RESEARCH PAPER

Pharmacological pre- and post-conditioning with the sphingosine-1-phosphate receptor modulator FTY720 after myocardial ischaemia–reperfusion

U Hofmann, K Hu, F Walter, N Burkard, G Ertl, J Bauersachs, O Ritter, S Frantz* and A Bonz*[†]

Department of Internal Medicine I, University Hospital Wuerzburg, Oberdürrbacherstrasse 6, Würzburg, Germany

Background and purpose: Our recent experiments demonstrated that the Sphingosine-1-phosphate (S1P) receptor agonist FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) improves recovery of function after myocardial ischaemia–reperfusion *ex vivo*. Therefore, we tested the hypothesis that pharmacological post-conditioning with FTY720 reduces infarct size after myocardial ischaemia–reperfusion *in vivo*.

Experimental approach: Myocardial ischaemia was induced in Wistar rats by ligation of the left coronary artery for 45 min. FTY720 (0.5 mg·kg^{-1}) was applied i.p. either once, before reperfusion, or twice, 24 h before myocardial ischaemia and before reperfusion. After 24 h reperfusion, we determined infarct size by triphenyltetrazolium chloride staining and granulocyte infiltration by immunohistochemistry. Tumour necrosis factor-α (TNF)-α concentration was determined by ELISA. S1P receptor expression was studied by Western blot. Calcium transients were evaluated in Indo-1-loaded cardiomyocytes.

Key results: In both groups, FTY720 significantly reduced lymphocyte count in peripheral blood. FTY720 treatment attenuated granulocyte infiltration and TNF-α protein expression in reperfused myocardium. However, both treatment regimens were not able to reduce infarct size. FTY720 increased mortality due to induction of fatal ventricular tachyarrhythmias when administered once before reperfusion, but protected against reperfusion arrhythmias when given 24 h prior to ischaemia. Pretreatment selectively down-regulated S1P$_1$ receptor expression within the myocardium. S1P receptor agonists did not induce calcium deregulation in cardiomyocytes.

Conclusions and implications: FTY720 applied during reperfusion did not reduce infarct size but increased mortality during myocardial ischaemia–reperfusion due to induction of arrhythmias. Pretreatment with FTY720 before ischaemia abrogated the deleterious pro-arrhythmic effects without reducing infarct size.
British Journal of Pharmacology (2010) **160,** 1243–1251; doi:10.1111/j.1476-5381.2010.00767.x

Introduction

With the finding that the same cardioprotective pathways described for preconditioning can effectively be induced by both ischaemic and pharmaceutical post-conditioning, there is now growing interest in developing pharmacological strategies to reduce infarct size and improve clinical outcome after reperfusion of acute myocardial infarction (Yellon and Downey, 2003). Recently, lysophospholipids and their receptors came into focus as therapeutic targets for improving tolerance to ischaemia and limiting reperfusion injury. Sphingosine-1-phosphate (S1P) is currently the best-characterized member of the growing family of lysophospholipid mediators. S1P is a physiological high-nanomolar constituent of serum and other extracellular fluids. S1P binds with low-nanomolar affinity to five related G-protein-coupled receptors S1P1–5 (An *et al.*, 1998; Jongsma *et al.*, 2009). S1P receptors are widely expressed on different cell types, and S1P affects multiple biological functions including cell migration, cytoskeletal organization, cell survival and differentiation

Correspondence: U Hofmann, Department of Internal Medicine I, University Hospital Wuerzburg, Oberdürrbacherstrasse 6, 97080 Würzburg, Germany. E-mail: hofmann_u2@medizin.uni-wuerzburg.de
*Both authors contributed equally.
[†]Current address: Center for Cardiology, Feldstr. 2a, 21335 Lüneburg, Germany.

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(Spiegel and Milstien, 2003). S1P receptors mediate a wide array of immunological effects, such as lymphocyte migration (Brinkmann *et al.*, 2004; Singer *et al.*, 2005; Chiba *et al.*, 2006), leukocyte adhesion and pro-inflammatory cytokine secretion (Bolick *et al.*, 2005; Theilmeier *et al.*, 2006; Whetzel *et al.*, 2006).

In the cardiovascular system, S1P was first characterized as modulator of endothelial cell function, such as vasopermeability (Gon *et al.*, 2005), vascular tone (Dantas *et al.*, 2003) and angiogenesis (English *et al.*, 2002). In cardiac myocytes, the S1P1 receptor is the predominant subtype expressed and the activation of this receptor attenuates adrenergic receptor-mediated contractility (Landeen *et al.*, 2008). Stimulation of the S1P3 receptor results in bradykardia by activating an inward rectifying K^+ current in atrial myocytes (Sanna *et al.*, 2004).

Sphingosine-1-phosphate confers protection against cardiomyocytes ischaemia–reperfusion injury both *ex vivo* (Karliner *et al.*, 2001; Tao *et al.*, 2007) and, especially as a physiological constituent of HDL particles, *in vivo* (Theilmeier *et al.*, 2006; Means *et al.*, 2007). Signals from S1P receptors activate pro-survival pathways, especially Akt signalling via the PI3 kinase pathway. S1P receptor signalling limits apoptosis and improves cardiomyocytes survival during hypoxia (Zhang *et al.*, 2007). Studies using pharmacological inhibitors or genetically modified mice demonstrated that protection occurred by S1P1, S1P2 or S1P3 receptors (Means and Brown, 2009). Besides anti-apoptotic effects, experimental studies have further demonstrated that the S1P receptor agonist FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) reduces endothelial permeability (Sanchez *et al.*, 2003; Dudek *et al.*, 2007). Increased permeability during ischaemia–reperfusion leads to tissue oedema, which hampers microvascular reperfusion and tissue oxygenation. Therefore pharmacological S1P receptor agonists might also favourably modulate the outcome after ischaemia–reperfusion by maintaining the endothelial barrier integrity.

