphogenesis, inflammation, or blood–brain barrier (BBB) integrity.⁴⁻⁶ Fingolimod has emerged as a new treatment for multiple sclerosis.^{7,8} The effectiveness of

fingolimod has also been shown by several groups in rodent models of ischemic brain injury.⁹⁻¹² These studies suggest that antiinflammatory mechanisms and vasculoprotection possibly underlie the beneficial effects of fingolimod after stroke.¹² We therefore hypothesized that combining fingolimod with tPA might reduce hemorrhagic transformation associated with delayed administration of tPA.

In this study, we evaluated the effect of fingolimod in a mouse model of thromboembolic stroke, in which the beneficial effect of tPA-induced reperfusion and hemorrhagic transformation associated with delayed administration are similar to those occurring in humans.

Material and Methods

Animals

C57BL/6 male mice (25–30 g, Charles River Laboratory) were maintained on a 12/12 hours light/dark cycle and fed ad libitum. Experiments were conducted according to protocols approved by the

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Table. Physiological Parameters

	Thrombin		Thrombin+tPA 30 Minutes		Thrombin+tPA 3 Hours	
	Saline	FTY720	Saline	FTY720	Saline	FTY720
Blood pressure (mm Hg)	84.3±6.0	86.1±7.3	86.0±7.3	86.9±7.0	85.7±7.7	82.7±5.0
pH (preischemia)	7.38±0.07	7.39±0.06	7.37±0.08	7.38±0.07	7.36±0.06	7.35±0.08
Pco ₂ (preischemia, mm Hg)	30.8±4.8	31.0±7.6	35.4±7.7	34.5±6.0	36.0±7.2	37.2±8.1
Po ₂ (preischemia, mm Hg)	142.7±35.5	146.2±23.3	160.3±34.5	131.7±31.5	133.9±26.3	173.7±25.9
pH (postischemia)	7.39±0.08	7.33±0.05	7.35±0.09	7.32±0.09	7.25±0.12	7.25±0.11
Pco ₂ (postischemia, mm Hg)	43.7±12.0	45.8±12.2	51.1±14.5	49.4±11.6	48.4±12.9	52.2±15.5
Po ₂ (postischemia, mm Hg)	132.2±38.6	119.3±38.4	142.4±37.1	109.8±34.4	115.7±30.4	145.8±14.6

Blood pressure was monitored during the entire surgical procedure.

Animal Research Committee of Massachusetts General Hospital and NIH guide for the Care and Use of Laboratory Animals.

Experimental Groups

Three cohorts were studied (Figure I in the online-only Data Supplement): (1) middle cerebral artery occlusion (MCAO) not treated with tPA (permanent occlusion); in this group, animals were treated (i.p.) with 0.5 mg/kg fingolimod or saline 45 minutes, 24 and 48 hours after occlusion. (2) MCAO+early tPA, in which tPA was administered intravenously 30 minutes after thrombin injection (transient occlusion); in this group, animals received fingolimod or saline 30 minutes (together with tPA), 24 and 48 hours after occlusion. (3) MCAO+delayed tPA, in which tPA was administered intravenously 3 hours after thrombin injection (transient occlusion); fingolimod or saline was administered 3 hours (together with tPA), 24 and 48 hours after occlusion.

Fingolimod and phosphofingolimod (generous gifts from Dr Volker Brinkmann, Novartis Institutes for Biomedical Research, Basel) were dissolved in saline and stored at 4°C for <48 hours.

Middle Cerebral Artery Occlusion

Experimental ischemia was carried out as described in References 13,14 (see the online-only Data Supplement for details). Isoflurane-anesthetized mice were placed in a stereotaxic frame, the skin between the right ear and eye was cut, the temporal muscle retracted, a craniotomy was performed over the artery bifurcation, the meninges were cut, and the middle cerebral artery (MCA), with its parietal and frontal branches, was exposed.

