Activation of Pak1/Akt/eNOS signaling following sphingosine-1-phosphate release as part of a mechanism protecting cardiomyocytes against ischemic cell injury

Emmanuel Eroume A Egom1,2, Tamer M. A. Mohamed3, Mamas A. Mamas2,5, Ying Shi3, Wei Liu6, Debora Chirico5, Sally E. Stringer2, Yunbo Ke4, Mohamed Shaheen2, Tao Wang2, Sanoj Chacko5, Xin Wang6, R. John Solaro4*, Farzin Fath-Ordoubadi5*, Elizabeth J. Cartwright2* and Ming Lei2*

1 Castle Hill Hospital, University of Hull, Department of Cardiology, Kingston-upon-Hull, HU16 5JQ
2 Cardiovascular Group, School of Biomedicine, The University of Manchester, Manchester, M13, 9NT UK.
3 Department of Cardiovascular Diseases, Union Hospital, Huazhong University of Sciences and Technology, Wuhan, Peoples` Republic of China
4 University of Illinois at Chicago, Department of Physiology and Biophysics, Center for Cardiovascular Research, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612, USA
5 Biomedical Research Centre, Central Manchester NHS Foundation Trust, Manchester, UK
6 Faculty of Life Sciences, 2 Faculty of Medical and Human Sciences, The University of Manchester, UK

Copyright © 2011 by the American Physiological Society.
Correspondence to:

Ming Lei, Cardiovascular Research Group, School of Bioscience, The University of Manchester, Manchester M13 9NT, Tel: 44-161-2751194 Fax: 44-161-2751183 E-mail: ming.lei@manchester.ac.uk.

*Joint senior authors

Word count: 7247
Abstract

Aim: We investigated whether plasma long chain sphingoid base (LCSB) concentrations are altered by transient cardiac ischemia during percutaneous coronary intervention (PCI) in humans, and examined the signaling through the sphingosine-1-phosphate (S1P) cascade as a mechanism underlying the S1P cardio-protective effect in cardiac myocytes.

Methods and results: Venous samples were collected from either the coronary sinus (n=7) or femoral vein (n=24) of 31 patients at 1, 5 min and 12 h, following induction of transient myocardial ischemia during elective PCI. CS levels of LCSB were increased by 1072% at 1 min, 941% at 5 min (n=7), while peripheral blood levels of LCSB were increased by 579% at 1 min, 617% at 5 min and 436% at 12 h (n=24). In cultured cardiac myocytes, sphingosine-1-phosphate (S1P), sphingosine (SPH) and FTY720, a sphingolipid drug candidate, showed protective effects against CoCl induced hypoxia/ischaemic cell injury, by reducing LDH activity. 25 nM FTY720 significantly increased phospho-Pak1 and phospho-Akt levels by 56% and 65.6% in cells treated with this drug for 15 mins. Further experiments demonstrated that FTY720 triggered NO release from cardiac myocytes is through Pertussis toxin (PTX)-sensitive PI3K/Akt/eNOS signaling. In ex vivo hearts, ischemic preconditioning was cardioprotective in wild type control mice (Pak1<sup>+/+</sup>), but this protection appeared to be ineffective in cardiomyocyte-specific Pak1 knockout (Pak1<sup>cko</sup>) hearts.

Conclusions: The present study provides the first direct evidence of the behaviour of plasma sphingolipids following transient cardiac ischemia with dramatic and early
increases in LCSB in humans. We also demonstrated that S1P, SPH and FTY720 have protective effects against hypoxic/ischaemic cell injury, likely a Pak1/Akt1 signaling cascade and NO release. Further study on a mouse model of cardiac specific deletion of Pak1 demonstrates a crucial role of Pak1 in cardiac protection against I/R injury.