For their high therapeutic potential as anti-inflammatory immune modulators several S1P receptor agonists have been developed. FTY720 is a substrate of sphingosine kinases and the phosphorylated metabolite acts as an agonist at four of the five S1P receptors, namely S1P1, S1P3, S1P4 and S1P5, but not S1P2 (Brinkmann, 2004). The compound is currently under investigation for solid organ transplantation and autoimmunity (Zhang *et al.*, 2007; Mansoor and Melendez, 2008). FTY720 further reduces both hepatic and renal ischaemia–reperfusion injury when administered before ischaemia (Anselmo *et al.*, 2002; Awad *et al.*, 2006). Pharmacological post-conditioning with FTY720 improves recovery of function after ischaemia–reperfusion in both rat and human myocardium (Hofmann *et al.*, 2009).

As the principle of pharmacological post-conditioning is highly attractive for clinical application, we tested the hypothesis that treatment with FTY720 reduces myocardial infarct size *in vivo*. However, we found that both pre- and post-conditioning with FTY720 were not able to reduce infarct size. Instead, treatment with FTY720 induced fatal arrhythmias when administered before reperfusion.

Methods

Animals and surgery

Male Wistar rats (body weight 350–400 g) underwent left anterior descending artery ligation for 45 min with reperfusion for 24 h. Briefly, after induction of anaesthesia (inhalation of 5% isoflurane) and intubation, animals were ventilated with a volume-cycled rodent respirator (1–2% isoflurane/air). Body temperature was maintained by placing animals on an adjustable heating pad. Ligation of the left coronary artery with 'soft pledges' on top of the vessel was performed with a 6-0 suture. After 45 min of ischaemia, reperfusion was allowed by opening the knot. Proper reperfusion was determined by visual inspection, changes in ventricular function and ECG changes (ST-segment elevation). Buprenophine (0.05 mg·kg^{-1}, Temgesic®, Essex Pharma, Munich, Germany) was given routinely i.p. for analgesia during isoflurane anaesthesia. The chest wall was closed with a continuous 6-0 prolene suture.

As malignant arrhythmias are very common in the rat model, animals were monitored by continuous ECG monitoring during the first hour. Every R-R interval corresponding to a heart rate <300 beats·min^{-1} or to a heart rate >500 min^{-1} was evaluated individually as bradykardia or tachycardia respectively. Ventricular tachycardia was defined as >3 consecutive ventricular premature beats, ventricular fibrillation was defined as signal that changed from beat to beat in configuration or rate or where QRS deflections could not easily be distinguished from another. (Opitz *et al.*, 1998)

The local governmental Committee on Animal Research has reviewed and approved the animal study protocol (Ref. Nr. 64/05).

Experimental design

Rats (*n* = 109) were randomly assigned to control treatment (control: NaCl 0.9%) or FTY720 (post) 0.5 mg·kg^{-1} (diluted in NaCl 0.9%) given i.p. to anaesthetized animals after ligature of the left coronary artery. The timing should allow resorption of the compounds into systemic circulation until start of reperfusion without reaching the area at risk (AAR) as long as the left coronary artery was ligated. We selected the dose based on the review of published experimental studies, which demonstrated protection in other ischaemia–reperfusion models. In pilot experiments, we treated sham-operated animals with increasing doses up to 5 mg·kg^{-1} and found no obvious toxicity, but a dose-dependant lymphopenic effect. In a third group (pre + post) of animals 0.5 mg·kg^{-1} FTY720 was given twice i.p., 24 h before infarct surgery and immediately before reperfusion.

After 24 h of reperfusion, infarct size was determined by planimetry after triphenyltetrazolium chloride (TTC) staining. Animals, which demonstrated an AAR < 30%, were excluded from further analysis (Table 1).

In six additional animals of each treatment group the infarcted area of the left ventricle (LV) was sectioned perpendicular to the long axis in three parts. The apical section was fixed in 10% formalin for histology. The mid ventricular ring was separated in infarct zone, border zone and septum and harvested in liquid nitrogen. The basal section was harvested in

Table 1 Distribution of the animals to the study groups

Treatment	Infarct-operated n=	Surviving 24 h n=	Excluded n=
Control	38	24	3
Post	42	22	5
Pre + post	22	19	1

OCT compound (Tissue Tec, Sakura Finetek, Staufen, Germany) for cryo-sectioning

Assessment of the AAR and infarct size

Area at risk and infarct size were assessed as recently described (Frantz et al., 2005). After 24 h, the animals were intubated again; the suture was re-occluded, and 5% Evans blue was injected through the jugular vein. Hearts were excised and sectioned transversely into five to six sections after freezing in OCT compound for 30 min at –20°C. Sections were then incubated in 1.5% TTC at 37°C. After TTC staining viable myocardium stains red and the infarcted areas appear pale. Sections were weighed, digitally photographed and the area of infarction of each section was determined by computerized planimetry (ImageJ imaging software). Tracing was performed under direct comparison with the original tissue sample for optimal delineation of different areas. All measurements were done within 24 h after staining for optimal colours and contrast of the specimen. The size of infarction was determined by the following equations: Weight of infarction = $(A_1 \times Wt_1)$ + ... + $(A_n \times Wt_n)$, where A is percent area of infarction of the section determined by planimetry and Wt is the weight of the individual section. For each heart, $n = 6$–7 sections were analysed. Percentage of infarcted LV = (weight of infarction/weight of LV) × 100. AAR as a percentage of LV = (weight of LV – weight of LV stained blue)/weight of LV. Animals developing an infarct size less than 30% of the AAR were excluded from the study.

