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Fingolimod provides long-term protection in rodent models of cerebral ischemia

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Abstract

Objective—The sphingosine-1-phosphate receptor agonist fingolimod (FTY720), that has shown efficacy in advanced multiple sclerosis clinical trials, decreases reperfusion injury in heart, liver and kidney. We therefore tested the therapeutic effects of fingolimod in several rodent models of focal cerebral ischemia. To assess the translational significance of these findings, we asked whether fingolimod improved long-term behavioral outcomes, whether delayed treatment was still effective, and whether neuroprotection can be obtained in a second species.

Methods—We used rodent models of middle cerebral artery occlusion and cell culture models of neurotoxicity and inflammation to examine the therapeutic potential and mechanisms of neuroprotection by fingolimod.

Results—In a transient mouse model, fingolimod reduced infarct size, neurological deficit, edema and the number of dying cells in the core and periinfarct area. Neuroprotection was accompanied by decreased inflammation, as fingolimod-treated mice had fewer activated neutrophils, microglia/macrophages, and ICAM-1-positive blood vessels. Fingolimod-treated mice showed a smaller infarct and performed better in behavioral tests up to 15 days after ischemia. Reduced infarct was observed in a permanent model even when mice were treated 4 hours after ischemic onset. Fingolimod also decreased infarct size in a rat model of focal ischemia. Fingolimod did not protect primary neurons against glutamate excitotoxicity or hydrogen peroxide, but decreased ICAM-1 expression in brain endothelial cells stimulated by TNFalpha.

Interpretation—These findings suggest that anti-inflammatory mechanisms, and possibly vasculo-protection, rather than direct effects on neurons, underlie the beneficial effects of fingolimod after stroke. S1P receptors are a highly promising target in stroke treatment.

Blood flow, excitotoxicity, peri-infarct depolarization, inflammation and apoptosis can affect brain ischemic stroke outcome, and have been targeted to improve stroke therapy[1]. Many agents modulating these processes were effective in animal models, but not in clinical trials.

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Sphingosine-1-phosphate (S1P) acts on five G protein-coupled receptors, regulating proliferation, apoptosis, adhesion, migration, cytoskeletal organization, differentiation/morphogenesis and inflammation[2]. S1P is a key player in protective mechanisms against hypoxia- or ischemia-mediated insults. S1P protects neonatal cardiac myocytes from hypoxic damage[3] and reduces ischemia/reperfusion-induced cardiac injury[4].

Fingolimod (FTY720) was first described in 1995, following a chemical derivatization program of myriocin[5]. *In vivo* actions of FTY720 are mediated by phosphorylated FTY720[6], an agonist at $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ receptors[6]. The pharmacokinetics of FTY720 have been extensively characterized[7], and it has shown clinical efficacy in phase 3 clinical trials involving multiple sclerosis patients[8, 9].

Because FTY720 protects from ischemia-reperfusion injury in liver[10, 11] and kidney[12–15], we hypothesized that FTY720 would improve outcome in models of brain ischemia. Our results, previously published in an abstract[16], indeed demonstrate that FTY720 treatment decreases lesion size, edema, cell death and inflammation and suggest that FTY720 might be effective in stroke.

Materials and Methods

These studies were approved by institutional review committee and conducted according to the NIH's "Guide for the Care and Use of Laboratory Animals".

Middle Cerebral Artery Occlusion (MCAo) in mice

C57BL/6 male mice (weighing 20 to 25 g, Charles River Laboratory, MA) were used in the experiments. The middle cerebral artery was occluded as reported previously[17] and described in Supplementary Methods.

Assessment of brain edema

Edema was assessed by measuring forebrain hemisphere water content using the formula: (wet–dry weight)/wet weight × 100. Twenty-four hours after MCAo, mice (n=6 per group) were decapitated and brain tissues were weighted to obtain wet weight. They were dried at 110°C for 48 h to determine dry weight.

Rat transient focal cerebral ischemia

The method was adapted from Yoshida et al.[18] as described in Supplementary Methods. Thirty minutes after reperfusion, FTY720 (1 mg/kg) or saline was administered intraperitoneally. Rats were euthanized 22 h after reperfusion. Infarct area was quantified with 2,3,5-triphenyltetrazolium chloride (TTC) staining.

Immunofluorescence staining and cell count

Intercellular adhesion molecule-1 (ICAM-1), Mac-1 (CD11b), myeloperoxidase (MPO), Iba-1 (specific for microglia and macrophage) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining were performed on sections of mice decapitated 48 hours after reperfusion. Cells were counted using the StereoInvestigator software (MBF Bioscience, Williston, VT) as described in Supplementary Methods.

In vitro ICAM-1 expression

A previously characterized human brain microvascular endothelial cell line was used[19]. Cells were seeded at 2×10^5 cells/well and cultured in RPMI 1640 (supplemented with 10% FBS, 10% NuSerum, 1 mM sodium pyruvate, MEM nonessential amino acids, MEM

vitamins, and 100 units/ml penicillin/streptomycin), grown in serum-free RPMI medium for 6 h before treatment with tumor necrosis factor (R&D Systems, Minneapolis, MN). Some wells were treated with FTY720, S1P (Avanti Polar Lipids, Alabaster, AL) or 4 mg/ml fatty acid free-BSA (control for S1P) for 18 h. Cells were harvested in 60 µl lysis buffer (Cell Signaling Technology, Beverly, MA). Samples were electrophoresed on 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Invitrogen, Carlsbad, CA). Membranes were blocked in 5% non-fat milk, incubated with anti-ICAM-1 antibody (0.5 µg/ml, Santa Cruz, Santa Cruz, CA) at 4°C overnight, and horseradish peroxidase-conjugated secondary antibody (1:4000, GE Healthcare, Piscataway, NJ) for 1 hour at room temperature. Enhanced chemiluminescence signal (GE Healthcare, Piscataway, NJ) was analyzed with MCID software. Data, normalized with β-actin (1:5000, Sigma), are the mean of four independent experiments.

