Sphingosine-1-Phosphate Receptor Agonist Fingolimod Increases Myocardial Salvage and Decreases Adverse Postinfarction Left Ventricular Remodeling in a Porcine Model of Ischemia/Reperfusion

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Background—Fingolimod, a sphingosine-1-phosphate receptor agonist, is used for the treatment of multiple sclerosis and exerts antiapoptotic properties. We hypothesized that sphingosine-1-phosphate receptor activation with fingolimod during acute myocardial infarction (MI) inhibits apoptosis, leading to increased myocardial salvage, reduced infarct size, and mitigated left ventricular (LV) remodeling in a porcine model of ischemia/reperfusion.

Methods and Results—Ischemia/reperfusion was induced in pigs by balloon occlusion of the left anterior descending artery, followed by reperfusion. Animals randomly received fingolimod or saline (control). In short-term experiments, fingolimod treatment activated the cardioprotective reperfusion injury salvage kinase and survivor activating factor enhancement pathways in the infarct border zone 24 hours after MI, leading to decreased cardiomyocyte apoptosis and reduced myocardial oxidative stress. These effects were abolished by specific inhibitors of both pathways, demonstrating that fingolimod-induced cardioprotection was mediated by reperfusion injury salvage kinase and survivor activating factor enhancement pathways. In long-term experiments, fingolimod significantly improved myocardial salvage, reduced infarct size, and improved systolic LV function measured by cardiac magnetic resonance 1 week and 1 month after MI. Importantly, fingolimod mitigated the development of adverse post-MI LV remodeling 1 month after MI. Specifically, fingolimod treatment led to a significant reduction in LV mass, LV dilatation, and neurohormonal activation, and it preserved LV geometry. Furthermore, fingolimod decreased interstitial fibrosis, cardiomyocyte hypertrophy, and chronic activation of Akt and extracellular receptor kinase 1/2 in the remote noninfarcted myocardium.

Conclusions—Sphingosine-1-phosphate receptor activation with fingolimod during acute MI reduced infarct size via the reperfusion injury salvage kinase and survivor activating factor enhancement pathways, improved systolic LV function, and mitigated post-MI LV remodeling. Our data strongly support a cardioprotective role for sphingosine-1-phosphate receptor activation during MI. (Circulation. 2016;133:954-966. DOI: 10.1161/CIRCULATIONAHA.115.012427.)

Key Words: apoptosis ■ cardiovascular diseases ■ ischemia ■ models, animal ■ myocardial infarction ■ reperfusion injury ■ ventricular remodeling

Clinical Perspective on p 966

Sphingosine-1-phosphate (SIP) has recently received attention for its cytoprotective effects, its antioxidant...
actions, and its potential to ameliorate I/R injury. S1P is a bioactive lyosphospholipid that is derived from the ubiquitous membrane lipid sphingomyelin. Recent studies demonstrate that S1P reduces I/R injury in the liver, kidney, and brain. Interestingly, S1P enhances cardiac myocyte survival during hypoxia in vitro and reduces infarct size in vivo in isolated heart preparations. Furthermore, initial in vivo experiments support a cardioprotective role of S1P in mice during acute MI.

Currently, fingolimod is the only US Food and Drug Administration–approved S1P receptor (S1P-R) agonist available for clinical use in humans. Fingolimod reduces relapse rates and magnetic resonance lesions in patients with relapsing-remitting multiple sclerosis. Interestingly, fingolimod-mediated activation of cardiac S1P-R may also have cardioprotective effects. Preliminary in vitro studies have demonstrated that fingolimod reduces cardiomyocyte apoptosis during hypoxic conditions. Moreover, fingolimod reduces I/R injury and improves myocardial function in isolated mouse and rat heart preparations. However, the cardioprotective effects of fingolimod have not yet been studied in vivo in large-animal models.

We hypothesized that S1P-R activation with fingolimod would reduce cardiomyocyte apoptosis in a porcine model of acute MI, leading to increased myocardial salvage, decreased infarct size, and improved LV contractility and remodeling.

Methods

Study Design

The study design is illustrated in Figure 1. The procedures were in compliance with the guidelines of the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai. Before MI, the animals underwent 2- and 3-dimensional (3D) echocardiography. Subsequently, the proximal left anterior descending artery was occluded with a percutaneous intracoronary balloon catheter for 60 minutes to induce myocardial ischemia and then to allow reperfusion.

In protocol 1 (short-term experiments), pigs underwent MI induction and were randomized to receive 15 minutes before reperfusion fingolimod (1 mg/kg IV) in 5 mL saline infused over 1 minute) or normal saline (control). Animals were euthanized 24 hours after MI for assessment of apoptosis, oxidative stress, and activation of the reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE; Janus kinase/STAT3) pathways. We found that phosphorylation of Akt, ERK1/2, GSK-3β, and STAT3 was considered statistically significant at values of P<0.05.

Results

Antiapoptotic and Antioxidant Effects of S1P-R Activation With Fingolimod on the Ischemic Border Zone Myocardium 24 Hours After MI

S1P-R activation with fingolimod resulted in a significant reduction in terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive nuclei in the ischemic border area, indicating decreased cardiomyocyte apoptosis (Figure 2A and 2B). However, simultaneous administration of wortmannin or AG490 abrogated this effect, indicating that S1P-R–induced reduction in apoptosis is mediated by the RISK and SAFE pathways. These results are further supported by a reduction in the activation of the proapoptotic proteins caspase-3 and p53 and an increase in the expression of the antiapoptotic proteins Bcl-2 and protein kinase C-ε in fingolimod-treated pigs (Figure 2D). Again, administration of the specific RISK and SAFE inhibitors abolished these cytoprotective effects of fingolimod. As a result of these antiapoptotic effects, fingolimod significantly reduced the infarct size determined by triphenyltetrazolium chloride (28.5±2.2% versus 36.9±2.5% of the LV for fingolimod and controls; P<0.05; Figure 2C). The reduction in infarct size was abolished by wortmannin and AG490 (infarct size, 35.1±1.8% and 38.4±1.9% of the LV, respectively).

Myocardial oxidative stress was reduced in the ischemic border zone of fingolimod-treated pigs. Nuclear oxidative stress assessed with 8-hydroxydeoxyguanosine staining was decreased in the fingolimod treatment arm (Figure 3A and 3B). The activity of the antioxidant enzyme superoxide dismutase was increased in fingolimod-treated animals (Figure 3C), which further supports the reduced oxidative stress in the fingolimod group.