Blood counts

After 24 h of reperfusion blood was drawn from the left A. carotis and harvested in EDTA-coated tubes. Differential leukocyte count was done by an automated analyser (Sysmex, XE2100, Norderstedt, Germany).

Tumour necrosis factor-α ELISA assay

Heart tissue stored at –80°C prior to analysis was lysed in standard radio-immunopreciptitation buffer (RIPA) with protease inhibitor (Cocktail III), phosphatase inhibitor (Cocktail II, each from Calbiochem, Darmstadt, Germany). Protein concentration in tissue lysates was determined by a commercial Bradford assay (Biorad, Munich, Germany). Serum and border-zone tissue tumour necrosis factor-α (TNF-α) protein was quantified by a commercial ELISA kit, as indicated by the manufacturer (Biosource, KRC 3011, distributed by Invitrogen, Darmstadt, Germany).

Immunohistochemistry

Cryostat sections (5 μm) were fixed in acetone. After quenching peroxidases by 0.3% H_2O_2 for 30 min the slides were immersed in 1% blocking serum. Then, the primary monoclonal mouse anti-rat granulocyte antibody (Clone HIS48, Acris, Herford, Germany), or a control immunoglobulin was applied over night. A biotin-conjugated goat-anti-mouse secondary antibody was applied for labelling by a peroxidase anti-peroxidase complex with diaminobenzidine as chromogen (Vectastain ABC Kit, Vector Laboratories, distributed by LINARS GmbH, Wertheim, Germany). Granulocyte infiltration in the infarct border zone was analysed by counting positive labelled cells on 4–5 high-power fields (400×). Results were presented as the mean count·mm^{-2} of two independent observers.

Western blot analysis

For Western blotting analysis, the tissue specimens from the septal myocardium were lysed in standard radio-immunoprecipitation buffer (RIPA) with protease inhibitor (Cocktail III), phosphatase inhibitor (Cocktail II, each from Calbiochem). After homogenization, protein determination was performed by a commercial Bradford assay (Biorad). The samples were heated at 90°C for 10 min. Forty microlitres of each sample was separated on 10% sodium dodecylsulfate polyacrylamide electrophoresis gels. Proteins were then transferred electrophoretically onto nitrocellulose membranes, while immersed in transfer buffer [25 mM, Tris(hydroxymethyl)-aminomethan, 192 mM glycine, 20% methanol, 0.037% SDS]. After transfer, non-specific binding was blocked by incubating membranes in blocking buffer (0.1% Tween-20, 5.0% dry milk) for at least 1 h. Protein samples were probed with a purified polyclonal anti-S1P$_1$ antibody (1:100), with a purified monoclonal anti-S1P$_3$ antibody (1:100) and a monoclonal anti-GAPDH antibody (1:5000), followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5000) or HRP-conjugated anti-rabbit (1:5000) for 1 h. Bands were visualized via enhanced chemiluminescence (Amersham Biosciences, distributed by GE Healthcare, Freiburg, Germany). Signals from scanned immunoblots were quantified desitometrically by Image J 1.32 software package (National Institute of Health, Bethesda, MD, USA). Results were normalized for GAPDH expression.

Measurement of cardiomyocyte Ca²⁺ transients

Adult ventricular mouse myocytes were isolated by liberase/trypsin digestion [for detailed procedure see Protocol PP00000125 from The Alliance for Cellular Signalling (AfCS)]. Whole-cell Ca^{2+} transients were measured in Indo-1-loaded, electrically paced (0.5 Hz) cardiomyocytes as described before (Kilic et al., 2005). Excitation was at 365 nm, and the emitted fluorescence was recorded at 405 and 495 nm. The ratio of fluorescence at the two wavelengths was used as an index of the cytosolic Ca^{2+} concentration. Data were collected at 20 Hz, and acquisition and processing were supported by Felix software (Felix version 1.1, Photon Technologies, Seefeld, Germany). After obtaining basal recordings for 10 min, myocytes were exposed to the indicated compounds and transients were continuously recorded for at least 15 min. For positive control, myocytes were stimulated with 100 nM isoproterenol at the end of the experiment.

Materials

FTY720 was provided by Novartis Pharma (Basel, Switzerland). Unless otherwise stated, all compounds were from Sigma (Deissenhofen, Germany). Animals were purchased from Harlan (Borchen, Germany). Antibodies: anti-S1P$_1$ and anti-S1P$_3$ were from Acris, anti-GAPDH from Milipore (HRP)-conjugated anti-rabbit (Milipore, Schwalbach, Germany) and (HRP)-conjugated anti-mouse were from GE Healthcare.

The drug and molecular target nomenclature conforms to the Guide to Receptors and Channels as recently published in revised form (Alexander *et al.*, 2009).