A micropipette (tip size: 30–50 μm), filled with 2 UI/μL mouse α-thrombin (Hematologic Technologies Inc) dissolved in 18% glycerol/saline, was placed in a micromanipulator and 0.5 μL of thrombin solution or vehicle was injected into the lumen of the artery bifurcation to induce the formation of a clot. The micropipette was removed 15 minutes later, when the clot had stabilized. Artery occlusion was considered successful when Laser Speckle Flowmetry showed a rapid and drastic fall of brain perfusion that remained stable during 80 minutes (mean reduction of 70% to 80%; Figure IIA in the online-only Data Supplement). To induce reperfusion, tPA (10 mg/kg; Alteplase, Activase) was administered i.v. (200 μL, 10% bolus, 90% perfusion during 40 minutes), 30 minutes (early recanalization), or 3 hours (delayed recanalization) after the injection of thrombin. In mice with early recanalization, we defined effective reperfusion when blood flow recovered to at least 75% of basal values (Figure IIB in the online-only Data Supplement).

Assessment of Lesion Volume and Histology

Assessment of lesion volume and histology is described in the online-only Data Supplement.

Neurological Deficit Evaluation

Neurological deficit was evaluated after ischemia using the grid and cylinder tests, described in the online-only Data Supplement.

Quantitative Evaluation of Evans Blue Extravasation

To evaluate the effect of fingolimod on blood-barrier damage Evans Blue extravasation was determined as described in the online-only Data Supplement.

Absolute Cerebral Blood Flow Measurement

Absolute cerebral blood flow measurement details are provided in the online-only Data Supplement.

In Vitro tPA Activity Analysis

In vitro interaction assay between tPA activity and fingolimod or P-fingolimod is described in the online-only Data Supplement.

Statistical Analysis

Mice were randomly allocated; treatment groups were coded with tail marks to assess infarct, hemorrhage area, and neurological deficit in a blinded fashion. The number of mice in each group was based on power analysis assuming a treatment effect of 30% and an SD of 25%. Total number of mice included and mortality during and after surgery are summarized in Table I in the online-only Data Supplement.

Data are expressed as mean±SD. For infarct, hemorrhage volumes, and Evans Blue extravasation, statistical difference between groups was calculated by analysis of variance. Statistical significance was evaluated for the grid test using a 2-way ANOVA. Statistical significance was evaluated in the cylinder test using 1-way ANOVA. *P*<0.05 was considered significant.

Results

Effect of Thrombin and tPA Administration on Cerebral Blood Flow

Blood pressure and blood gases were stable in all groups under both basal and ischemic conditions (Table). No significant differences in these parameters were observed between treated and nontreated mice.

In all groups, local injection of thrombin into the MCA caused an immediate drop (to ≈10% of baseline) of cerebral blood flow. In animals not treated with tPA (permanent occlusion), the occlusion was stable during 80 minutes (as assessed by laser speckle flowmetry; Figure 1A) and for at least 4 hours (assessed by [¹⁴C]iodoantipyrine autoradiography in a separate cohort of 4 mice; Figure 1B).

Treatment with tPA 30 minutes after thrombin injection induced a gradual reperfusion starting around 30 minutes after its administration (Figure 1A). No significant differences in reperfusion time were observed between animals treated and nontreated with fingolimod. In mice receiving tPA after