In vitro neuroprotection experiments

Primary neurons were prepared from the cortex of E14-16 CD1 mouse embryos[20]. Neuroprotection experiments were performed on day 10 in culture. Cells were exposed to Glutamate (100 µM) in Neurobasal medium for 5 minutes, washed and treated with medium, or medium supplemented with FTY720 or S1P for 24 hours. Cells were stained with Calcein AM (Invitrogen), fixed in 4% PFA, and stained with DAPI. Images were acquired in three random fields, and healthy-looking cells were counted by an investigator blinded to the treatment groups. The effect of FTY720 and S1P on hydrogen peroxide (H_2O_2) toxicity was assessed by exposing neurons to 30 µM H_2O_2 for 30 minutes.

Statistics

Values are given as mean±SEM. Neurologic score is given as median. Mean values of body weight, blood pressure, blood gases, rCBF, infarct size, cell counts, vessel length measurements, brain water content measurements between the vehicle and the FTY720-treated groups were compared by using Mann-Whitney *U* test.

Results

Physiological parameters and cerebral blood flow

Body weight did not differ between groups (not shown). There was no significant difference between cortical blood flow values measured by a laser Doppler during MCA occlusion and 30 minutes after reperfusion in animals receiving vehicle, 0.5 or 1 mg/kg FTY720 (Figure 1A). Heart rate, blood pressure, blood gases and rectal temperature, measured in separate cohorts of mice (Table 1) did not differ between saline- and FTY720-treated mice (1 mg/kg, i.p.). To further examine whether FTY720 has an effect on cerebral blood flow during focal ischemia, we used non-invasive laser speckle flowmetry in a separate group of mice[21]. CBF deficit was similar in mice treated with vehicle or FTY720 (1 mg/kg) 1h before distal middle cerebral artery occlusion.

FTY720 dose-dependently decreases infarct size and neurological deficit

FTY720 significantly and dose-dependently decreased the corrected infarct size assessed 48 hours after reperfusion (Figure 2). FTY720 significantly improved the neurological deficit in the 1 mg/kg FTY720-treated group compared with the vehicle-treated group. No significant effect was observed at the lower dose of FTY720.

Reduction of brain water content after transient focal cerebral ischemia by FTY720 Treatment

One day after reperfusion, cerebral ischemia increased brain water content of the ischemic hemisphere from 76.7%±2% to 83.5%±3% (Figure 2C). FTY720 treatment significantly reduced the increase of brain water content to 76.4%±2% in the ischemic hemisphere ($p < 0.05$).

Effect of FTY720 treatment on apoptosis *in vivo* and *in vitro*

DNA strand breaks, presumably an index of apoptotic cell death[22], were labeled by TUNEL staining. Forty eight hours after reperfusion, numerous TUNEL-positive cells were seen in periinfarct area and ischemic core, and very few were observed in ipsilateral intact and contralateral area (Figure 3). FTY720 (1 mg/kg, i.p.) significantly reduced the number of TUNEL-positive cells in the periinfarct area and ischemic core ($p< 0.05$).

Because most TUNEL positive cells after MCAo are neurons[23], and we found no overlap between MPO and TUNEL staining (ruling out that TUNEL positive cells represent neutrophils in our study) (Supplemental Figure 5), we used primary neurons (briefly exposed to Glutamate or H_2O_2) to examine whether the decreased in TUNEL-positive cells observed in FTY720-treated mice was due to a direct protective effect on target cells or was indirectly caused by a decreased release of toxic mediators. Cells were then treated with culture medium alone, or medium supplemented with various concentrations of FTY720 or S1P for 24 hours. Both Glutamate and H_2O_2 treatment reduced cell viability (assessed by Calcein staining) to about 25%. Neither FTY720 nor S1P treatment was able to rescue these cells (Figure 3D). Similar results were obtained using two different assays (MTT and LDH release) to confirm the lack of direct neuroprotective effect of FTY720.

Attenuation of ICAM-1 expression by FTY720 treatment

ICAM-1 positive blood vessels were seen mainly in the periinfarct area and ischemic core 48 h after reperfusion (Figure 4). Lower levels of immunoreactivity were seen in parenchymal cells, probably corresponding to microglia and leukocytes[24]. FTY720 treatment significantly reduced the total length of ICAM-1-labeled vessels measured in the periinfarct, ipsilateral intact and contralateral areas.

In order to determine whether the reduction of ICAM-1 expression was a direct effect of FTY720 on blood vessels, we examined the effect of FTY720 and S1P on ICAM-1 protein expression induced by Tumor Necrosis Factor-α in microvascular endothelial cells *in vitro* (Figure 4). FTY720 (0.6 μM) and S1P (1 μM) significantly reduced ICAM-1 up-regulation in endothelial cells by approximately 50%.

Attenuation of postischemic microglial activation and neutrophil infiltration by FTY720 treatment

Numerous MPO-positive cells, presumably neutrophils, were seen in periinfarct area and ischemic core 48 h after reperfusion, and very few were observed in the ipsilateral intact and contralateral area (Figure 5). FTY720 treatment (1 mg/kg, i.p.) significantly reduced neutrophil infiltration in all the areas investigated ($p< 0.05$).