Statistical analysis

All data are expressed as mean and standard error of mean (SEM). Statistical analysis was performed using WinStat software (Benecke & Schwippert; Staufen, Germany), on Excel spreadsheets. Multiple comparisons were done by ANOVA analysis for repeated measurements. Comparison of variables between two groups was performed by using the unpaired Student's *t*-test. *P*-values <0.05 were considered significant. Mortality rates were compared between groups by a χ^2-test. Survival was analysed by a Cox-Mantel Log-rank test.

Results

Blood count

FTY720 induces a dose-dependent lymphopenia in peripheral blood. Therefore, we performed differential blood counts 24 h after ischaemia–reperfusion to verify the effectiveness of treatment. In all experiments, FTY720 (0.5 mg·kg^{-1}) induced leukopenia by significantly reducing the number of lymphocytes in peripheral blood (Figure 1). Lymphocyte count was not different between pre + post and post group. When compared with the control group, FTY720 did not affect absolute neutrophile counts in peripheral blood.

Mortality and infarct size

Survival was significantly different between groups due to differences in mortality during ischaemia and early reperfusion (Figure 2A). Overall mortality was 37% (*n* = 38), 50% (*n* = 42) and 14% (*n* = 22) in control, post and pre + post group respectively. All post animals that have died within 1 h after ligation showed fatal ventricular tachycardias or ventricular fibrillation (Figure 2C and D). Importantly, most arrhythmias occurred in the post group before initiation of reperfusion. Mortality was 24%, 39%, 14% for control, post and pre + post group during the first hour from start of ligation (45 min of ischaemia + 15 min of reperfusion). During the following 23 h of reperfusion two animals died in the control and three in the pre + post group.

After 45 min of ligation and 24 h of reperfusion, the left ventricular (LV) area affected by coronary ligation (AAR) was not significantly different between the experimental groups (AAR/LV: 41%, 43% and 41% in control, post and pre + post). Neither post-conditioning nor combined the pre + post treat-
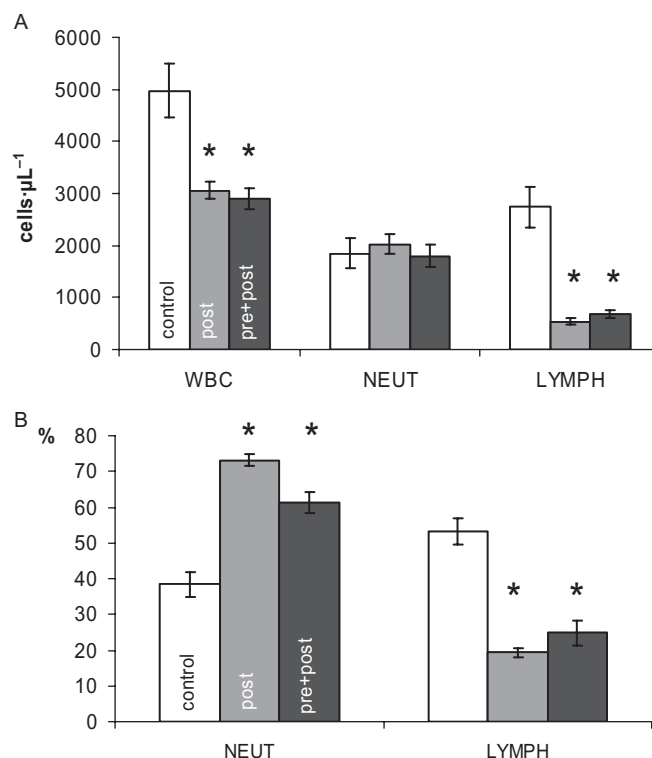

Figure 1 Differential blood count. Blood was drawn after 24 h and analysed for absolute leukocyte numbers (A) in control (white bars), post (grey bars) and pre + post (black bars) treated animals. The percentage of granulocytes (NEUT) and lymphocytes (LYMPH) of the total leukocyte count (WBC) was calculated from these data (B). *P < 0.05 versus control

ment procedures were able to reduce infarct size (infarct area/AAR: 58%, 59% and 61% in control, post and pre + post) (Figure 3).

Myocardial inflammation

During early reperfusion, granulocytes make up the major leukocyte population in the infarct border zone and substantially contribute to local expression of pro-inflammatory cytokines. Therefore, we quantified granulocyte numbers in the infarct border zone. FTY720 treatment significantly reduced granulocyte infiltration in the infarct border zone 24 h after I/R (295.9 ± 37.6 mm^{-2} post, 345.0 ± 22.3 mm^{-2} pre + post, P < 0.05 vs. 432.6 ± 30.3 mm^{-2} control, Figure 4).

The pre + post regimen significantly reduced TNF-α protein expression in the infarct border zone (pre + post vs. control 0.69 ± 0.04 vs. 0.94 ± 0.10 pg·mg^{-1}, P < 0.05, Figure 5A). Serum TNF-α was quantified by ELISA from blood drawn after 24 h. There was no significant difference between groups, albeit the pre + post regimen tended to reduce serum levels (Figure 5B).