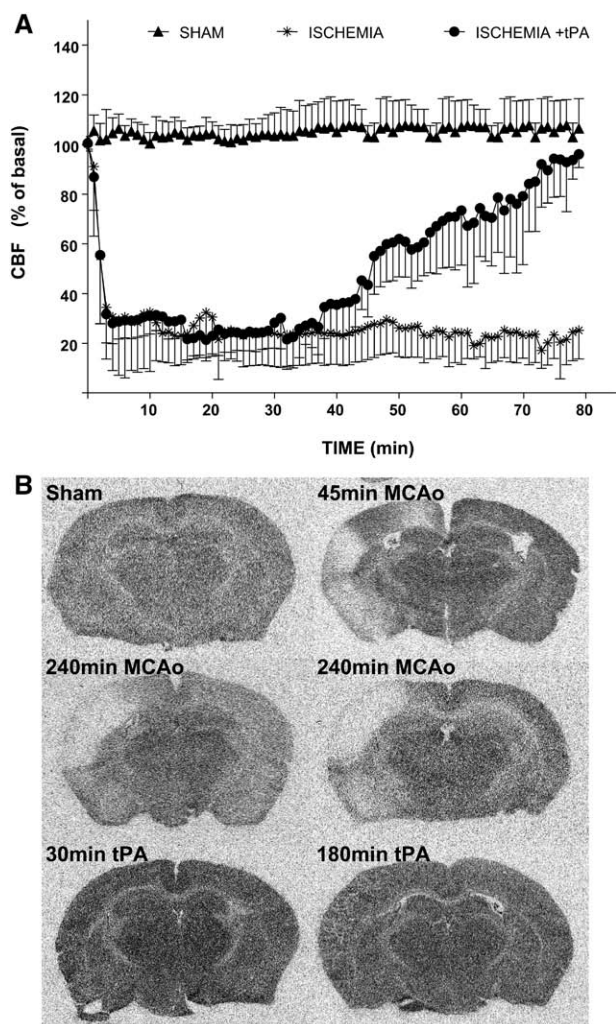


Figure 1. **A**, Analysis of cerebral blood flow by laser speckle flowmetry (LSF) in the sham group (18% glycerol injection), in animals receiving thrombin injection (ischemia) and animals with early tissue plasminogen activator (tPA)-induced recanalization (ischemia+tPA). Cerebral blood flow is expressed as percentage of basal level. **B**, Regional cerebral blood flow using [¹⁴C]iodoantipyrine autoradiography was performed 45 minutes after 18% glycerol injection (sham, n=2); 45 minutes (n=2) or 240 minutes (n=4) after MCA occlusion (note that the ischemic area extends beyond the territory of the occluded MCA branch, probably because of the occurrence of cortical spreading depression/periinfarct depolarization); 4 hours after tPA injection in mice undergoing early (30 minutes, n=2) or late (180 minutes, n=2) tPA recanalization.

3 hours, the cerebral flow was only measured during the first 80 minutes of the procedure. The occlusion was stable during this period.

The effectiveness of reperfusion in mice with transient occlusion (see Material and Methods) was confirmed by [¹⁴C]iodoantipyrine autoradiography. [¹⁴C]iodoantipyrine autoradiography of brains obtained 4 hours after tPA administration confirmed that the clot was completely dissolved in both transient ischemic groups.

In the sham group, local administration of thrombin vehicle (18% glycerol) did not alter cerebral blood flow. Mice in which baseline cerebral flow was altered as result of the

craniotomy were not included in the study (Table I in the online-only Data Supplement). No persistent cortical spreading depression events were observed after thrombin injection in any of the 3 groups studied.

Effect of Fingolimod on Infarct Volume and Neuronal Deficit

Permanent occlusion caused a cortical infarct of 27.3 ± 6.1 mm³ after 3 days. Ischemia was associated with neurological deficits evaluated using the cylinder and grid walking tests on day 3. In the absence of tPA treatment, infarct volume was significantly reduced (17.5 ± 9.1 mm³, $P < 0.05$) when animals received 3 injections of fingolimod, 45 minutes, 24 and 48 hours after thrombin (Figure 2). Reduction of infarct volume by fingolimod was paralleled by better functional outcome (Figure 3).

Early vessel recanalization with tPA reduced infarct volume (18.0 ± 5.5 mm³) and improved functional outcome, compared with the permanent ischemic group. Fingolimod administered in combination with tPA, and 24 and 48 hours after occlusion further improved the neuronal deficit in both behavioral tests, but did not decrease lesion volume beyond the effect of tPA alone (15.7 ± 6.7 mm³).