The extent of neutrophil recruitment was determined by immuno-fluorescent staining of the Mac-1 α-chain (CD11b), which is rapidly up-regulated on neutrophils after activation. At 48 hours after reperfusion, numerous activated neutrophils, positively stained by the anti-Mac-1 antibody were seen in both periinfarct area and the ischemic core, and very few were observed in the ipsilateral intact and contralateral area. FTY720 treatment significantly

reduced the number of activated neutrophils in the periinfarct area and ischemic core ($p < 0.05$; Figure 5B).

Iba-1-positive activated microglia/macrophages, amoeboid in shape, showing hypertrophy and proliferation, were mainly observed in periinfarct area and ipsilateral intact area. FTY720 treatment significantly reduced the number of activated microglia/macrophages when compared to vehicle-treated group in periinfarct area, ipsilateral intact area and ischemic core (Figure 5C). On the contralateral side, we mainly observed resting microglia with small somas, thin and branched processes, with a homogeneous distribution. FTY720 treatment had no significant effect on the number of resting microglia in any area investigated (Figure 5D).

Long-term effects of FTY720 on lesion size and behavioral deficit and effect in other rodent stroke models

The oral efficacy of fingolimod was recently established in two large-scale, phase III clinical trials on Multiple Sclerosis patients[8, 9]. In order to assess the potential for oral and the long-term efficacy of the drug in stroke, mice received 3 mg/kg FTY720 dissolved in 200 μL saline 2 hours after reperfusion, at 24 hours and once again at 48 hours (these time points were chosen to test the hypothesis that FTY720 targets early, presumably inflammatory, processes, and minimize the possibility that S1P receptor activation might initiate repair mechanisms[25]). Mice were sacrificed at 14 days and infarct size was measured on eosin-hematoxylin frozen sections. There was a potent and highly significant reduction in infarct size in FTY720- vs. vehicle-treated mice (20 ± 6 vs. 68 ± 16 mm^3; $p<0.001$). Neurological deficit and motor function in the wire grip test [26] were assessed at day 1, 3, 7, 10 and 14 (Figure 6A). There was an overall statistically significant difference in neurological deficit score between vehicle- and FTY720-treated mice ($p<0.001$, Friedman repeated measures ANOVA on ranks). Compared with vehicle-treated mice, wire-grip test performance was significantly improved in FTY720-treated mice over the experimental period ($p<0.05$) (Figure 6B). Mortality was 1/11 and 3/11 in the vehicle and the FTY720 group, respectively.

Having shown that FTY720 treatment had a beneficial effect on long-term histological and functional outcomes, we then examined its effectiveness in another species and in a permanent model in order to address some of the criteria proposed by the Stroke Therapy Academic Industry Roundtable (STAIR)[27]. Rats underwent 2-h MCAo as described in Methods. FTY720 (1 mg/mg, i.p. 30 minutes after reperfusion) significantly decreased infarct volume measured 22 h after reperfusion (Figure 6C). Mortality was 0/9 and 1/10 in the vehicle and FTY720 group, respectively. In a permanent mouse MCAo model, 1 mg/kg FTY720 significantly decreased infarct size when administered 2 or 4 hours after the beginning of the occlusion (compared to time-matched saline-treated mice) (Figure 6D). In the 2-h study, mortality was 2/13 and 4/13 in the vehicle and FTY720 group, respectively; in the 4-h study, mortality was 3/13 and 4/14.

Discussion

These results show that FTY720 reduces infarct size in several rodent models of brain ischemia. In a transient model, FTY720-treated mice showed significantly attenuated neurological deficit, decreased edema, and decreased number of dying cells in the core and periinfarct area. We also found a reduced number of activated neutrophil and microglia/macrophages, and fewer ICAM-1 positive blood vessels. Protection by FTY720 was long-lasting and functionally relevant, because treated mice showed a smaller infarct and performed better in behavioral tests up to 15 days after ischemia. Of particular translational significance, protection was observed in a permanent model even when FTY720 was

administered 4 hours after filament insertion, and protection was seen in more than one species.

Various aspects of the response to stroke have been targeted in rodent models [1]. Because S1P plays a role in many cell processes[2], and S1P receptors are found in all brain cell types[28], virtually any known protective mechanisms could, at least in theory, be involved in FTY720-mediated protection. Animal models of ischemic brain injury suggest a neuroprotective effect of hypothermia, with most studies showing little efficacy when hypothermia is instituted beyond a 3-hour time window[29]. Although direct intracerebroventricular S1P application decreases core temperature in mice[30], FTY720 treatment had no effect on rectal temperature in the present study and a previous study in rats[7]. Increasing blood flow is the only strategy that has improved outcome clinically. Our laser Doppler data show that FTY720-treated mice had the same level of initial reperfusion as mice receiving saline. Although performed in a distal occlusion model, laser speckle experiments strongly indicate that FTY720 does not act by altering the area of blood flow deficit up to 60 minutes after artery occlusion. An effect on blood flow via reduction of the microvascular “no reflow” phenomenon is possible[31]. Leukocyte adhesion has been demonstrated in different models of cerebral ischemia as early as 30 minutes after reperfusion[32]. Because adhesion is mediated by interaction between β2-integrins on leukocytes with ICAM-1 on cerebral endothelial cells, and we found that FTY720 decreased ICAM-1 expression both *in vivo* and *in vitro*, FTY720 treatment may have increased microvascular patency at later time points.