S1P receptor expression

As FTY720 is well known for modulating S1P receptor signalling by receptor down-regulation, we analysed S1P$_1$ and S1P$_3$ receptor expression in non-infarcted left ventricular myocar-
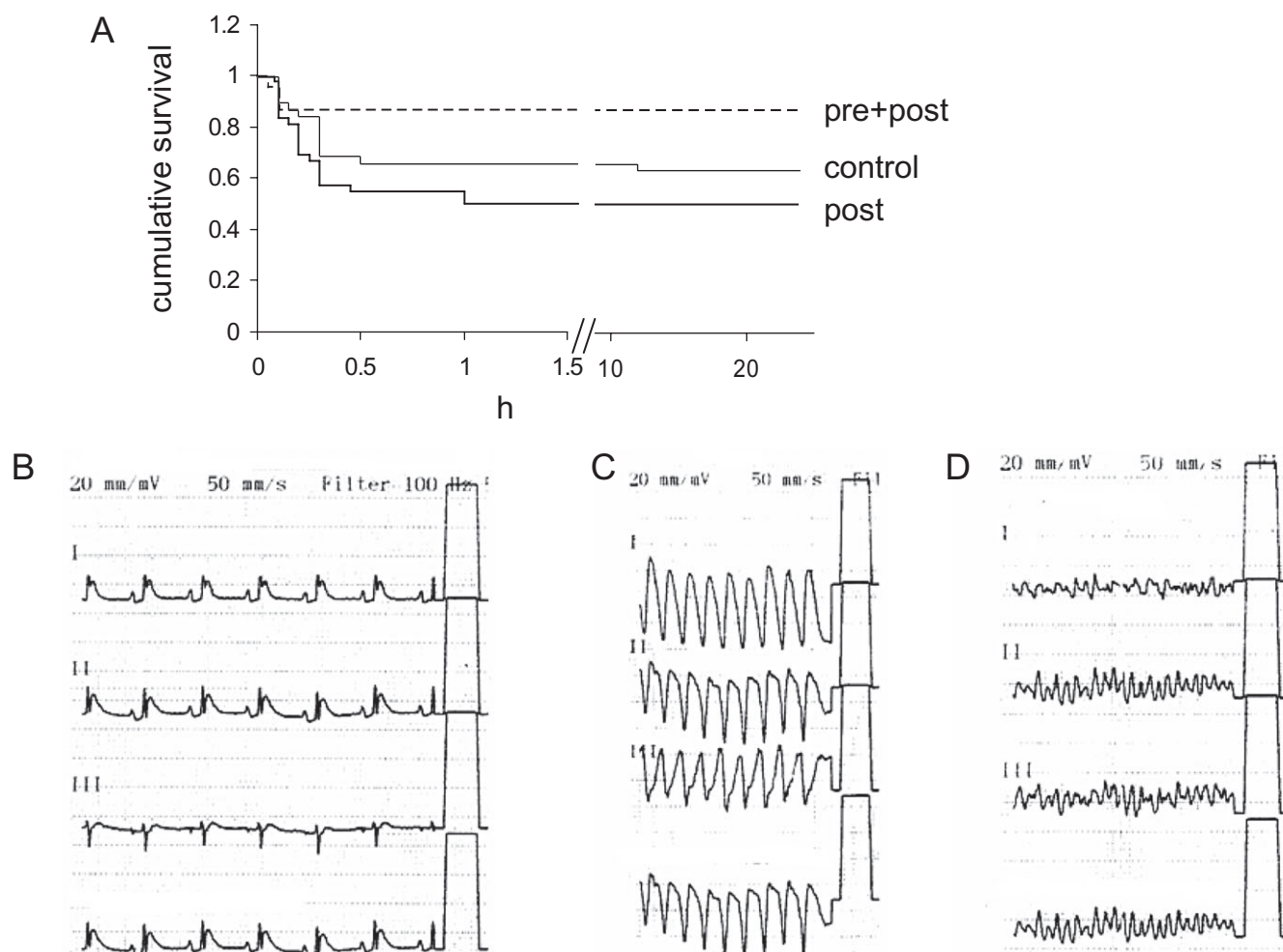
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Figure 2 Survival. (A) Kaplan-Meier survival plot for rats treated with 0.9% NaCl (control) and 0.5 mg·kg^{-1} FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) once, before reperfusion (post), or twice, 24 h before ischaemia and before reperfusion (pre + post). Survival was significantly different between groups ($P < 0.05$). (B–D) Representative ECG tracings from infarcted animals showing ST-segment elevation (B). FTY720 when administered for the first time after coronary ligature (post regimen) increased the incidence of ventricular tachycardias (C) and ventricular fibrillation (D).

dium. Western blotting demonstrated that FTY720 treatment significantly reduced S1P$_1$ expression (Figure 6A). Expression of S1P$_3$ was not different between groups after 24 h of reperfusion (Figure 6B).

Effect of S1P receptor agonist on cardiomyocyte calcium metabolism

S1P was reported to induce both a rise in diastolic calcium and spontaneous calcium transients in neonatal cardiomyocytes, just as can be observed during ischaemia–reperfusion (Nakajima *et al.*, 2000). Therefore, we studied the effect of FTY720 on intracellular calcium transients in adult cardiomyocytes. FTY720, up to 500 nM, did neither affect the diastolic calcium level nor the calcium transient amplitude. We further tested its phosphorylated active form (FTY720-P concentration range tested: 5–500 nM), the selective S1P$_1$ agonist SEW2871 (10 µM) and S1P (5–500 nM). None influenced intracellular calcium transients in electrically paced

cardiomyocytes. We also studied intracellular calcium in unstimulated resting cardiomyocytes and found no change in response to S1P receptor stimulation. When the compounds were applied to cardiomyocytes in buffer supplemented with 0.1% bovine serum albumin, each induced a considerable rise in diastolic calcium. However, the effect could be reproduced by 0.1% bovine serum albumin alone. Representative calcium transient recordings are presented in Figure S1.