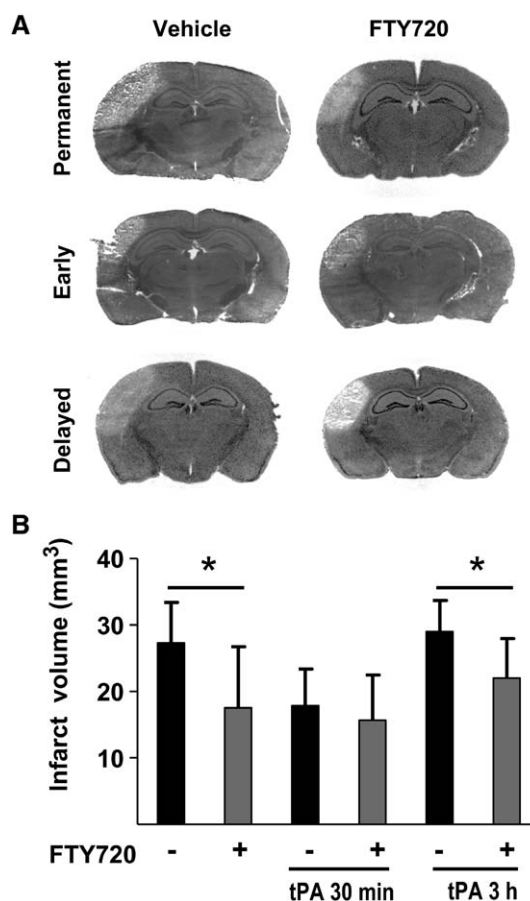


Figure 2. **A**, Representative Hematoxylin and Eosin (H&E)-stained sections illustrating the effect of vehicle or fingolimod (0.5 mg/kg FTY720) on infarct volume in animals with permanent occlusion, early recanalization (tissue plasminogen activator [tPA] 30 minutes after occlusion) and delayed recanalization (tPA 3 hours after occlusion). **B**, Infarct volumes were measured 3 days after occlusion. Data are means \pm SD; n=9 to 10; * $P < 0.05$.

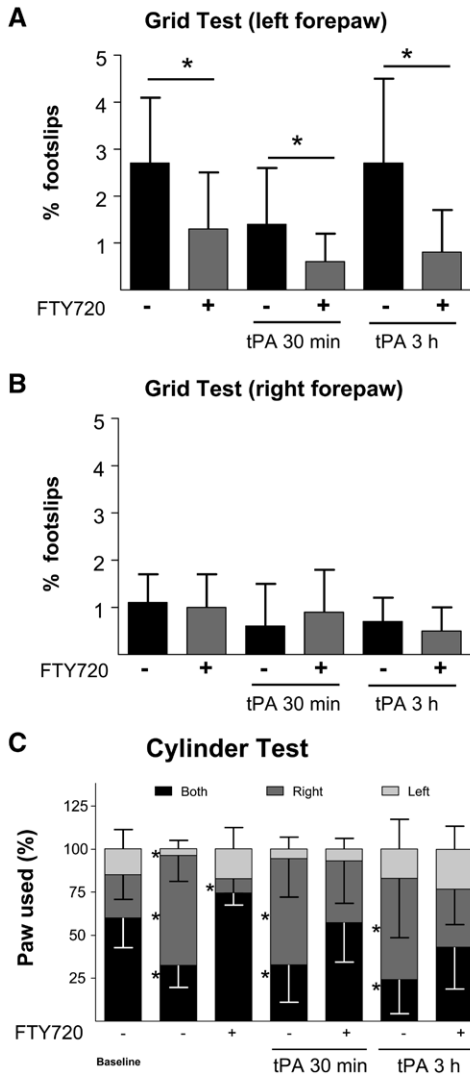


Figure 3. Effect of vehicle and fingolimod (FTY720, 0.5 mg/kg) on functional deficit, evaluated by the grid-walking test in the 3 experimental groups tested. Panels **A** and **B** show the percentage of footslips for the left and right forepaws. The grid-walking test was performed 3 days after occlusion. **C**, Shows the effect of vehicle and fingolimod (FTY720, 0.5 mg/kg) on functional deficit, evaluated by the cylinder test in the 3 experimental groups tested. This test was performed before surgery (baseline) and 3 days after occlusion. Animals treated with FTY720 show a behavior close to baseline. Data are means±SD; n=9 to 10; *P<0.05.

Delayed administration of tPA had no beneficial effect on infarct (29.0±4.7 mm³) and neuronal deficit. Both parameters were similar to those seen in animals with permanent occlusion. Administration of fingolimod in combination with tPA and 24 and 48 hours after occlusion reduced infarct volume (22.0±5.9 mm³, P<0.05), and improved both functional outcome measures.

Effect of Fingolimod on Hemorrhagic Transformation and Blood Barrier Breakdown

Permanent occlusion induced by thrombin and early recanalization were associated with only minor hemorrhages in the ischemic region at 3 days (total volume: 0.27±0.33 mm³ and 0.17±0.38 mm³ in the permanent and 30 minutes tPA groups).