Leukocyte accumulation causes tissue injury by several mechanisms in addition to occlusion of the microvasculature: generation of oxygen free radicals, release of cytotoxic enzymes, cytokines and chemoattractants. Free radicals generated by activated neutrophils can also damage the microvascular endothelium, resulting in edema and a deleterious rise in intracranial pressure. In addition, FTY720, once phosphorylated by sphingosine kinase 2, acts directly on endothelial S1P receptors to maintain the integrity and functionality of endothelial cells, decreasing vascular permeability[33]. Both mechanisms (decreased neutrophil activation and direct action on endothelial S1P receptors) might explain why FTY720-treated mice showed decreased edema at 24 hours in our study.

FTY720 reduced TUNEL staining *in vivo*, presumably corresponding to neuronal death[23]. But neither FTY720 nor S1P protected neurons in *in vitro* models of excitotoxicity and oxidative stress-induced cell death. This observation, taken together with the fact that we observed fewer activated neutrophils and microglia/macrophages in treated mice, suggests that FTY720 might decrease tissue damage by limiting the levels of cytotoxic agents, rather than by a direct neuroprotective effect.

Phosphorylated FTY720 binds to lymphocyte $S1P_1$ receptors leading to receptor internalization and degradation[34], resulting in the loss of T cell response to S1P produced by the lymphatic endothelium[35], inhibition of egress from secondary lymphoid tissues and peripheral lymphopenia[34]. The decrease in circulating lymphocytes is associated with a reduction in T-cell infiltration at the sites of inflammation in several allograft and autoimmune disease models[36]. FTY720 mitigates the effects of hepatic ischemia/reperfusion injury by reducing T cell infiltration[37]. Although a similar protective role by FTY720 is well documented in kidney ischemia/reperfusion injury, there is controversy as to whether this protection is associated with an effect on T-lymphocyte infiltration[14, 38]. In brain, there is a growing body of evidence supporting a role for T-lymphocytes in the tissue injury following ischemic stroke[39]. Mice deficient in CD4+ or CD8+ T-lymphocytes exhibit a smaller infarct, fewer adherent leukocytes and platelets in the cerebral venules, and improved neurological outcome as early as 24 hours after reperfusion[39]. Cytotoxic T lymphocytes infiltrate the

ischemic infarct within 1 h of reperfusion after transient middle cerebral artery occlusion[40]; this study also showed that the serine esterase granzyme B released by cytotoxic T lymphocytes mediates ischemia-associated neuronal death. These data, taken together with the peripheral lymphopenic effects of FTY720, further indicates that lymphocyte depletion may underlie the beneficial effects of FTY720 reported here.

It is likely however that FTY720 acts in brain ischemia via multiple mechanisms, as discussed in the context of multiple sclerosis[28]. Direct effects on endothelium may be relevant. For instance, we have shown that FTY720 protects primary rat brain endothelial cells from oxygen/glucose deprivation-induced cell death via Akt-mediated mechanisms[41]. And, in the present study, FTY720 decreased ICAM-1 expression by acting directly on cultured endothelial cells, suggesting that FTY720 might decrease *in vivo* leukocytes binding to endothelial cells, and hence improve blood vessel patency and inflammation.

The large number of neuroprotective agents effective in preclinical studies contrasts with the fact that only a couple of agents have shown clinical efficacy. Our study addresses most of the recommendations issued by the Stroke Therapy Academic Industry Roundtable to decrease the gap between preclinical and clinical studies[27]: "*The ideal neuroprotective drug should demonstrate efficacy in at least 2 species, in at least 2 laboratories that use different models, is effective in both permanent and transient focal ischemia, and improves short term and long-term histological and functional outcomes, even when administered several hours after the onset of ischemia*." Another study showed that FTY720 reduced infarct size in a mouse model of cerebral ischemia, but the drug was administered at the time of occlusion[42]. More clinically relevant, a recent study showed that, when administered immediately after reperfusion, FTY720 reduced infarct volume and improved neurological score at 24 and 72 hours after middle cerebral artery occlusion in rats[43].

We are aware of very few agents associated with a robust protection when administered as late as 4 hours after the beginning of a permanent ischemia[44–49]. Because of the high mortality rate in the permanent model after 24 hours, we did not investigate treatment effect beyond that time. However, the extended therapeutic window of FTY720, its long-lasting effects (up to 15 days after treatment), taken together with the fact that it has been extensively characterized preclinically and clinically, suggests that FTY720 might be beneficial not only in multiple sclerosis, but is also an excellent candidate to investigate in advanced preclinical stroke studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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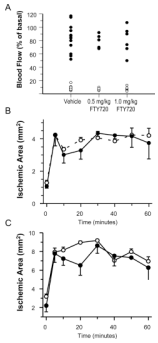

Figure 1.

Effects of FTY720 on cerebral blood flow. Regional cerebral blood flow was measured using a flexible laser Doppler probe placed over the temporal bone after removal of part of the temporalis muscle; relative blood flow laser Doppler flow probe during MCA occlusion (open circles) and 30 minutes after reperfusion (filled circles) in animals receiving vehicle, 0.5 mg/kg or 1 mg/kg FTY720 (i.p.) are shown in panel A (n=12 for vehicle, n=6 for FTY720). In a separate cohort of mice, we used laser speckle flowmetry to study the spatio-temporal characteristics of CBF changes during focal cerebral ischemia in mice pre-treated with either saline (open circles) or FTY720 (1 mg/kg, i.p.; filled circles) 1h before distal middle cerebral artery occlusion. Laser speckle imaging was started 1 min before distal middle cerebral artery occlusion and continued throughout the experiment. Ischemic CBF deficit was analyzed over time by quantifying the area of cortex with either severe (0% to 20% residual CBF, shown in panel B) or moderate CBF reduction (21% to 30% residual CBF, shown in panel C). 60 minutes after distal MCA occlusion, the area of severely ischemic cortex (i.e. with ≤20% residual CBF) was 3.7±1.0mm^2 in FTY-treated mice (n=4), compared to 4.2±0.5 mm^2 in the saline-treated group (n=4) (Figure 1B); the area of mildly ischemic cortex was 6.3±0.5 mm^2 in FTY-treated mice, compared to 6.9±1.3 mm^2 in saline-treated mice (Figure 1C).