Key words: LCSB, FTY720, ischemia
1. INTRODUCTION

Sphingolipids are biologically active lipids that play important roles in various cellular biological processes (12). Recent studies indicate that myocardial sphingosine (SPH) levels are elevated in animal models of myocardial infarction (47, 52) and have suggested that SPH have an important role in protection against ischaemic injury (19). Sphingosine-1-phosphate (S1P) has also been shown to be an important mediator of cardiac ischaemic pre- and post-conditioning in both pharmacological and knockout animal studies (15, 26). FTY720 (fingolimod), a sphingolipid drug candidate displaying structural similarity to S1P, demonstrated a protective effect in preventing organ ischemia/reperfusion (I/R) injury in an animal model (7, 11). Thus, it appears highly likely that these sphingolipid molecules and their analogues have the potential to act as therapeutic modulators of cardiac responses to myocardial injury. The underlying detailed key mechanism(s) and signaling pathway(s) for their cardioprotective effect, however, remain poorly understood (18).

A recent study suggests that the activation of Akt underlies the protective effects of S1P receptor agonist treatment after myocardial ischemia–reperfusion, which opens the door for understanding the key signaling mechanism(s) for S1P cardio-protection (11). Another significant clue as to the mechanism came from experiments in a mammalian cell line, which demonstrated that p21 activated kinase (Pak1), a Ser/Thr kinase downstream of small G-proteins, is activated by sphingosine and several related LCSBs in a time- and dose-dependent manner (2). We recently also showed that FTY720 prevents arrhythmias induced by ischemia/reperfusion injury by activation of signaling through the Pak1 and Akt cascade (7). However, to date, neither the signaling
pathways mediating these effects, nor an *in vivo* metabolism for endogenously released S1P has been established during acute cardiac ischaemic conditions. Accordingly, we designed both an *in vivo* study to determine whether plasma sphingolipids measured as LCSB concentrations were altered by transient cardiac ischemia following temporary coronary artery occlusion during percutaneous coronary intervention (PCI) in humans and *in vitro* experiments to examine the cell-survival pathways regulated by S1P agonist FTY720 in cardiomyocytes.
2. METHODS AND MATERIALS

2.1. Human subjects study protocol

Blood samples were obtained from 31 patients aged 40 to 73 years old undergoing elective PCI to native coronary arteries at the Manchester Heart Centre, Manchester, UK. The study complies with the Declaration of Helsinki, that the locally appointed ethics committee has approved the research protocol and that informed consent has been obtained from the subjects. Demographic data follows: men (97%); Caucasions (87%); normal left ventricular (LV) function (100%); normal renal function (100%). Incidence of risk factors: hypertension (53%); diabetes (15%); hyperlipidaemia (85%); smokers (39%) and body mass index (BMI) (27.4±4.8 kg/m²). Medication: anti-platelet therapy (100%); β-adrenoceptor blockers (82%); Angiotensin-Converting Enzyme (ACE) inhibitors (65%); statins (100%); nitrates (22.5%) and Ca²⁺-channel antagonists (29.5%). Only patients with angiographic single vessel disease undergoing elective PCI were used in this study that had documented normal left ventricular and renal function. Patients with a previous history of coronary artery bypass graft (CABG), valvular heart disease or recent myocardial infarction/acute coronary syndrome (ACS) were excluded as were PCI procedures in patients with chronic total occlusions.

Procedures were performed via the femoral artery through standard 6Fr sheaths and peripheral venous samples were collected through a 6Fr femoral venous sheath. Coronary sinus sampling was performed using a 6Fr Amplatz Left-1 catheter (AL-1) during PCI. Predilation of the target lesions was performed with angioplasty balloons inflated between 14 to 22 Atmospheres (mean 15 Atmospheres) for a period of between 28 to 40 seconds (mean 31.1 sec). Control venous blood samples were obtained either
from the CS (7 patients) or via the femoral venous sheath (24 patients) once the guide
catheter and guide wire were in position prior to the PCI procedure. Balloon inflations
of between 30 seconds and 1 min were performed to predilate the target lesions. Serial
venous samples were then collected from either the CS or femoral vein at 1 min and 5
min post balloon inflation. PCI was then completed as per routine at our centre. 12
hours post-procedure samples were taken from a peripheral vein to measure LCSB
levels. Blood samples were immediately dispensed into 3 ml EDTA tubes with 2-
chloroadenosine (0.05 mmol/L) and procaine hydrochloride (0.154 mol/L) and
equilibrated at 4 °C.