Discussion and conclusions

Reducing infarct size by pharmaceutical interventions as an adjunct to classical reperfusion interventions would be an attractive therapeutical principle. Therefore, we investigated whether FTY720, the first orally bioavailable S1P receptor agonist, might be effective in reducing infarct size. As expected, FTY720 treatment reduced peripheral lymphocyte
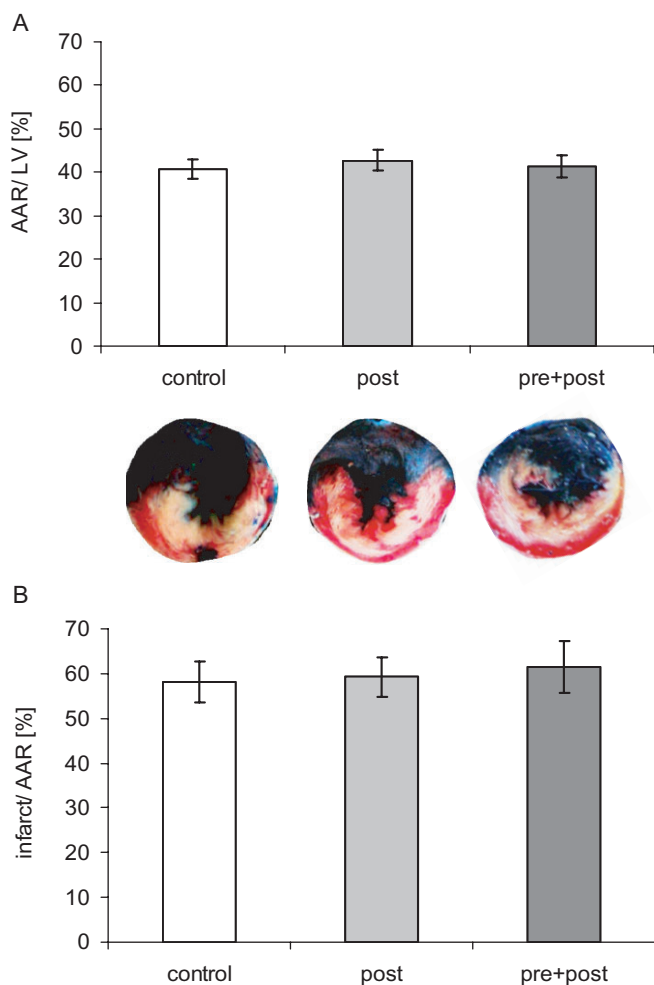
Figure 3 Infarct size. (A) Staining negative for Evans blue delineates the area at risk (AAR) within the left ventricle (LV). (B) triphenyltetrazolium chloride stains vital tissue red and enables calculation of the infarcted tissue within the AAR. Neither AAR/LV nor infarct area/AAR was different between groups.
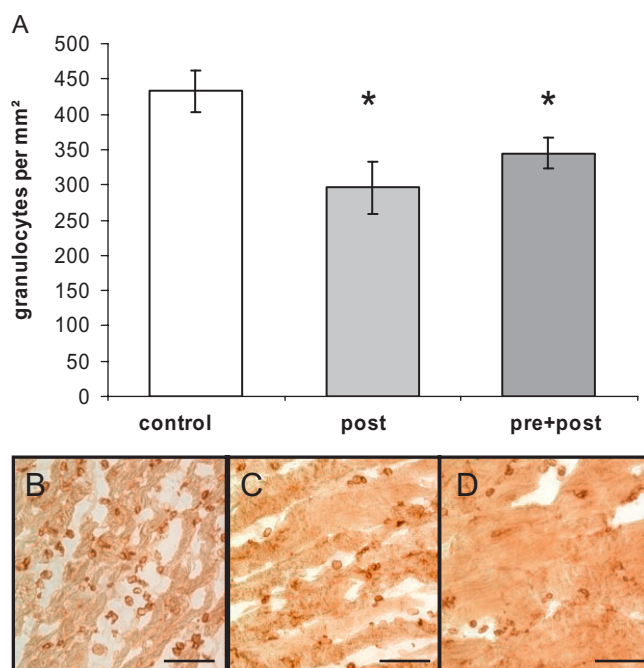

Figure 4 Granulocyte tissue infiltration was evaluated by counting positively stained cells within the infarct border zone of control (B), pre + post (C) and post (D) treated animals. Bar represents 100 μm. FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) significantly reduced number of granulocytes/400× field (*$P < 0.05$ vs. control) (A).

counts. However, in contrast to our previous *ex vivo* findings (Hofmann *et al.*, 2009), the compound was not able to reduce infarct size. Instead, in animals post-conditioned with FTY720 a significantly increased mortality was found. This was mainly caused by induction of fatal arrhythmias during ischaemia and early reperfusion. As the pro-arrhythmic effect might only be a first dose effect and preconditioning treatment might rather be more effective than pharmacological post-conditioning, we decided to treat another group of animals (pre + post group) with FTY720 24 h before surgery. Indeed, this treatment regimen reduced mortality, but still failed to reduce infarct size. In either group most animals died within the first hour from ventricular tachycardia. Unfortunately, we did not comprehensively assess incidence and characteristics of arrhythmias, but our analysis clearly indicated that the mortality from arrhythmias was significantly higher in the post group.