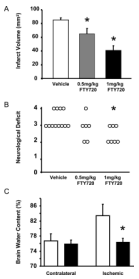

Figure 2.

Effect of FTY720 post-treatment on infarct size, neurological deficit, and edema following 90-min MCAo in mice. A: Mice treated at reperfusion and at 24 hrs with saline (n=12), 0.5 mg/mg (n=5) or 1 mg/kg FTY720 (n=7) (i.p.) were decapitated after 48 hours. Infarct area was measured on six hematoxylin-stained frozen sections. Both doses of FTY720 significantly reduced infarct size. Only the higher dose improved neurological deficit assessed just before sacrifice (panel B). C: for assessment of brain edema, a separate cohort of mice (n=6 per group) was treated at the time of reperfusion with saline (open bars) or 1 mg/kg FTY720 (solid bars). Brain edema was assessed at 24 hours post reperfusion by measuring brain water content using wet weight minus dry weight method. FTY720 treatment significantly reduced the increase of brain water content to control levels in the ischemic hemisphere.

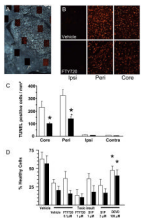

Figure 3.

Effect of FTY720 treatment on cells death *in vivo* and *in vitro*. Mice were treated at reperfusion (following 90-min MCAo) and at 24 hrs with saline or 1 mg/kg FTY720 (i.p.) (n=7/group) and were decapitated after 48 hours. Analysis of cells exhibiting DNA fragmentation was performed using a fluorescein-based terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) to label double-stranded DNA breaks. TUNEL-labeled cells were counted in randomly selected fields of view by an investigator blinded to the treatment groups. For detailed analysis of the distribution of immunoreactive cells, we divided the coronal brain sections into ischemic core, peri-infarct area, ipsilateral intact area and contralateral area. A shows the distribution of counting fields ipsilateral to the lesion in a representative coronal brain section. B show representative TUNEL staining pattern and intensity in ipsilateral intact areas, the peri-infarct areas and cores of a vehicle- (top) and an FTY720-treated mouse (bottom). C: Quantitative analysis shows that FTY720 (1 mg/kg, i.p.; solid bars) significantly reduced the number of TUNEL positive cells in the periinfarct area (140±17 vs. 324±47 cells / mm2) and ischemic core (100.6±9 vs. 230.2±48 cells / mm2) when compared to vehicle-treated group (open bars; $p < 0.05$). D: Primary cortical neurons were exposed to 100 μM Glutamate for 5 minutes (open bars) or to 30 μM H_2O_2 for 30 minutes (solid bars). Cells were then treated with culture medium alone, or medium supplemented with various concentrations of FTY720 or S1P for 24 hours. Cells were then stained with Calcein AM, fixed in 4% PFA, and stained with DAPI. Healthy-looking cells were counted in three random fields by an investigator blinded to the treatment groups. Both Glu and H_2O_2 treatment reduced cell viability to about 25%. Neither FTY720 nor S1P treatment was able to rescue these cells (as a positive control, 100 μM z-DEVD-FMK significantly increased the number of healthy-looking cells, compared to vehicle-treated cells in both Glu and H_2O_2-induced toxicity experiments, $p<0.05$; 4 independent experiments performed in triplicate). Neuronal viability assays after H_2O_2 exposure using the MTT and LDH assays: neurons were exposed to H_2O_2 for 30 min as described in Supplemental Methods. Vehicle, S1P, FTY720 or phospho-FTY720 was then added to the cultures. Cell viability was assessed after 24 hours using the MTT (E) and LDH (F) assays as described in Supplemental Methods. None of the FTY720 or S1P concentrations tested were able to rescue neurons from H_2O_2-induced cell death.