Only patients with angiographic single vessel disease undergoing elective PCI
were used in this study that had documented normal left ventricular and renal function.
Patients with a previous history of CABG, valvular heart disease or recent myocardial
infarction/acute coronary syndrome were excluded as were PCI procedures in patients
with chronic total occlusions.

2.2. Spectrophotometry analysis of long-chain base fraction

Procedures for the quantitative analysis of LCSB have been based on the determination
of the long chain base of sphingosine and the analysis of other sphingolipid components.
A direct analysis for sphingosine is the preferred analytical approach to sphingolipid
analysis since all known sphingolipids contain one molecule of sphingosine per
molecule of sphingolipid. As all glycosphingolipids and sphingomyelin contain one
mole of long chain base, the total amount of these lipids can be appreciated by the
amount of fatty bases present in the hydrolyzed extract. Several procedures may be used
but the procedure reported here were based on the determination of the long chain base
of SPH as described by Siakotos et al (13, 44).

2.3. Isolation and culture of rat ventricular cardiomyocytes

Neonatal rat ventricular cardiomyocytes were prepared and cultured from 2- to 3-day-old rats, as described previously (34). Experiments were performed after further 24 h cultivation.

2.4. Simulated ischemia model

Cells were washed with PBS before addition of 1 ml ischemia buffer (in mM: 130 NaCl, 10 KCl, 0 Glucose, 0.6 MgCl$_2$, 1.8 CaCl$_2$, 1 NaHCO$_3$, 0.6 NaH$_2$PO$_4$, 10 HEPES, pH 6.6, gassed with 100% nitrogen for more than 30 min before the experiment was started). Subsequent experiments were performed using 24 h exposure to 100 µM cobalt chloride (CoCl$_2$). Cobalt has been widely used as an ischemia injury mimic in cell culture (49).

2.5. Langendorff perfused hearts ischemic preconditioning study

Langendorff perfused hearts with ischemic preconditioning (IPC) protocol in cardiomyocyte-specific Pak1 knockout mice (Pak1$^{cko}$) and their wild type control mice (Pak1$^{ff}$). Generation of Pak1$^{ff}$ and Pak1$^{cko}$ mice were described elsewhere by Liu et al.(27), hearts were equilibrated for 20 min and then subjected to 3 short cycles of IR, each consisting of 2 min of global ischemia and 2 min of reperfusion, followed immediately by 30 mins global ischemia and 30mins global reperfusion.

2.6. Measurement of lactate dehydrogenase activity
The cells were subjected to global ischemia or CoCl$_2$ treatment for 20 min or 24 h respectively. After the completion of simulation, the buffer was gently collected for lactate dehydrogenase (LDH) determination using a spectrophotometric LDH enzyme assay kit (Cayman Chemical Co. Cambridge, UK) as described previously (28).

2.7. Determination of intracellular nitric oxide bioavailability

Intracellular nitric oxide (NO) bioavailability was measured as described previously (14). Fluorescence was detected with a live cell imaging Leica AS MDW inverted fluorescence microscope (Leica Microsystems (UK) Ltd) in which the cells were kept at 37°C.

2.8. Western blotting analysis

Western blot analysis was conducted as described previously (14) using specific antibodies described below.

2.9. Antibodies

Rabbit anti-Pak1, rabbit anti-phospho-Pak1 Thr423, rabbit anti-Akt and anti-phospho-Akt Thr308 residue, were all purchased from Cell Signaling Technology, Hitchin, UK.