No significant difference was observed between groups. Fingolimod treatment did not affect hemorrhage volume in either group (Figure 4).

Delayed recanalization caused a significant increase in hemorrhage volume (1.10±0.62 mm³) compared with animals not treated with tPA (P<0.05) and animals treated with tPA 30 minutes (P<0.05) after occlusion. Hemorrhagic transformation was significantly reduced (0.32±0.44 mm³) when tPA was administered in combination with fingolimod (P<0.05). The same effects were observed after normalization of these data by infarct volumes.

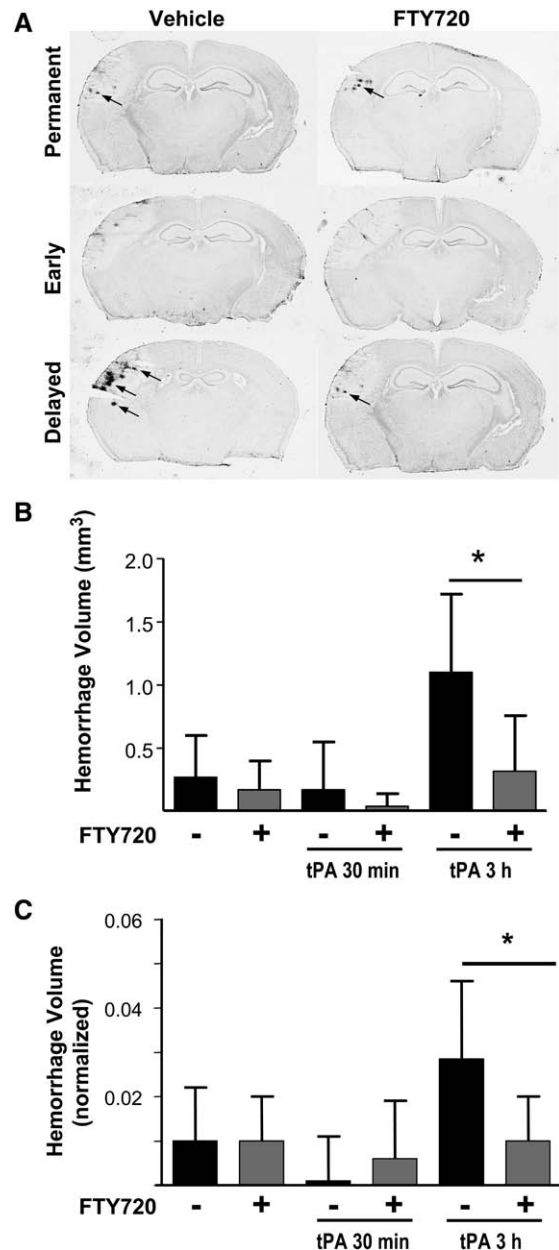


Figure 4. **A**, Representative 3, 3'-diaminobenzidine (DAB)-stained sections illustrating the effect of vehicle or fingolimod (0.5 mg/kg FTY720) on hemorrhage volume in animals with permanent occlusion, and animals with early or delayed recanalization. **B**, The volume of hemorrhages (small arrows in **A**) was analyzed 3 days after occlusion. Panel **C** represents the ischemic region normalized for infarct volumes. Data are means±SD; n=9 to 10; *P<0.05.