Besides its agonistic effect, FTY720 down-regulates $S1P_1$ receptors but not $S1P_3$ receptors (Graler and Goetzl, 2004). Accordingly, we could demonstrate that $S1P_1$ receptor

expression is down-regulated in the pre + post group after 24 h of reperfusion. $S1P_1$ receptor stimulation has previously been reported to aggravate reperfusion arrhythmias (Tsukada *et al.*, 2007). Therefore, the dichotomous effect of FTY720, $S1P_1$ receptor stimulation with first administration and $S1P_1$ receptor down-regulation after repeated administration, offers a plausible explanation for our finding that FTY720 increased mortality in the post but reduced mortality in the pre + post treatment group: After first dosing FTY720 likely aggravates reperfusion arrhythmias by stimulating $S1P_1$ receptors on cardiomyocytes. After repeated administration FTY720 protects against pro-arrhythmic $S1P_1$ receptor-mediated effects of S1P, which is endogenously released by local ischaemia and thrombocyte activation (Sano *et al.*, 2002; Dahm *et al.*, 2006).

It remains unclear however, by which mechanism $S1P_1$ agonists induce arrhythmias. Potential cardiac side effects of the drug FTY720 have been studied extensively in animal model as well in man. FTY720 induces a moderate reduction in heart rate but does not affect repolarization in healthy subjects (Schmouder *et al.*, 2006). Therefore, we did only pilot experiments in sham-operated animals mainly for assessment of the dose- and time-dependent effect of i.p. delivered doses on lymphocyte count. Here we could not find any indication that FTY720 induced arrhythmias. However, there is some experimental evidence that S1P receptors might elicit cardio-depressant and pro-arrhythmogenic effects, which might become relevant in ischaemia-reperfused myocardium. $S1P_1$ receptor stimulation induces calcium overload in neonatal cardiomyocytes (Nakajima *et al.*, 2000). Intracellular

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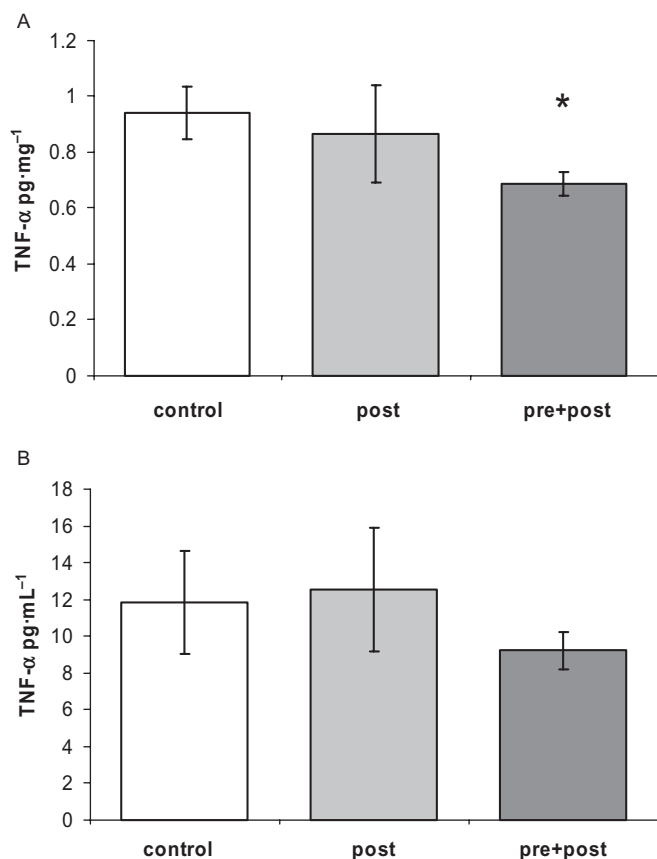U Hofmann et al
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Figure 5 Tumour necrosis factor (TNF)-α protein expression. (A) The pre + post regimen significantly reduced TNF-α protein levels in the infarct border zone (*$P < 0.05$ vs. control). (B) Serum TNF-α was quantified by ELISA from peripheral blood drawn after 24 h from the left A. carotis.

calcium overload due to Na^+/Ca^{2+} exchanger reverse mode activity and sarcoplasmatic reticulum dysfunction is generally supposed to be a main cause for induction of arrhythmias in ischaemia-reperfused myocardium. The exact effects of S1P receptor subtypes on ion conductance and calcium homeostasis are still incompletely understood, but the existing literature (Means and Brown, 2009) indicates that a $S1P_1$ receptor-mediated effect on cardiomyocytes calcium metabolism was the most likely mechanism for the observed pro-arrhythmic effect. Therefore, we hypothesized that $S1P_1$ receptor agonism during early reperfusion adds to reperfusion arrhythmias by aggravating calcium overload, whereas pretreatment with FTY720 attenuates pro-arrhythmic effects by $S1P_1$ receptor down-regulation. In order to test this hypothesis, we studied calcium transients in adult cardiomyocytes stimulated with S1P, FTY720, its active form FTY720 phosphate, and with the selective $S1P_1$ agonist SEW2871. However, we were not able to observe an effect on both diastolic calcium and calcium transient amplitude, as has been reported for neonatal cardiomyocytes (Nakajima et al., 2000). The discrepancy might earliest rely on differences in intracellular signalling between neonatal and adult cardiomyocytes. The response to $S1P_1$ receptor ligands might also be different in the in vivo ischaemia-

reperfused myocardium, but we would rather conclude from our data, that $S1P_1$ receptor induced calcium deregulation is not the most likely mechanism for the pro-arrhythmogenic effect of FTY720.