To understand the mechanism of fingolimod-mediated cardioprotection, we assessed the short-term effects of fingolimod on the 2 pathways regulating the protection of cardiomyocytes against I/R injury: the RISK (involving Akt/ERK/GSK-3β) and SAFE (involving Janus kinase/STAT3) pathways. We found that phosphorylation of Akt, ERK1/2, GSK-3β, and STAT3 was
significantly increased in the ischemic border zone of fingolimod-treated pigs 1 day after MI (Figure 2D and 2E and Figure I in the online-only Data Supplement), indicating that fingolimod activated both the RISK and the SAFE pathways. Simultaneous administration of wortmannin reduced the fingolimod-induced phosphorylation of Akt/ERK/GSK-3β (ie, it inhibited RISK pathway activation; Figure 2D and 2E), whereas coadministration of AG490 decreased the fingolimod-mediated phosphorylation of STAT3 (ie, it inhibited SAFE pathway activation; Figure 2D and 2E). Importantly, our results demonstrate that fingolimod-induced cardioprotection is mediated via activation of the SAFE and RISK pathways because the antiapoptotic (Figure 2A, 2B, 2D, and 2E), the antioxidant (Figure 3), and the infarct size--reducing (Figure 2C) effects of fingolimod were abrogated with the simultaneous inhibition of the SAFE or RISK pathways.

SIP-R Activation With Fingolimod Improved Myocardial Salvage and Reduced Infarct Size

One week after MI, infarct size was significantly smaller in the fingolimod arm in terms of both absolute infarct mass and percentage of infarcted LV myocardium (Figure 4A and 4B and Table 1). Furthermore, the transmurality index was significantly lower in the fingolimod-treated animals (Figure 4C and Table 1). Importantly, the area at risk was similar between both groups (Figure 4D and 4E and Table 1). As a result, fingolimod treatment resulted in a 5-fold increase in myocardial salvage (Figure 4F and Table 1) compared with controls.

The differences in infarct size persisted at 1 month (Table 1). The reduction in infarct size at 1 month after MI was also confirmed by triphenyltetrazolium chloride staining (23.3±3.8% versus 29.7±4.9% of the LV for fingolimod and control pigs respectively; \( P = 0.02 \)). There was an excellent correlation between infarct size at 1 month quantified by late gadolinium enhancement and by triphenyltetrazolium chloride staining (\( r = 0.95, P = 0.01 \)), indicating the validity of our CMR data.

SIP-R Activation With Fingolimod Improved LV Systolic Function

Our CMR results show that the reduction in infarct size in the fingolimod group was accompanied by an increase in LV ejection fraction (LVEF) at both 1 week and 1 month after MI (Table 1 and Figure 5A), with a greater improvement in LVEF between the 2 time points (absolute change, 3.9±1.8% for fingolimod versus −0.2±2.2% for controls; \( P = 0.001 \); Figure 5B). Contractile reserve at 1 month determined by dobutamine CMR was also enhanced in the fingolimod animals compared with controls (absolute increase in LVEF, 12.0±5.6% versus 2.9±3.7%; \( P = 0.01 \); Figure 5C).
Figure 2. Fingolimod (FIN) treatment before reperfusion reduced myocardial apoptosis in the border zone of the ischemic myocardium 24 hours after myocardial infarction, which is mediated by activation of the reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways. A, Representative images of DAPI (nuclei in blue) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL; apoptotic nuclei in green) immunofluorescence. TUNEL-positive nuclei were quantified from merged (Continued)
Quantification of LVEF by 3D echocardiography showed results comparable to those of CMR, with no significant differences at baseline before MI but with significantly better LVEF in the fingolimod group at 1 week and 1 month (Table 2). Similar to the CMR data, LVEF in fingolimod-treated pigs improved further from 1 week to 1 month, whereas it remained unchanged in control pigs. Furthermore, LV mechanics assessed by 3D speckle-tracking echocardiography also demonstrated better preservation of longitudinal, radial, and circumferential strains in fingolimod-treated pigs (Figure 6 and Table 2).

Invasive hemodynamics further corroborated the superior LV systolic performance in the fingolimod group demonstrated by significantly higher peak LV pressure rate of rise (dP/dtmax), stroke work, contractility index, and LV end-systolic pressure than controls (Figure 5D–5F and Table 3).

**SIP-R Activation With Fingolimod Reduced Adverse Post-MI LV Remodeling 1 Month After MI**

The administration of fingolimod mitigated adverse LV remodeling. CMR-determined LV mass was significantly lower in fingolimod animals at both 1 week and 1 month after the MI induction (Table 1 and Figure 7A). Fingolimod-treated animals also exhibited a lower echocardiography-calculated relative wall thickness index 1 month after MI (0.34±0.03 versus 0.40±0.05; P=0.03), which supports lower compensatory hypertrophy in the treatment group.

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**Figure 2 Continued.** Images. B, Quantification of TUNEL-positive nuclei demonstrated a significant reduction in apoptotic cells in fingolimod-treated pigs. C, Triphenyltetrazolium chloride (TTC)-determined infarct size was smaller in fingolimod-treated pigs than in control pigs. D and E, Western blot analysis revealed less activation of proapoptotic proteins (cleaved caspase-3, phosphorylated [p]-p53), more activation of antiapoptotic proteins (Bcl-2, p-protein kinase Cε [PKC-ε]), and increased activation of both the RISK (p-Akt, p-extracellular receptor kinase [ERK], p-glycogen synthase kinase 3-β [GSK3-β]), and SAFE (p-signal transducer and activator of transcription 3 [STAT3]) pathways in the ischemic myocardium of fingolimod-treated pigs. Representative Western blot images are shown in D; quantification is shown in E. WORT indicates wortmannin. *indicates P<0.05.
A trend for reduced LV volumes in the fingolimod-treated group was noted with both CMR and 3D echocardiography that reached statistical significance for LV end-systolic volume at 1 month (Tables 1 and 2 and Figure 7B). This finding demonstrates lower LV dilatation in the fingolimod-treated animals. Furthermore, LV geometry measured by the LV sphericity index was also more preserved in fingolimod-treated animals (1.61±0.11 versus 1.38±0.13, respectively; \(P=0.02\); Figure 7C and 7D), thus demonstrating decreased architectural changes in LV geometry in the treatment arm.

These anatomic changes were accompanied by a reduction in plasma metanephrine levels (285.1±366.8 versus 774.2±158.4 pg/mL for fingolimod and control pigs, respectively; \(P=0.02\); Figure 7E), which indicates less sympathetic neurohormonal activation in fingolimod-treated animals. Similarly, the serum aldosterone concentration was lower in the fingolimod group (46.4±29.9 versus 117.1±61.7 pg/mL; \(P=0.03\); Figure 7F).