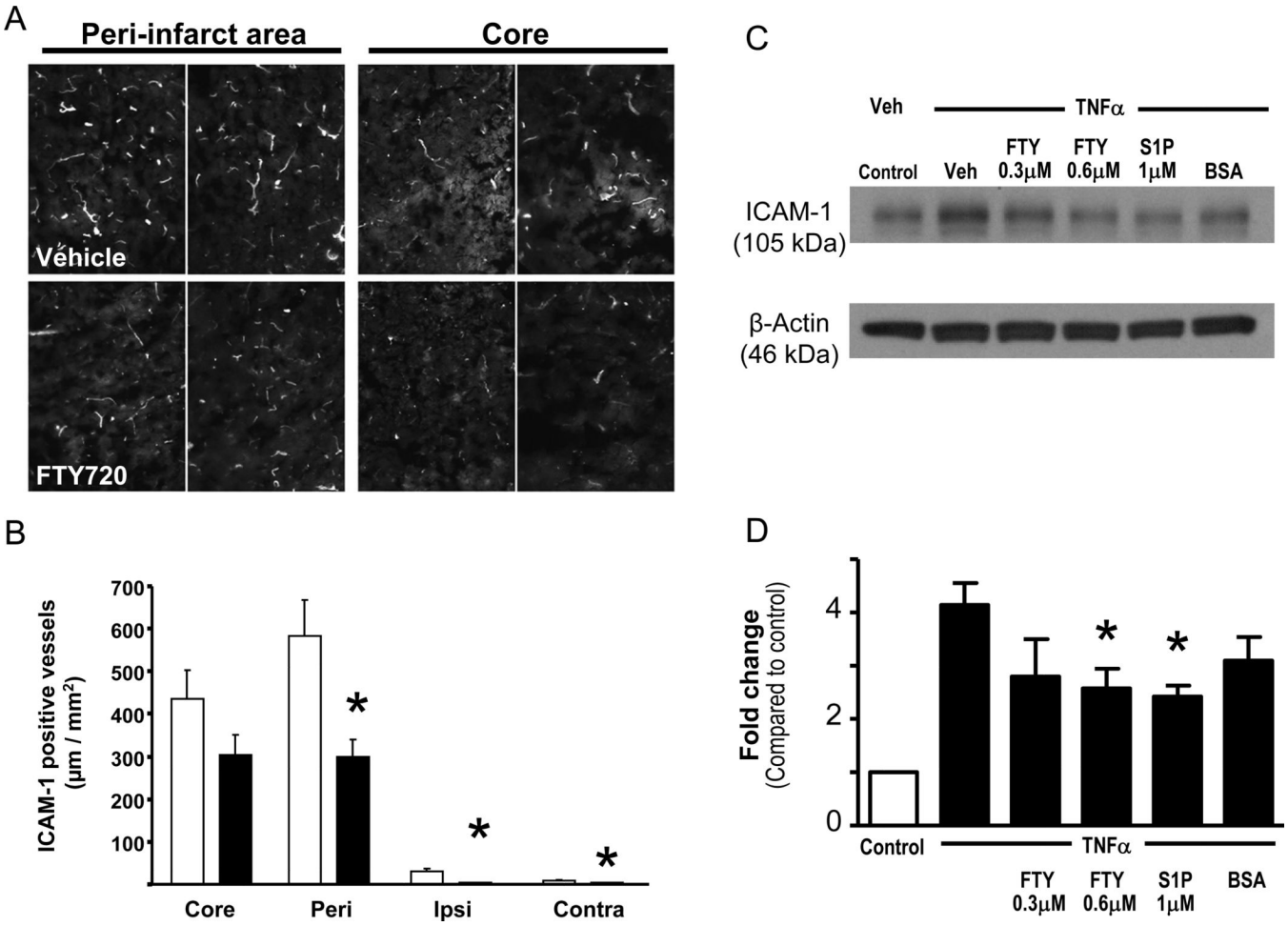

Figure 4.

Effect of FTY720 treatment on endothelial expression of ICAM-1 *in vivo* and *in vitro*. A: representative ICAM-1 staining pattern and intensity in two peri-infarct and two core areas of a vehicle- (top) and an FTY720-treated mouse (bottom). Mice were treated at reperfusion (following 90-min MCAo) and at 24 hrs with saline or 1 mg/kg FTY720 (i.p.) and were decapitated after 48 hours (n=7/group). B: the length of vessels stained by the ICAM-1 antibody per area was measured by an investigator blinded to the treatment groups as described in Methods. This length was significantly shorter in the periinfarct (298.6±43 vs. 583.93±83 μm / mm^2), ipsilateral intact (3.94±1 vs. 29.26±8 μm / mm^2) and contralateral areas (3.61±1 vs. 8.87±1 μm / mm^2) of FTY720-treated mice (solid bars) than saline-treated mice (open bars). C: Human brain microvascular endothelial cells treated *in vitro* with tumor necrosis factor (TNF-α) showed enhanced ICAM-1 protein expression as shown on this representative Western blot (see panel D for conditions). D: Western blots were quantified by image analysis: FTY720 (0.6 μM) and S1P (1 μM) significantly reduced ICAM-1 up-regulation in endothelial cells by approximately 50%; there was no effect of 4 mg/ml BSA treatment (S1P vehicle) (4 independent experiments).