Ischaemic (Zatta et al., 2006) as well as pharmacological post-conditioning (Huhn et al., 2008) has been demonstrated to be able to reduce infarct size in in vivo ischaemia–reperfusion models in the rat. Furthermore, Egom et al. could demonstrate previously that FTY720 reduces infarct size and protects against reperfusion arrhythmias ex vivo (Egom et al., 2009). However, we could not find an infarct size limiting effect of FTY720 in our in vivo model. There are several reasonable explanations for this: first, reperfusion after 45 min of ischaemia produces a great infarct within the AAR. If we would have studied shorter periods of ischaemia, as has often been done in comparable in vivo studies, we might have detected an infarct sparing effect. In our opinion however, longer periods of ischaemia are clinically more relevant.

Second, it could be a matter of dosing. However, most studies showing a beneficial effect of FTY720 against renal ischaemia–reperfusion injury used comparable doses in rats or mice (Ortiz et al., 2003; Kaudel et al., 2006; Delbridge et al., 2007). We did not further try higher doses for post-conditioning, as we would expect even higher mortality due to pro-arrhythmic effects.

Third, proper timing might be crucial for efficacy. Our initial protocol with FTY720 application just before start of reperfusions reflects pharmacological post-conditioning. However, this clinically most promising therapeutical principle might not work with S1P receptor modulators. The protective effects of S1P receptor stimulation were mainly demonstrated in ex vivo models for preconditioning. Therefore, we performed another series of experiments in which animals were additionally pretreated 24 h before ischaemia (pre + post). In this group, we could demonstrate significantly reduced mortality. However, there was also no significant effect on infarct size. We would not suggest that the negative results have to be ascribed to inappropriate timing of treatment, as both regimens (post and pre + post) were ineffective in reducing infarct size.

Fourth, the immuno-modulating properties of the compound might be rather disadvantageous. Induction of T-cell lymphopenia might be unfavourable, because there are particular subsets of T-cells that can be able to favourably modulate the immune response in ischaemia–reperfusion (Liesz et al., 2009; Monteiro et al., 2009). We suggest that these immunological effects contribute to the obvious discrepancy between ex and in vivo effects on cardiac injury. In conclusion, treatment with the S1P receptor modulator FTY720 was not able to reduce infarct size after myocardial ischaemia–reperfusion in vivo. FTY720 increased mortality due to induction of arrhythmias as first dose effect when administered during myocardial ischaemia. Therefore, pharmacological S1P receptor agonists in general might not be the ideal post-conditioning agent. Despite the promising effects in ex vivo models, especially the potential confounding effects on cardiomyocyte electrophysiology might pose a major obstacle for the therapeutic use of S1P receptor agonists in myocardial ischaemia–reperfusion.
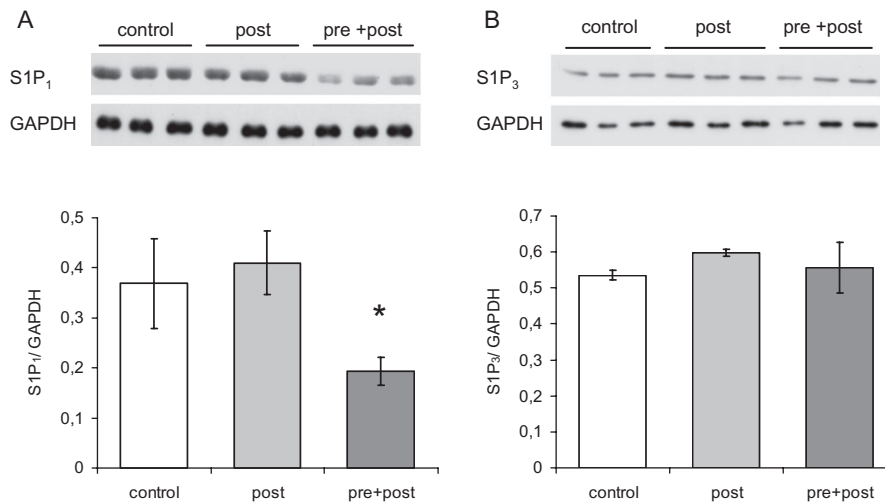
Figure 6 Sphingosine-1-phosphate (S1P) receptor expression after 24 h of reperfusion was analysed by Western blotting. S1P$_1$ receptor expression (A) was significantly lower in the pre + posts treatment group (*$P < 0.05$ vs. post group). S1P$_3$ receptor expression was not different between groups (B).

Acknowledgements

Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Calcium transients were recorded in Indo-1-loaded cardiomyocytes under baseline conditions (control) and after stimulation with $S1P_1$ receptor agonists. Representative transients were generated by averaging the Indo-1 signal ratio of 10 consecutive single transients. (A) FTY720 did neither affect diastolic nor systolic signal height, unless cardiomyocytes were superfused with buffer containing 0.1% BSA. (B) An analogous rise in diastolic calcium could be demonstrated by supplementing buffer with 0.1% BSA. (C) The active phosphorylated compound FTY720-P, akin to the other $S1P_1$ receptor agonists tested (SEW2871 and S1P, data not shown), did not influence transients. Isoproterenol stimulation at the end of the experiment always confirmed that myocytes' intracellular calcium transients were responsive to exogenous stimulation. FTY720, S1P receptor agonist, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride; S1P, sphingosine-1-phosphate.

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