**Figure 4.** Fingolimod treatment reduced infarct size and improved myocardial salvage 1 week after myocardial infarction. A, Representative cardiac magnetic resonance (CMR) short-axis image for late gadolinium enhancement (LGE). Hyperintense (white) areas by LGE depict infarcted myocardium. B, LGE-derived infarct size was significantly smaller in fingolimod-treated pigs. C, Transmurality index was also significantly smaller in fingolimod-treated animals. D, Representative CMR short-axis image of T2-weighted spin-echo images; hyperintense (white) areas indicate the presence of edema, thereby identifying area at risk (AAR). E, The AAR was similar between both groups. F, Sphingosine-1-phosphate receptor activation with fingolimod significantly improved myocardial salvage.

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<tr>
<th>Table 1. CMR Results 1 Week and 1 Month After MI</th>
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<td>Infarct size (LGE), % of LV</td>
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<td>Transmurality index</td>
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<td>Area at risk, % of LV</td>
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<td>Salvaged myocardium, % of AAR</td>
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<td>LV ejection fraction, %</td>
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<td>LV stroke volume, mL</td>
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AAR indicates area at risk; CMR, cardiac magnetic resonance; LGE, late gadolinium enhancement; LV, left ventricular; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; and MI, myocardial infarction.
Amelioration of adverse remodeling was found by histological analysis of the remote, noninfarcted myocardium. Picrosirius Red staining demonstrated a reduction in collagen deposition in the interstitial myocardium in fingolimod-treated pigs compared with controls (8.1±0.7% versus 9.6±1.6% of myocardium, respectively; P=0.03; Figure 8A–8C). Furthermore, fingolimod-treated animals exhibited smaller cardiomyocyte perimeters (156.5±17.9 μm versus 176.0±7.9 μm; P=0.03) as measured by vinculin immunohistochemistry (Figure 8D–F). This indicates less compensatory hypertrophy in the fingolimod-treated group on a cellular level.

Cardiomyocyte hypertrophy in the setting of LV remodeling is mediated by activation of Akt and ERK1/2. Consistent with the results of our LV mass measurements by CMR and our histomorphometric analysis, S1P-R activation with fingolimod improved LV mass by cardiac magnetic resonance (CMR; A–C) and invasive hemodynamics (at 1 month after MI; D–F). Fingolimod-treated pigs showed improved LV systolic function compared with controls. A, LV ejection fraction (LVEF) at 1 week and 1 month after MI. B, Recovery of LVEF from 1 week to 1 month after MI. C, Contractile reserve assessed by dobutamine CMR at 1 month after MI. D, Peak LV pressure rate of rise (dP/dt max). E, Contractility index. F, Stroke work.

| Table 2. Three-Dimensional Echocardiography Results 1 Week and 1 Month After MI |
|------------------|------------------|---------------------|
|                  | Control          | Fingolimod         | P Value |
| LVEF at baseline, % | 71.59±3.03       | 71.50±1.22         | 0.95    |
| LVEF at 1 wk, %     | 33.02±3.99       | 40.34±4.61         | 0.01    |
| LVEF at 1 mo, %     | 34.24±5.26       | 44.71±6.96         | 0.008   |
| LVEDV at baseline, mL | 43.81±4.38     | 40.97±5.90         | 0.33    |
| LVEDV at 1 wk, mL   | 66.96±5.23       | 62.32±7.01         | 0.18    |
| LVEDV at 1 mo, mL   | 98.02±19.44      | 81.03±15.39        | 0.09    |
| LVESV at baseline, mL | 12.4±1.15      | 11.67±1.73         | 0.37    |
| LVESV at 1 wk, mL   | 45.06±7.25       | 37.30±5.29         | 0.07    |
| LVESV at 1 mo, mL   | 65.27±17.35      | 45.33±12.64        | 0.02    |
| Peak global longitudinal strain at baseline, % | −21.70±1.22 | −22.03±2.72 | 0.77 |
| Peak global longitudinal strain at 1 wk, %     | −9.38±2.31       | −14.43±1.63        | 0.0005  |
| Peak global longitudinal strain at 1 mo, %     | −11.35±1.70      | −16.34±1.95        | 0.0002  |
| Peak global circumferential strain at baseline, % | −27.18±4.08   | −24.5±5.3          | 0.37    |
| Peak global circumferential strain at 1 wk, %   | −10.85±2.24      | −17.06±4.88        | 0.01    |
| Peak global circumferential strain at 1 mo, %   | −11.86±3.81      | −17.46±2.96        | 0.001   |
| Peak global radial strain at baseline, %        | 128.24±37.93     | 120.21±31.41       | 0.67    |
| Peak global radial strain at 1 wk, %            | 29.68±8.02       | 55.95±18.64        | 0.005   |
| Peak global radial strain at 1 mo, %            | 32.77±10.59      | 62.51±20.45        | 0.005   |

LVEDV indicates left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESV, left ventricular end-systolic volume; and MI, myocardial infarction.
fingolimod resulted in a significant reduction in the activation of Akt and ERK1/2 compared with controls (Figure 8G–8I and Figure II in the online-only Data Supplement).

Discussion
This study examined the cardioprotective effects of the S1P-R agonist fingolimod in a porcine model of myocardial I/R. The main finding of our study is that S1P-R activation with fingolimod during acute MI leads to significantly increased myocardial salvage, reduced infarct size, and improved systolic LV function. The underlying mechanism for the decrease in infarct size is a reduction in cardiomyocyte apoptosis and oxidative stress mediated by activation of the SAFE and RISK pathways. Importantly, treatment with fingolimod mitigated adverse post-MI LV remodeling at both a macroscopic and microscopic level. To the best of our knowledge, this in vivo study is the first to indicate a cardioprotective effect of the S1P-R agonist fingolimod after MI in a large-animal model.

Evidence from previous preclinical studies suggests that S1P represents a promising pharmacological target to mitigate myocardial I/R injury. In both neonatal12 and adult13 rat ventricular cardiomyocytes, S1P enhanced cardiomyocyte survival in vitro during hypoxia. S1P also induced resistance to I/R injury in ex vivo isolated wild-type mouse14 and rat15 hearts. Consistent with this, the hearts of sphingosine kinase–null mice, which lack the key enzyme for S1P synthesis, showed more severe I/R injury than control hearts,26,27 whereas adenoviral gene transfer of sphingosine kinase protected against I/R injury.16 Similarly, knockout mice for S1P-R exhibited larger infarct size than control mice.17,28 Interestingly, S1P metabolism is a key mediator in preconditioning and postconditioning, 2 established strategies for cardioprotection. In fact, preconditioning and postconditioning reduced MI size in wild-type hearts, but neither was cardioprotective in sphingosine kinase– or S1P-R–null hearts.15,26,27,29 Together, these results point to a promising role of S1P in preventing I/R injury. We decided to use fingolimod in our studies because it is the only S1P-R agonist currently approved for clinical use, which could therefore facilitate the translation of our results into clinical practice.