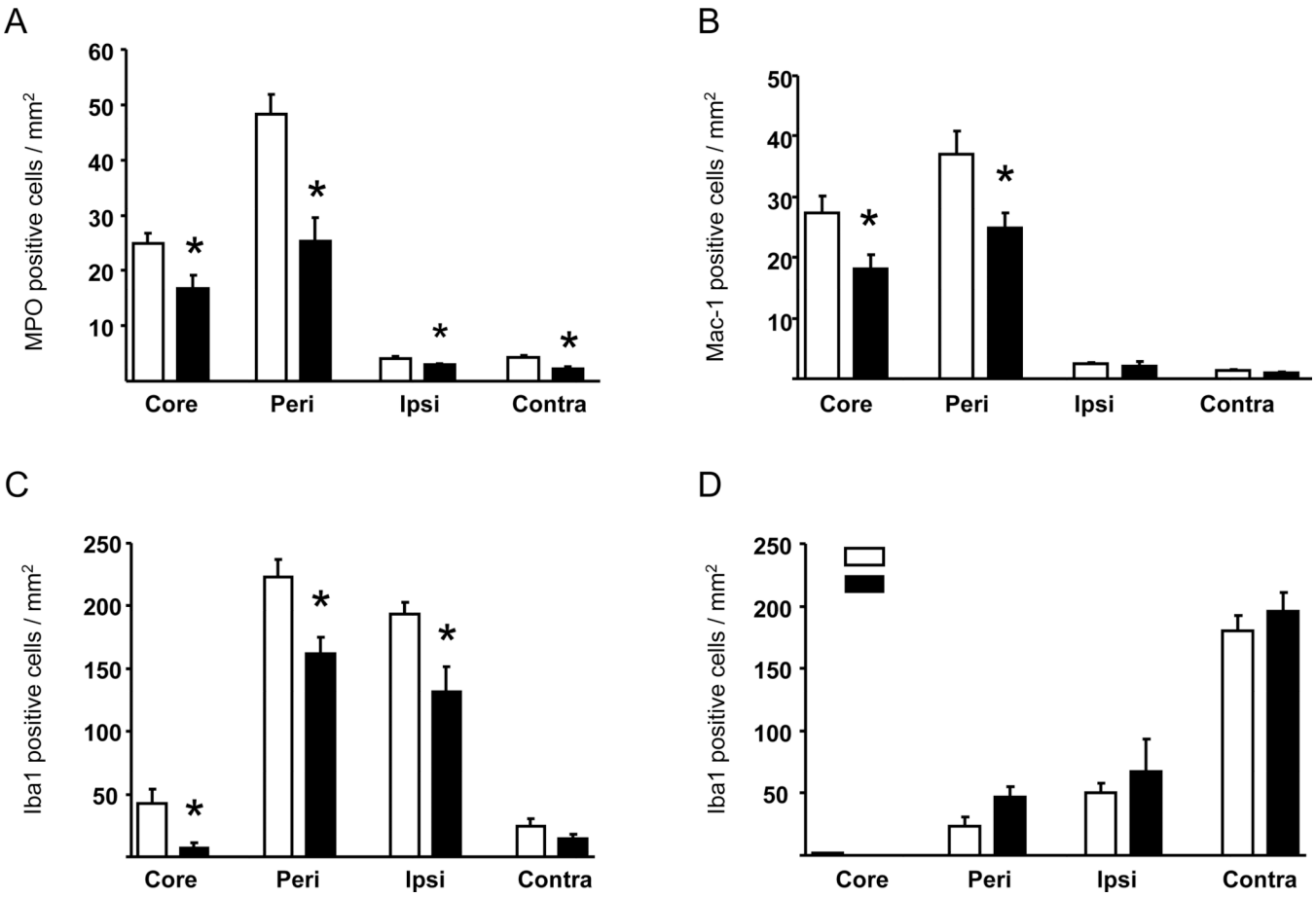

Figure 5.

Effect of FTY720 treatment of the expression of inflammatory markers 48 hrs after 90-min MCAo. Mice were treated at reperfusion (following 90-min MCAo) and at 24 hrs with saline (open bars) or 1 mg/kg FTY720 (i.p.) (solid bars) (n=7/group). Immunohistochemistry and cell counting were performed as described in Methods. A: FTY720 treatment (1 mg/kg, i.p.) significantly decreased the number of cells immuno-reactive for myeloperoxidase (MPO, a marker enzyme for neutrophils) in all the areas: periinfarct area (25±4 vs. 48±4 cells / mm^2), ischemic core (16±3 vs. 25±2 cells / mm^2), ipsilateral intact area (3±0.3 vs. 4±0.4 cells / mm^2) and contralateral area (2.3±0.3 vs. 4.3±0.3 cells / mm^2) ($p < 0.05$). B: Mac-1 is an adhesion protein involved in neutrophil extravasation during inflammation, its expression and activity are greatly increased after neutrophil activation. FTY720 significantly decreased the number of Mac-1 labeled cells in the periinfarct area (24±3 vs. 37±4 cells / mm^2) and ischemic core (18±2 vs. 27±3 cells / mm^2). Iba1 is specifically expressed in macrophages/microglia and is upregulated during the activation of these cells. Microglia were considered activated when they exhibited stout, partially retracted processes extending from the cell perikarya. C: FTY720 treatment decreased the number of Iba1-positive cells with an “activated morphology” in the periinfarct area (162±13 vs. 223±14 cells/mm^2), ipsilateral intact area (131±20 vs. 193±10 cells / mm^2) and ischemic core (7±4 vs. 42±11 cells / mm^2), but did not affect the number of Iba1-positive cells exhibiting a resting morphology (D).