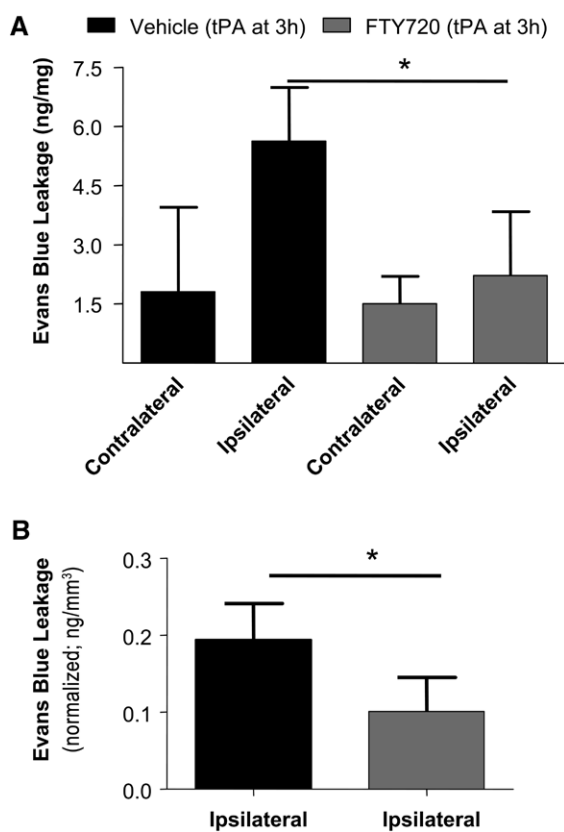


Figure 5. **A**, Effect of fingolimod (0.5 mg/kg FTY720) on Evans Blue extravasation in animals with delayed recanalization determined in contralateral and ipsilateral hemisphere of control and treated group. **B**, Shows Evans Blue extravasation in ischemic region normalized for infarct volume. Evans Blue extravasation was determined 24 hours after occlusion. Data are means \pm SD; n=6; * P <0.05.

To further examine the effect of fingolimod on vascular integrity, we examined Evans Blue extravasation 24 hours after occlusion in mice receiving delayed tPA (Figure 5). We found that tPA increased Evans Blue leakage in the ipsilateral region (5.6 ± 1.4 ng Evans Blue/mg tissue) compared with the contralateral region (1.8 ± 2.1 ng Evans Blue/mg tissue). The ipsilateral increase was not observed when tPA was administered in combination with fingolimod (2.2 ± 1.6 ng Evans Blue/mg tissue, ipsilateral region, P <0.05).

The extent of Evans Blue leakage 3 and 48 hours after tPA administration was similar to that observed at 24 hours (data not shown), and the effect of fingolimod was only investigated at 24 hours.

To demonstrate that the increased Evans Blue leakage was caused by tPA and was not because of ischemic damage or reperfusion injury, we assessed Evans Blue leakage in mice with permanent thrombin-induced occlusion, and in mice in which transient occlusion (3 hours) was induced using a vessel clip¹⁵; but we did not observe an ipsilateral increase in Evans Blue extravasation, and therefore did not study the effect of fingolimod in either cohort (Figure III in the online-only Data Supplement). No differences in the pattern of Evans Blue leakage were observed after normalizing these data for infarct volume.

In Vitro Analysis Interaction of Fingolimod/P-Fingolimod and tPA Activity

In order to expand on the observation that fingolimod treatment did not affect the reperfusion time course of thrombolytic treatment compared with vehicle group (Figure 1), we used an in vitro assay to confirm that fingolimod or P-fingolimod did not interfere with the enzymatic activity of tPA. Fingolimod and P-fingolimod, tested in a range of concentrations between 0.01 μ mol/L and 100 μ mol/L, did not modify the activity of tPA, at variance with leupeptin, used as a positive control (Figure IV in the online-only Data Supplement).

Discussion

This study shows that fingolimod attenuates the neurological deficit and reduces infarct volume in a model of thromboembolic MCA occlusion. In this model, in combination with thrombolytic therapy, fingolimod improves the neurological outcome with early tPA administration, whereas with delayed administration of tPA, fingolimod both improves neurological outcome and reduces the infarct volume.

Fingolimod has been recently suggested to be a potential new treatment for ischemic brain injury, based on several studies using rodent models of brain ischemia⁹⁻¹² (1 study, however, failed to detect an effect,¹⁶ suggesting variations possibly because of experimental conditions). When protective effects by fingolimod were observed, the mechanisms of action were not clearly established. Thus, when fingolimod was tested in a rat model of transient ischemia by filament occlusion of the MCA, it reduced neuronal injury and improved behavior by activation of Akt (protein kinase B) and ERK (extracellular signal-regulated kinase) via SIP_1 receptors, preventing neuronal apoptosis.¹⁰ In a mouse study, the protective efficacy of fingolimod was associated with a reduction of inflammatory response and a direct neuroprotective effect mediated by inhibition of apoptotic death, but preservation of BBB was not observed.⁹ We have recently described that in a transient mouse model, fingolimod reduced infarct size, neurological deficit, and edema.¹² This protective effect was accompanied by decreased inflammation. However, at variance with the neuroprotective effect previously described,^{9,10} fingolimod did not protect primary neurons against glutamate excitotoxicity or hydrogen peroxide, but it decreased intercellular adhesion molecule 1 (ICAM-1) expression in brain endothelial cells stimulated by tumor necrosis factor- α . The findings of the later study suggest that anti-inflammatory mechanisms, and possibly vasculoprotection, rather than direct effects on neurons, are the most plausible mechanisms that underlie the beneficial effects of fingolimod.¹²