Opening of the mitochondrial permeability transition pore is the final step in apoptosis in I/R injury and triggers oxidative stress. Consequently, prevention of mitochondrial permeability transition pore opening reduces infarct size.30,31 The main molecular pathways inhibiting mitochondrial permeability
transition pore opening are the RISK (Akt/ERK/GSK3-β) and SAFE (Janus kinase/STAT3) signaling cascades,\textsuperscript{3,25,32} and previous in vitro data suggest that S1P can activate them.\textsuperscript{15,21,33} Our results demonstrate that fingolimod induced significantly greater phosphorylation of Akt, ERK1/2, GSK3-β, and STAT3 24 hours after MI compared with controls. Therefore, in a porcine model of I/R, fingolimod activated both the RISK and SAFE pathways in the acute phase of MI. Consistent with this, fingolimod-treated pigs demonstrated a reduction in apoptosis, as shown by decreased activation of the proapoptotic proteins caspase-3 and p53, a reduction in terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive cells, and an increase in the prosurvival Bcl-2 and protein kinase C-ε proteins at 24 hours after MI. Furthermore, fingolimod treatment also results in reduced oxidative stress. Specifically, our results show that fingolimod decreased 8-hydroxydeoxyguanosine, a marker of nuclear oxidative stress, and enhanced the activity of the antioxidant enzyme superoxide dismutase. Importantly, the antiapoptotic effects and the reduction in oxidative stress as a result of S1P-R activation were abolished by the RISK inhibitor wortmannin and the SAFE inhibitor AG490, which indicates that the cardioprotective actions of fingolimod are mediated by both the RISK and SAFE pathways.

Previous studies have demonstrated benefits of immunosuppression in the prevention of I/R injury.\textsuperscript{34} Interestingly, fingolimod is an immunomodulator that regulates lymphocyte trafficking from blood and peripheral tissues to lymph nodes and reduces the egress of lymphocytes from the lymph nodes.\textsuperscript{5,7,18} In this context,

Table 3. Hemodynamic Data by Micromanometer Conductance Catheterization 1 Month After MI

<table>
<thead>
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<th>Metric</th>
<th>Control</th>
<th>Fingolimod</th>
<th>P Value</th>
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<tr>
<td>End-systolic pressure, mmHg</td>
<td>95.00±10.88</td>
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<td>dP/dtmax, mmHg/s</td>
<td>1056.15±261.03</td>
<td>1865.88±147.46</td>
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<td>Contractility index, 1/s</td>
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<td>19.06±3.11</td>
<td>0.002</td>
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<tr>
<td>Stroke work</td>
<td>2395.67±401.80</td>
<td>3423.91±799.46</td>
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\(dP/dtmax\) indicates peak pressure rate of rise; and MI, myocardial infarction.

Figure 7. Fingolimod treatment mitigated anatomic post–myocardial infarction (MI) left ventricular (LV) remodeling. A, Fingolimod treatment led to significantly lower LV mass measured by cardiac magnetic resonance, indicating less compensatory hypertrophy. B, Fingolimod-treated animals presented with lower LV end-systolic volume (LVESV) and a trend toward reduced LV end-diastolic volume (LVEDV) compared with the control group. C, The sphericity index was calculated as the ratio of LV length to LV diameter measured in the 4-chamber view on 2-dimensional echocardiography. D, Fingolimod-treated animals showed significantly less deformation in the LV shape after MI as indicated by a higher sphericity index. E, Fingolimod-treated animals presented with lower plasma metanephrine levels, thus indicating less sympathetic overdrive. F, Fingolimod-treated animals presented with lower plasma aldosterone levels.
a recent study using a murine model of spontaneous obstructive coronary atherosclerosis demonstrated improved mortality and reduced infarct size ex vivo with fingolimod treatment.35 The authors found that fingolimod reduced CD4 and CD8 cells and increased regulatory T cells, which suggests that the immunosuppressive effects of fingolimod contribute to its cardioprotective properties. Our study did not address the effect of fingolimod on the immune system; therefore, we are unable to comment on its contribution to improved myocardial salvage in our animals. Nonetheless, the impact of the immunomodulatory and anti-inflammatory effects of S1P-R activation on cardioprotection warrants further investigation.

Limiting MI size is of utmost clinical importance because it is a key determinant of morbidity and mortality. Infarct size has been related to outcomes in patients with ST-segment-elevation MI and is an independent predictor of impaired cardiovascular prognosis, even after accounting for LVEF.36,37 In our study, both CMR and postmortem triphenyltetrazolium chloride staining showed a significant reduction in MI size in the fingolimod group. One previous study in rats found no difference in MI size in animals treated with fingolimod,38 but differences in study design and animal models most likely explain the disparity in outcomes. Because some variation in coronary anatomy and thus MI size among animals is

Figure 8. Fingolimod treatment reduced histological and molecular left ventricular (LV) remodeling in the remote noninfarcted LV myocardium 1 month after myocardial infarction. A through C, Measurement of diffuse interstitial myocardial fibrosis. Representative images of Picosirius Red staining in bright-field microscopy (A and B; collagen in red, cardiomyocytes in yellow) of a fingolimod-treated pig (A) and a control pig (B). C, Fingolimod-treated pigs showed significantly less interstitial fibrosis in the remote noninfarcted myocardium than controls. D through F, Measurement of cardiomyocyte size. Representative images of vinculin immunohistochemistry (cardiomyocyte membrane in green, DAPI for nuclei in blue) depicting the cardiomyocyte size of fingolimod-treated (D) and control (E) animals. F, Fingolimod-treated pigs showed significantly less cardiomyocyte hypertrophy than controls. G through I, Assessment of the activation of extracellular receptor kinase (ERK) 1/2 and Akt. Western blot for phosphorylated (p) and total ERK1/2 and Akt (G). Fingolimod-treated animals had significantly less activation (phosphorylation) of ERK1/2 (H) and Akt (I) than controls.
always present, we calculated the degree of myocardial salvage, which takes the actual myocardium at risk into account. Furthermore, myocardial salvage has been reported to independently predict adverse cardiovascular outcome in patients, even after adjustment for infarct size.\textsuperscript{39,40} Importantly, we found that myocardial salvage was nearly 5-fold greater in fingolimod-treated pigs than in control animals.