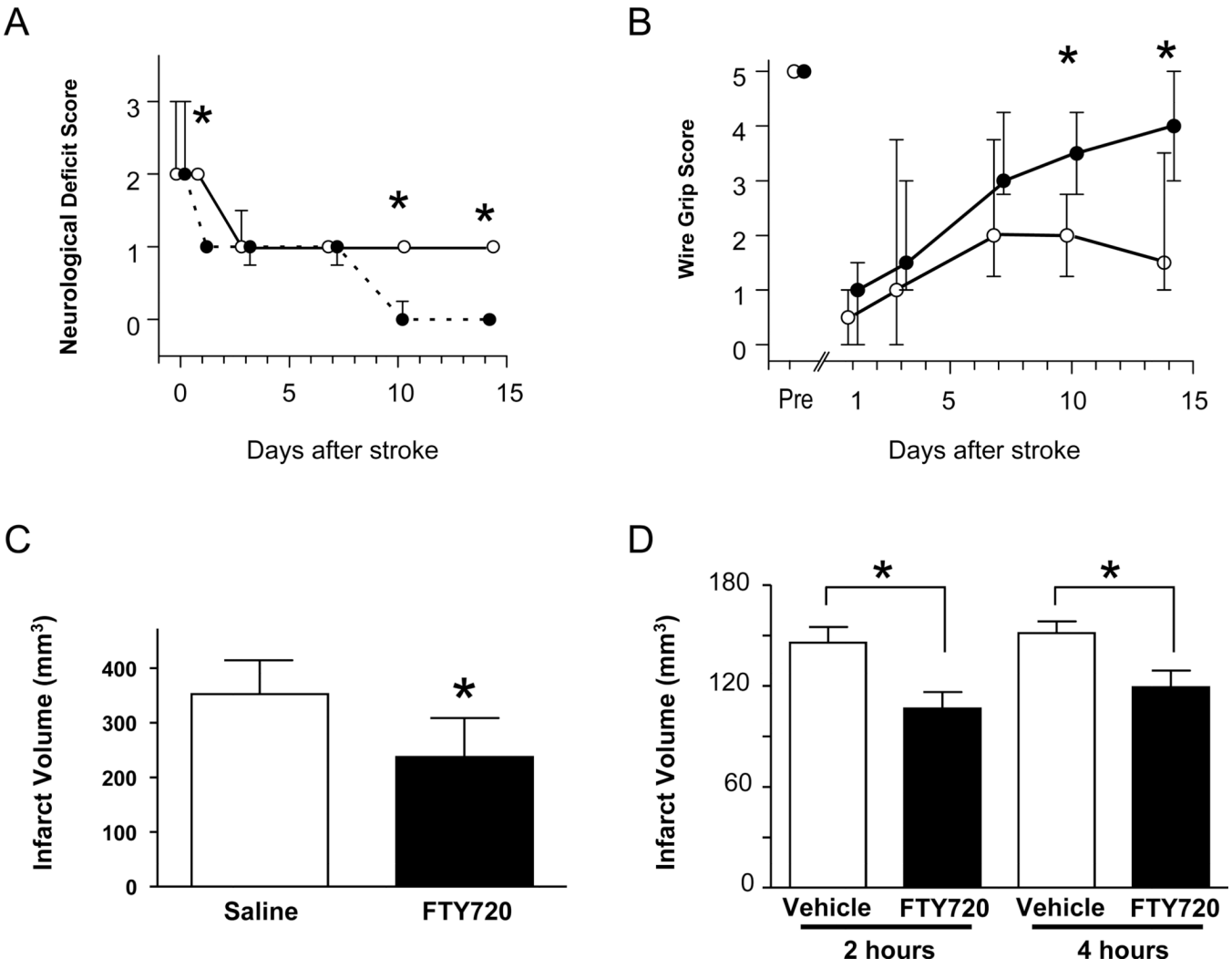

Figure 6.

Long term effects of FTY720 treatment and effect in other rodent stroke models. Mice received 3 mg/kg FTY720 2 hours after reperfusion (following 90-min MCAo), at 24 hours and once again at 48 hours. Neurological deficit and motor function in the wire grip test were assessed at day 1, 3, 7, 10 and 14 (baseline wire grip performance was also assessed the day before MCAo). A: There was an overall statistically significant difference in neurological deficit score between vehicle- (open circles; n=10) and FTY720-treated mice (filled circles; n=8) ($p<0.001$, Friedman repeated measures ANOVA on ranks). When days were analyzed individually, there was a difference between vehicle- and FTY720-treated mice on day 1 ($p=0.01$), day 10 ($p = 0.02$), and day 14 ($p = 0.006$), but not on day 0, 3 and 7 (Mann-Whitney Rank Sum Test). B: Compared with vehicle-treated mice (open circles), wire-grip test performance was significantly improved in FTY720-treated mice (filled circles) over the experimental period ($p<0.05$); when days were analyzed individually, there was a difference between vehicle- and FTY720-treated mice on day 10 ($p=0.05$) and 14 ($p=0.04$). C: Sprague-Dawley rats underwent 2-hour MCAo as described in Methods. FTY720 (1 mg/mg, administered i.p. 30 minutes after reperfusion) significantly decreased infarct volume measured 22 hours after reperfusion (n=9/group). D: In a permanent mouse MCAo model, 1 mg/kg FTY720 significantly decreased infarct size when administered

either 2 or 4 hours after the beginning of the occlusion (compared to time-matched saline-treated mice) (n=9/group).

Table 1

Physiological Variables in mouse MCAo.

		n	pH	pCO_2 (mmHg)	pO_2 (mm Hg)	MABP (mmHg)	Rectal Temperature (°C)
Vehicle	Before	5	7.40±0.01	37.3±2.7	110.4± 6.5	95.4± 3.9	38.5 ± 0.6
	During	5	7.40±0.03	35.9±1.89	137.8±11.3	82.6± 7.5	
	After	5	7.30±0.04	39.7±2.2	122.4±15.0	76.6± 5.9	37.1± 0.3[1]
FTY720	Before	5	7.40±0.02	38.8±0.9	116.1±4.7	86.9± 4.8	38.4± 0.6
	During	5	7.40±0.02	41.0±3.0	151.1±7.3	78.7± 3.9	
	After	5	7.30±0.03	43.8±9.8	114.5±14.0	71.7± 6.1	37.1± 0.6[1]

Values were measured 10 minutes before and during MCA occlusion and once again 30 minutes after reperfusion. FTY720 (1 mg/kg) or the corresponding amount of vehicle (200 μl) was administered intraperitoneally at the time of reperfusion.

[1] Rectal temperature was measured 90 minutes after reperfusion.