3.0. Statistical analysis

Data are expressed as means ± SE. For spectrophotometry data, repeated measure one-way analysis of variance (ANOVA) was used to compare values of measurements between groups. When analysis of variance revealed the existence of a significant difference among values, Tukey’s test was applied to determine the significance of a difference between selected group means.
3. RESULTS

3.1. Spectrophotometry analysis of PCI induced changes to plasma LCSB levels

To determine if sphingolipids are involved in pathophysiological processes associated with acute cardiac ischemia, we measured LCSB concentrations in transient cardiac ischemia following temporary coronary artery occlusion during PCI in humans.

Plasma levels of LCSB in patients at baseline (pre-balloon inflation) and at different time points after balloon inflation were analysed by spectrophotometry. Significant alterations were found in plasma levels of LCSB sampled from the CS in 7 patients, and peripheral veins in 24 patients, following induction of transient myocardial ischemia by balloon occlusion of target lesion. At baseline, LCSB levels from CS and peripheral blood samples averaged 1.7±0.9 µM (n=7) and 0.03±0.005 µM (n=31), respectively. There was a significant increase in LCSB levels at 1 and 5 min, compared with baseline levels, both in CS blood (Figure 1A) and peripheral blood (Figure 1B).

LCSB levels in CS and peripheral blood at different time points are shown in Figure 1. At 1 min following balloon inflation in the CS, levels of LCSB increased by 1072 % compared with baseline levels (n=7, all P<0.001), whereas in peripheral blood, levels of LCSB increased by 579 % compared with baseline levels (n=24, all P<0.001). Peripheral sphingolipid levels were consistently very much lower than CS levels. At 5 min after balloon inflation in the CS blood, levels of LCSB increased by 941 % compared with baseline levels (n=7, all P<0.001), while in peripheral blood, levels of LCSB increased by 617 % compared with baseline levels (n=24, all P<0.001). At 12 h following the PCI procedure, in peripheral blood, levels of LCSB increased by 436 % compared with baseline levels (n=24, all P<0.001, 95% C.I.). These results implicate
an important role of sphingolipids in pathophysiological processes that occur during early cardiac ischemia. It is known that ischemia/reperfusion (I/R) injury can be significantly minimized by a cardiac self-protective mechanism called ischaemic preconditioning, a phenomenon describing a brief period of myocardium I/R that significantly reduces injury resulting from subsequent long-term I/R. Therefore the release of LCSB might be involved in such a self defense system.

3.2. The effects of S1P, SPH and FTY720 on cell viability in in vitro hypoxic and ischaemic cell models

The effects of S1P, SPH and FTY720 on the viability of myocytes subjected to hypoxia and ischemia were first examined using in vitro cell models. Viability was gauged by LDH activity, a stable enzyme normally found in the cytosol of all cells, but rapidly released upon damage to the plasma membrane. The increase of LDH in the culture supernatant fraction provided a measurement of the number of lysed/damaged cells.

As shown in Figure 2A, after 24 h exposure with 100 µM CoCl₂ to induce hypoxia, LDH release increased 10-fold from 2.55±0.16 mU/ml in control conditions to 25.17±0.20 mU/ml (n=5, p<0.01). In contrast, when the cells were subjected to a 24 h CoCl₂ treatment in the presence of 25 nM of S1P, FTY720 or SPH respectively (Figure 2A), there was a significant reduction of LDH released compared to cells treated with CoCl₂ alone. LDH decreased by 45.0% in the S1P treated group, 44.7% in the FTY720 treated group and 91 % in the SPH treated group. This indicates a protective effect against CoCl₂ induced hypoxia by these molecules.

As shown in Figure 2B, a 20 min exposure to ischaemic solution alone increased LDH release from 1.56±0.11 at base line to 2.55±0.16 mU/ml (n=5, p<0.01). When the
cells were subjected to a 20 min ischaemic solution treatment in the presence of 25 nM S1P, 25 nM FTY720 or 25 nM SPH respectively (Figure 2B), LDH release was reduced by 40.0%, 47.5% and 45.1 % compared with the cells treated with ischaemic solution alone, which indicates that these molecules display protective effects against ischaemic induced cell injury.