The reduction in infarct size with fingolimod was paralleled by an improvement in LV systolic performance. Fingolimod-treated pigs showed higher LVEF at 1 week and 1 month after MI, enhanced recovery of LVEF from 1 week to 1 month, and significantly greater contractile reserve than in controls. These findings are important because both better LVEF and the presence of contractile reserve are associated with improved outcomes.\textsuperscript{36,41} Although LVEF is widely available and is the most commonly used index of LV systolic function, it presents several known caveats, including its load dependency. Thus, we measured strain echocardiography, which allows a more detailed evaluation of myocardial mechanics. In fact, strain imaging predicts clinical outcomes better than LVEF in patients with previous MI.\textsuperscript{42,43} Our data clearly indicate that fingolimod treatment during acute MI improved 3D longitudinal, 3D circumferential, and 3D radial strains compared with controls. The improvement in LV systolic function was also supported by the results of our invasive hemodynamic measurements.

Chronic systolic heart failure and post-MI adverse LV remodeling are also important determinants of patient morbidity and long-term outcomes.\textsuperscript{44} Our data are the first to demonstrate that fingolimod not only reduces I/R injury and decreases MI size but also mitigates the development of ischemic cardiomyopathy and adverse cardiac remodeling after MI. The anatomic features of post-MI adverse LV remodeling include dilatation, compensatory hypertrophy, and a change in sphericity. In our study, the hearts of fingolimod-treated pigs dilated less than those of controls. Specifically, LV end-systolic volume, a strong predictor of adverse outcomes even after adjustment for LVEF and MI size,\textsuperscript{45,46} was significantly smaller in the fingolimod-treated group.

Hypertrophy of the remote noninfarcted myocardium develops as an initially important compensatory mechanism, but prolonged cardiac hypertrophy with a subsequent increase in LV mass is a maladaptive process associated with worse outcomes.\textsuperscript{37} The cardioprotective effects of fingolimod were associated with significantly lower LV mass after MI, suggesting a reduced need for this compensatory mechanism. The reduced hypertrophy was confirmed at the histological level, demonstrating smaller cardiomyocytes in fingolimod-treated pigs. Furthermore, interstitial fibrosis in the remote nonischemic myocardium, another histological trademark of LV remodeling,\textsuperscript{34,45} was also decreased by S1P-R agonism.

Akt and ERK1/2 signaling are important stimulants for cardiomyocyte growth and survival. Although short-term activation of Akt and ERK1/2 results in reduced apoptosis, long-term activation of Akt and ERK1/2 leads to hypertrophy. The latter is one of the key molecular features of adverse LV remodeling and is associated with detrimental effects.\textsuperscript{49,50} In our studies, phosphorylation of Akt and ERK1/2 was increased in the ischemic border zone 24 hours after MI in fingolimod-treated pigs, which is a signal of cardioprotection in that setting. In contrast, the phosphorylation of Akt and ERK1/2 was reduced in the remote noninfarcted myocardium in fingolimod-treated pigs 1 month after MI, thus heralding less remodeling. Taken together, these findings support enhanced prosurvival signaling mediated by S1P-R in the acute phase and reduced adverse remodeling in later stages.

Finally, long-term activation of the neurohormonal response, especially of the sympathetic nervous system and the renin-angiotensin-aldosterone system, is a major molecular hallmark of adverse LV remodeling. In fact, the levels of both plasma catecholamines\textsuperscript{51} and aldosterone\textsuperscript{52} are important predictors of cardiovascular mortality in post-MI patients. In the present study, we focused on degradation products of plasma catecholamines, that is, metanephrines, because they offer higher stability and reflect long-term neurohormonal activity.\textsuperscript{53} Interestingly, circulating plasma levels of metanephrines and aldosterone were significantly reduced in the fingolimod-treated group. These findings suggest an interruption of the pathological neurohormonal cycle and are consistent with the observed amelioration in LV remodeling in fingolimod-treated pigs.

One of the main strengths of our study is the use of an in vivo large-animal model. The pig heart with regional myocardial ischemia and reperfusion is of unique translational value.\textsuperscript{54} Thus far, all available evidence for the involvement of S1P and fingolimod in cardioprotection has been derived from in vitro studies or rodent models. Rodents, however, differ significantly from larger mammals in heart rate and temporal and spatial infarct development, and they may even vary in their preferential cardioprotective signaling.\textsuperscript{55} Therefore, the translation of findings for cardioprotection from rodent to larger mammal hearts is not trivial and cannot be taken for granted.

Limitations

First, coronary artery disease in patients is frequently associated with comorbidities such as hypertension, diabetes mellitus, and hyperlipidemia, which are not reflected in this animal model. Second, we do not know how strong the effects of S1P-R on cardiac remodeling would be on the background of standard post-MI medical therapy. Furthermore, our study did not examine whether the ability of fingolimod to improve LV systolic function and to prevent post-MI remodeling would be further enhanced with long-term S1P-R activation. In our long-term experiments, fingolimod was given for only 3 days; therefore, the initial reduction in infarct size most likely explains the observed improvement in systolic function and reduction in adverse remodeling. Further studies are needed to specifically address this issue. It is also worth noting that we did not include a sham MI group. A sham group would have allowed us to differentiate between the changes in LV volumes over time caused by animal growth and those changes resulting from post-MI LV remodeling. However, the direct comparison of fingolimod-treated pigs with nontreated animals allowed us to assess the effects of fingolimod on infarct size, myocardial function, and LV remodeling, mimicking the experimental conditions of a clinical scenario in MI patients. Accordingly, we believe that the investigation of S1P-R activation in patients with acute MI is an important next step to further evaluate the potential clinical efficacy of this treatment strategy.
Conclusions
Our data support the concept that pharmacological activation of S1P-R with fingolimod can reduce the detrimental effects of acute MI in an experimental setting. Fingolimod treatment improved myocardial salvage and reduced infarct size, leading to a robust improvement in systolic LV function, enhanced intrinsic LV mechanics, and mitigation of LV remodeling in a large-animal model. These cardioprotective effects of fingolimod in the acute setting are mediated by activation of both the SAFE and RISK pathways, resulting in a reduction of myocardial apoptosis and oxidative stress. Our findings highlight the therapeutic potential of S1P-R signaling in the peri-infarct period and warrant further investigations.

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Disclosures
None.

References


Acute myocardial infarction (MI) remains an important clinical problem, and early reperfusion by percutaneous coronary intervention is currently the most successful treatment to limit infarct size. Unfortunately, reperfusion is frequently insufficient to achieve adequate myocardial salvage, and paradoxically, reperfusion in itself can induce cardiomyocyte injury, increase oxidative stress, and trigger apoptosis, a phenomenon called ischemia/reperfusion injury. Therefore, novel pharmacological interventions are needed to further reduce infarct size, to preserve left ventricular function, and to ameliorate adverse post-MI remodeling.

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**Clinical Perspective**

Acute myocardial infarction (MI) remains an important clinical problem, and early reperfusion by percutaneous coronary intervention is currently the most successful treatment to limit infarct size. Unfortunately, reperfusion is frequently insufficient to achieve adequate myocardial salvage, and paradoxically, reperfusion in itself can induce cardiomyocyte injury, increase oxidative stress, and trigger apoptosis, a phenomenon called ischemia/reperfusion injury. Therefore, novel pharmacological interventions are needed to further reduce infarct size, to preserve left ventricular function, and to ameliorate adverse post-MI remodeling.