In the 3 treatment paradigms used in the present study, fingolimod improved neurological deficit, but did not achieve a significant reduction of infarct volume when administered in combination with tPA 30 minutes after occlusion (Figure 2). Previous clinical studies have observed that tPA treatment administered within 3 hours of symptom onset decreases the neuroinflammation that follows stroke.¹⁷ Although further studies are necessary to confirm this hypothesis, we speculate that the reduction of inflammatory response induced by early tPA administration might have masked the beneficial effect of fingolimod on infarct size.

Vasculoprotection and preservation of BBB integrity are other important processes associated with the activation of SIP receptors,^{18,19} and have been implicated in the effects of fingolimod in studies unrelated to stroke.²⁰ SIP receptor activation stimulates the recruitment of endothelial proteins that form adherens junctions, thereby creating a tighter contact between endothelial cells, enhancing BBB integrity.²⁰

Early tPA administration is the optimal therapeutic strategy to rescue still viable ischemic tissue, and to improve the outcome in patients with acute ischemic stroke. But studies based on magnetic resonance perfusion/diffusion imaging (mismatch concept)²¹ suggest that there might be patients who could benefit from thrombolysis beyond 3 hours, even if they present a higher risk of BBB breakdown and hemorrhagic transformation. In agreement with these clinical data and also with previous experimental studies in rats and mice, we observed that tPA administered 30 minutes after thrombin injection (early administration) reduced ischemic injury without BBB damage, whereas BBB breakdown and hemorrhagic transformation appeared when tPA was given 3 hours after occlusion (delayed administration).

Of note, the dose of 10 mg/kg tPA used in the current study is higher than the clinical dose (0.9 mg/kg). The fibrinolytic system in rodents has been known for 30 years to be about 10 times less sensitive to tPA than in humans.²² The vast majority of stroke studies in rodents have therefore been performed with 10 mg/kg tPA. In our study, significant hemorrhages were not seen with early tPA. We therefore assume that the hemorrhages we observed were the consequence of the delayed administration rather than of the high dose of tPA, and we believe that fingolimod-induced prevention of BBB damage caused by delayed tPA might indeed be clinically significant. Similarly, mice were treated with rather high doses of fingolimod (0.5 mg/kg bodyweight/d), but it is a dose commonly used to treat mice with experimental autoimmune encephalomyelitis (a model of multiple sclerosis).^{23,24}

It is possible that reduced hemorrhagic transformation and Evans Blue extravasation could be the mere consequence of reduced infarct volume in fingolimod-treated mice after delayed rtPA, as a direct effect of fingolimod on BBB damage was not demonstrated. But this is unlikely, as normalized data per infarct volume (Figures 4 and 5) or lack of Evans Blue leakage in mice with permanent occlusion or mice with transient occlusion (3 hours) induced using a vessel clip (Figure III in the online-only Data Supplement) suggest that BBB damage was not associated with infarct size and was mediated by delayed tPA administration, confirming the vasculoprotective effect of fingolimod.

Several treatments, some promising, have been tested in combination with tPA (see²⁵ for review) to reduce its neurotoxicity, risk of hemorrhage, and reperfusion injury, or to increase neuroprotection and increase therapeutic time window. One of the advantages of fingolimod with respect to other treatments is that, in addition to reducing the risk of hemorrhage, fingolimod is itself protective, which is likely to synergize with the benefits of thrombolytic therapy, as we have observed in our study.

In conclusion, in this study, we have confirmed the protective efficacy of fingolimod as a treatment against ischemic

stroke in a novel model thromboembolic occlusion model, and we have shown the potential applicability of this treatment in combination with tPA to reduce the risk of brain hemorrhage.

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Disclosures

None.

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