Fingolimod, a sphingosine-1-phosphate receptor agonist, is used for the treatment of multiple sclerosis and exerts antipapoptotic properties. In our experiments using a porcine model of ischemia/reperfusion, sphingosine-1-phosphate receptor activation with fingolimod during acute MI significantly increased myocardial salvage, reduced infarct size, and improved systolic left ventricular function. We demonstrated that the underlying mechanism for the decrease in infarct size is a reduction in cardiomyocyte stress, and trigger apoptosis, a phenomenon called ischemia/reperfusion injury. Therefore, novel pharmacological interventions are needed to further reduce infarct size, to preserve left ventricular function, and to ameliorate adverse post-MI remodeling.

Fingolimod during acute MI significantly increased myocardial salvage, reduced infarct size, and improved systolic left ventricular function. We demonstrated that the underlying mechanism for the decrease in infarct size is a reduction in cardiomyocyte stress, and trigger apoptosis, a phenomenon called ischemia/reperfusion injury.
Sphingosine-1-Phosphate Receptor Agonist Fingolimod Increases Myocardial Salvage and Decreases Adverse Postinfarction Left Ventricular Remodeling in a Porcine Model of Ischemia/Reperfusion

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS:

Yorkshire pigs (weight 25 kilogram) were housed in AAALAC accredited facilities, and all procedures were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee. All experiments were performed in anesthetized animals.

Acute Myocardial Infarction

Twelve hours prior to the induction of the myocardial infarction (MI), all animals received 150 mg of clopidogrel orally. On the day of the infarction anesthesia was induced with telazol (6 mg/kg i.m.), and buprenorphine (0.1 mg/kg i.m.) was administered for pain control. Anesthesia maintenance was achieved by intravenous administration of propofol (10 mg/kg/hr). The pigs were premedicated with heparin 3000 units IV, amiodarone 75 mg IV, and atropine (0.04 mg/kg). Normal saline solution was given as a 20 mL/kg bolus followed by a continuous infusion of 2 ml/kg/hr. The maintenance fluids also contained 75 mg amiodarone, 10 mEq potassium chloride and atropine (0.04 mg/kg).

MI was induced as previously described by our group\textsuperscript{1-3}. Arterial access was obtained through the common femoral artery by percutaneous puncture. A 7.5 French arterial sheath was placed in Seldinger technique. A 7 French hockey-stick coronary guide catheter was then advanced into the ascending aorta and the left main coronary artery was engaged. A baseline coronary angiogram was performed. Under fluoroscopy guidance a coronary guidewire was advanced into the LAD over which a coronary balloon dilatation catheter (TREK OTW
Coronary Dilation Catheter 4.0 x 15 mm, Abbot Vascular) was then placed in the proximal LAD prior to the first septal perforator branch. Subsequently, acute MI was induced by occlusion of the proximal LAD for 60 minutes via inflation of the coronary dilation balloon to 10 atm. The animals were connected to an EKG and invasive blood pressure monitoring system throughout the ischemia period. Close attention was paid to early detection of ventricular arrhythmias in order to provide immediate defibrillation. After 60 minutes of total ischemia, the balloon was deflated and removed from the LAD. Patency and reperfusion of the LAD were documented with a final coronary angiogram. The arterial sheath was then removed and manual pressure was applied for 15-20 min to obtain hemostasis. The post-procedural care included cephalosporin (25 mg/kg), buprenorphine (0.1 mg/kg), and normal saline containing amiodarone (75 mg/500 ml) infused at 50 mL/hr for 12 hours.

Study medications and study groups

**Protocol 1** (Figure 1, Main Manuscript): We studied four groups of animals:

1) Fifteen minutes before reperfusion, fingolimod (Selleckchem, Houston, TX) at the dose of 1mg/Kg (as previously reported\(^4-7\)) was administered intravenously (diluted in 5mL of saline and administered in a one-minute infusion) to the treatment group.

2) The control animals received saline (vehicle, 5mL) intravenously at the same timepoint.

3) An additional group of pigs received concomitant administration of fingolimod (same protocol as previously) and wortmannin (inhibitor of the RISK pathway). Wortmannin was administered at a dose of 20μg/Kg as previously reported\(^8,9\), diluted in 5mL of saline, and administered in a one-minute IV infusion at 40 minutes of occlusion (ie. five minutes before fingolimod administration).
4) A fourth group of animals received concomitant administration of fingolimod (same protocol as previously) and AG490 (inhibitor of the SAFE pathway). AG490 was administered intracoronarily at a dose of 9μg/kg/min starting 10 minutes before ischemia, with 1 mg/kg total dose, as previously reported\textsuperscript{10,11}.

MI was induced in twenty two initial pigs (7 control, 5 FIN, 6 FIN+AG490, 4 FIN+wortmannin). However, two control pigs and two pigs of the FIN+AG490 group died within a few hours of MI of ventricular fibrillation. Therefore, the final number of analyzed animals 24 hours post-MI was 5, 5, 4, and 4, respectively.

**Protocol 2** (Figure 1, Main Manuscript): Animals were randomized to:

1) **Fingolimod treatment group**: Fifteen minutes before reperfusion, fingolimod at the dose of 1mg/Kg (as previously reported\textsuperscript{4,7}) was administered intravenously (diluted in 5mL of saline and administered in a one-minute infusion) to the treatment group. Additionally, fingolimod treatment (1mg/Kg) was continued once daily for three days.

2) **Control group**: The control animals received a saline (vehicle) injection at that time point at the same timepoint.

Twenty additional animals underwent MI induction, and fourteen animals survived the procedure (seven in each group). Six animals died from refractory ventricular arrhythmias within 45 minutes of LAD occlusion and did not receive any treatment.

**Euthanasia**

Within one hour upon completion of the imaging procedures, the animals were euthanized by intravenous injection of potassium chloride (20mEq through a femoral venous sheath). Then the hearts were removed, and the LV was cut along the short axis into 7 slabs of
the same thickness. Tissue samples for histopathological validation were collected immediately from one of the slides, at the level of the papillary muscle insertion. Specifically, samples were taken from the remote myocardium, the border zone and the scar, and they were snap frozen in liquid nitrogen for molecular studies or embedded in the OCT medium for histological studies. We visualized the final infarct size by staining the remaining six slices with 2,3,5-triphenyltetrazolium chloride. High-resolution digital images from all slices were acquired, and areas of infarction (stained in white) and of normal myocardium (stained in brick-red) were blindly quantified with ImageJ software (National Institutes of Health, Bethesda, MD). The MI volume was expressed as a percentage of the total LV myocardium.

**Cardiac Magnetic Resonance Imaging (CMR)**

CMR studies were performed with a 3.0 Tesla magnet (Achieva, Philips Medical Systems, Netherlands). Steady-state free precession short axis images (TR 3.6 ms, TE 1.6 ms, flip angle 45°, field of view 250 x 250 mm, SENSE factor 3, voxel size 1 x 1 x 5 mm, no gap, number of averages 3, bandwidth 1286 Hz, 12 lines per segment) from the LV base through LV apex were used for the quantification of LV volumes, ejection fraction and mass. Subsequently, a T2-weighted, short tau inversion recovery sequence (STIR, TR 2-3 heartbeats, TE 60 ms, time-interval 210 ms, field-of-view 250 x 250 mm, SENSE factor 3, voxel size 1 x 1 x 5 mm, number of averages 3, bandwidth 926 Hz, echo-train length 16) was used for edema assessment to measure the area at risk. Late gadolinium enhancement (LGE) was performed 15 minutes after the administration of gadolinium (Magnevist, 0.2 mmol/kg) using an inversion-recovery fast gradient echo sequence (TR 9 ms, TE 5.4 ms, TI optimized to null normal myocardium, gating factor 3, field-of-view 250 x 250 mm, pixel size 1 x 1 x 5 mm, SENSE factor 3, number of
averages 3, bandwidth 232 Hz, TFE factor 16). After LGE, dobutamine was infused IV and SSFP cine imaging was repeated for assessment of contractile reserve.

All CMR images were blindly analyzed using commercially available software (Extended MR Workspace, Philips Medical Systems, Netherlands). Epicardial and endocardial contours were traced in each SSFP cine image to obtain LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), LV ejection fraction (LVEF) and LV mass; by convention, papillary muscles were included in the LV cavity. For assessment of area at risk, endocardial and epicardial borders were manually traced in each contiguous short-axis view. The area at risk was defined as the hyperintense area on T2-weighted images. A myocardial area was regarded as hyperintense when the signal intensity was higher than 3 standard deviations of the signal intensity in remote, normal myocardium\(^1,2\). Care was taken to exclude slow flowing blood within the trabeculae. LV scar size was measured by LGE and expressed as a percentage of the LV mass; the absolute MI size also was quantified in grams (calculated as volume multiplied by myocardial density \([1.05 \text{ g/cm}^3]\)). Infarct was defined as myocardium with signal intensity was higher than 3 standard deviations of that in remote, normal myocardium\(^1,2,13\). The LV was divided into 16 segments and each segment was assigned a score from zero to four, accounting for the percentage of the myocardium that was scarred (0 – no scar, 1 – less than 25% scar, 2 – 25 to 50% of scar, 3 – 50 to 75% of scar, 4 – more than 75% of scar). The final score (scar transmurality index) was calculated as the sum of all 16 segments. The salvaged myocardium was calculated as the difference between area at risk and LGE-derived infarct size. The salvaged myocardium index was calculated as \([\text{Area at risk (g)} – \text{infarct size (g)}] / \text{Area at risk (g)}\)\(^1,14,15\), and expressed as a percentage of the LV myocardium.
**Echocardiography**

The animals were maintained under anesthesia with a continuous propofol infusion (10 mg/kg/hr) throughout the echocardiogram. 2D-echocardiography studies were performed with an iE33 system with a S5 transducer (Philips Medical Systems, Andover, MA), according to the recommendations of the American Society of Echocardiography/European Society of Echocardiography\(^\text{16,17}\). Image analysis, measurements, and quantifications were performed directly on the iE33 system. Full volume 3D-echocardiographic cine images were acquired with the same echocardiography system using an X3 transducer (Philips Medical Systems, Andover, MA). Special care was taken to include the entire LV cavity within the pyramidal scan volume. The imaging settings were optimized for endocardial visualization, and at least five data sets were acquired and stored digitally for offline analysis. Subsequently, images were blindly analyzed using the commercially available Q-Lab software (Philips Medical Systems, Andover, MA). The 3D endocardial surface was semi-automatically reconstructed and tracked in 3D space throughout the cardiac cycle. Subsequently, the endocardial surface was manually adjusted as necessary.

To assess global LV deformation, we performed blind 3D speckle tracking analysis utilizing 4D LV-Analysis software (TomTec Imaging Systems, Germany). Apical two-chamber, four-chamber, and short-axis views at different levels of the LV were automatically selected at end-diastole. Non-foreshortened apical views were identified by finding the largest long-axis dimensions. The 3D endocardial surface was automatically reconstructed and tracked in 3D space throughout the cardiac cycle. Subsequently, the endocardial surface was manually adjusted when necessary. The LV was automatically divided into 16 three-dimensional segments using standard segmentation. Global 3D longitudinal (LS), circumferential (CS), and radial strain (RS)
were measured and averaged over the 16 segments. In our model, the intra and interobserver variability obtained for 3D-strains was as follows: 3D-LS 6.4±4.3% and 8.3±7.2%; 3D-CS 7.3±6.9% and 9.8±6.9%; 3D-RS 8.3±5.9% and 10.4±7.9%. This variability is well within the range of previously reported data. The intraobserver intraclass correlation coefficients for 3D-LS, 3D-CS and 3D-RS were 0.96 (CI 95%: 0.88-0.99), 0.95 (CI 95%: 0.88-0.98) and 0.94 (CI 95%: 0.85-0.98), respectively. The corresponding interobserver intraclass correlation coefficients for 3D-LS, 3D-CS and 3D-RS were 0.92 (CI 95%: 0.80-0.97), 0.90 (CI 95%: 0.79-0.96) and 0.89 (CI 95%: 0.71-0.96), respectively.

The LV sphericity index was calculated as the ratio of major LV End-Dyastolic Diameter (LVEDD)/minor LVEDD in apical 4-chamber view. Relative wall thickness was calculated as 2 x Posterior Wall Thickness / LVEDD.

**Micromanometer Conductance Catheterization**

LV invasive PV loops were obtained at one month post-MI, immediately before sacrifice, to assess hemodynamic differences between both groups. For hemodynamic catheterization, we accessed the femoral artery with 8F sheath. A 7F Millar Micro-Tip catheter system (Millar Instruments Inc., Houston, TX) was zeroed and balanced in warm saline and guided to the LV apex into the aorta and the left ventricle (LV). Steady-state PV loops were acquired during a short breath hold. We determined the following parameters: systolic pressure, end-diastolic pressure, peak LV pressure rate of rise (dP/dt)max, and stroke work; the contractility index was calculated as (dP/dt)max/(systolic – end-diastolic pressure). MPVS Ultra (Millar Instruments) was used to acquire analog data and convert it to digital data. Data analysis was performed using
iox2 (Emka Technologies, Falls Church, VA). The mean of at least 3 consecutive cardiac cycles was calculated for each measurement.

**Plasma levels of metanephrines and aldosterone**

One month post-MI, plasma samples were obtained 15 minutes after insertion of the arterial sheath, when the animal was intubated under no current pain and in a steady state regarding anesthesia. As previously reported, plasma metanephrines and aldosterone concentrations were analyzed by enzyme immunoassay (EIA) (Rocky Mountain Diagnostics, Colorado Springs, Co).

**Apoptosis, oxidative stress, and cardioprotective pathways**

Heart samples obtained 24 hours post-MI were embedded in OCT, cut in 8-µm cryosections and used to study apoptosis and oxidative stress in the ischemic border area. Apoptosis was detected by a TUNEL-based apoptosis detection kit, following manufacture instructions (ApopTag® Fluorescein In situ Apoptosis Detection Kit, Millipore). Then, a mounting medium containing DAPI was used for nuclei counterstain. The percentage of TUNEL-positive nuclei relative to total nuclei was determined in a blinded manner by counting on ten randomly chosen 40x fields per coverslip for each myocardium.

Frozen tissue was crushed and homogenized in RIPA buffer (Sigma-Aldrich) with complete protease inhibitor (1 tablet per 10 ml, Roche) and using the MP homogenate system (FastPrep homogenizer). After 20 minutes of centrifugation at 30,000 g, the insoluble portion was removed and the soluble portion was stored at -80°C. Aliquots containing 50 µg of total protein were diluted in 4x sample buffer (40% β-mercaptoethanol, 8% sodium dodecyl sulfate,
40% glycerol, 0.025% bromophenol blue, and 0.25 mmol/L Tris, pH 6.4), separated by electrophoresis on 12% polyacrylamide gel, transferred onto nitrocellulose membranes, and incubated with primary antibodies: anti-cleaved caspase-3, anti-total caspase-3, anti-phospho p53, anti-total p53, anti-Bcl-2, anti-phospho PKC-ε, anti-total PKC-ε, anti-phospho Akt, anti-total Akt, anti-phospho GSK-3β, anti-total GSK-3β, anti-phospho ERK1/2, anti-total ERK1/2, anti-phospho STAT3, and anti-total STAT3 at 1:1000 dilution (all from Cell Signaling) overnight at 4°C. Peroxidase-conjugated monoclonal secondary antibodies (Amersham) at 1:5000 dilution for one hour at room temperature were used for chemoluminescence development. Monoclonal GADPH antibody (Sigma-Aldrich) was used as a loading control. Bands were visualized with the ECL-Plus chemiluminescence system (Amersham), and autoradiography films were analyzed using the validated Image J software (National Institutes of Health, Bethesda, MD).

We assessed 8-hydroxydeoxyguanosine (8-OHdG) and superoxide dismutase (SOD) as markers of oxidative stress. 8-OHdG is one of the markers of DNA and RNA damage. Cardiac frozen OCT-sections were sliced (8-μm) and placed 15 minutes at room temperature, immersed in PBS-T and blocked (1% BSA and 1% goat serum in PBS) 20 and 30 minutes respectively. The sections were incubated with primary antibody (anti-8-OHd, Santa Cruz Biotechnology, Inc) at 1:100 dilution overnight at 4°C. The slices were rinsed with PBS for 10 minutes and incubated with secondary antibody (Alexa 488) at 1:200 dilution for 1 hour at room temperature. After rinse the sections 2x10 minutes, the slices were mounted with mounting media containing DAPI. The average nuclear fluorescence was measured in five 40X fields by Image Pro Plus. SOD catalyzes the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide as part of the cellular antioxidant mechanism. Total SOD activity in heart tissue
homogenates was determined by ELISA following manufacture instructions (Superoxide Dismutase Assay kit, Cayman Chemical Company).

**Histological and molecular assessment of LV remodeling**

Heart samples obtained one month post-MI in the remote non-ischemic myocardial tissue were embedded in OCT were cut in 8-µm cryosections and used to study both myocardial fibrosis and cardiomyocyte size, the main histological characteristics of adverse LV remodeling. To analyze the cardiomyocyte size, each sample was immunostained for vinculin (Sigma-Aldrich). Three sections of each sample were permeabilized with Triton 0.3% and blocked with BSA 5%. Vinculin antibody was incubated overnight at 4°C at a dilution of 1:100 and for 1 hour at room temperature with the secondary antibody, Alexa Fluor 488 Goat Anti-mouse dilution of 1:500 (Invitrogen). Sections were mounted with DAPI medium (Vector Lab) and digitally imaged (Zeiss Axioplan2 microscope and Zeiss AxioVision software, Micro-optik).

Cardiomyocyte area and size were quantified with Image J software. A minimum of 10 fields per section were recorded at x40 magnification.

For interstitial fibrosis quantification, three sections of each sample were stained with Picrosirius Red (Spectrum Chemical) according to manufacturer´s specifications. Picrosirius red images were recorded using polarized light by a Zeiss Axioplan2 microscope and Zeiss AxioVision software (Micro-optik). The area of myocardium positive for Picrosirius red was analyzed by quantitative morphometry using the validated software Image ProPlus 7.0 (Media Cybernetics Inc, Bethesda, MD).

The activation of the molecular pathways leading to cardiomyocyte hypertrophy was studied by Western blot as previously described. A piece of homogenized tissue from 1 month
post-MI remote LV was dissolved in cold RIPA lysis buffer (as previously described) and 50 μg of proteins separated on polyacrylamide gels, transferred to membranes and incubated with specific primary antibodies: incubated with primary antibodies: anti-phospho Akt, anti-total Akt, anti-phospho ERK1/2, and anti-total ERK1/2 at 1:1000 dilution (all from Cell Signaling) overnight at 4°C.
Supplementary Figure 1: Fingolimod treatment prior to reperfusion reduced myocardial apoptosis in the border zone of the ischemic myocardium 24 hours post-MI. Western blot analysis revealed less activation of pro-apoptotic proteins (cleaved caspase-3, p-p53), more
activation of antiapoptotic proteins (Bcl-2, p-PKC-ε) and increased activation of both the RISK (p-Akt, pERK, p-GSK3-β) and SAFE (p-STAT3) pathways in the ischemic myocardium of fingolimod-treated pigs compared with control animals.
Supplementary Figure 2: Fingolimod treatment reduced molecular LV remodeling in the remote non-infarcted LV myocardium one month post-MI. Western blot analysis revealed that fingolimod-treated animals had significantly less activation (phosphorylation) of ERK1/2 and Akt than controls.
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