3.3. FTY720 induces Pak1 and Akt activation

Different cardioprotective signaling pathways converge to Pak1 and PI3-kinase-mediated Akt activation. Thus, we focused on Akt, and Pak1, a Ser/Thr kinase downstream of small G-proteins, to clarify the key signaling mechanism(s) for the observed sphingolipid cardio-protection effect. Pak1 and Akt activation were assessed by quantifying the levels of (Thr423)-phosphorylated Pak1 and (Thr308)-phosphorylated Akt in FTY720- treated cardiomyocytes by Western blotting. Compared with control non-treated myocytes, cells treated with 25 nM FTY720 for 15 min showed an increase in phospho-Pak1 and phospho-Akt levels by 56% and 65.6% respectively ($P < 0.05$ vs. control - Fig 3A,B,E,F). There was no significant difference in the levels of total Pak1 and total Akt expression levels between non-treated and treated myocytes with FTY720 (Fig. 3A,B, E,F).

3.4. FTY720 stimulates nitric oxide (NO) production via a Pertussis toxin (PTX)-sensitive PI3K/Akt/eNOS cascade

To determine whether FTY720-mediated Pak1 and Akt activation, and NO release, was through Gi, myocytes were treated with 100 ng/ml PTX overnight and then stimulated with 25 nM FTY720 for 15 min. As shown in Figure 3C,D,G,H, FTY720 induced a 1.6-fold increase in Pak1 phosphorylation, and a 1.45-fold increase in Akt.
phosphorylation relative to vehicle. After PTX treatment, FTY720-mediated activation of Pak1 was reduced by 94%, and activation of Akt was reduced by 55%. FTY720 induced NO release was abolished (data not shown).

Different cardioprotective signaling pathways also converge to release NO (29,30). We thus determined how S1P/FTY720 induced NO production that results in cardioprotection. We detected NO release using DAF-FM, an NO sensitive dye. As shown in Figure 4A, B, (red arrows), myocytes exposed to 25 nM FTY720 and 25 nM SEW2871 (a specific S1P1 receptor agonist) displayed NO vesicles localized in specific areas near the cell membrane which disappear over time. However, NO vesicles were not observed when cells were pre-incubated for 1 hour with 1 mM L-NAME (a potent inhibitor of eNOS, iNOS, nNOS) or with 1 μM L-NIO (a potent inhibitor of eNOS) or for 30 min with 1μM of A6730 (a Akt1/2 kinase inhibitor, Fig. 4C,F,G) or with 50 μM of LY294002 (a PI3K inhibitor, Fig. 4H) respectively. When cells were pre-treated with 10 μM L-NIL, a potent and selective inhibitor of iNOS and 1 μM SMLT, a potent inhibitor of nNOS for 60 mins the appearance of FTY720 induced NO vesicles was still observed, although these were fewer in number (Fig. 4D,E), suggesting that the effect of FTY720 is mainly through PI3K/Akt/eNOS signaling pathway. This ultimate finding is indirect evidence that FTY720 might trigger the production and the release of NO by cells.

A disruption of Pak1 sensitizes the myocardium to ischemia/reperfusion induced ventricular arrhythmias

Ischemic preconditioning (IPC) is a short period of IR that rescues hearts from subsequent long term IR injury. Reported triggers are adenosine, bradykinin, opioids,
and S1P (4, 14, 16). Evidence suggests a definitive role of sphingolipid metabolites, including S1P, as important members of the IPC-mediated intracellular signaling process (14). In our previous study, we provided evidence that FTY720 prevents I/R injury associated arrhythmias and the cardio-protective effect of FTY720 is likely to involve activation of signaling through the Pak1. However, there is no direct evidence for the involvement of Pak1 in IPC signaling. Thus we took advantage of our recently developed mouse model of cardiac-specific deletion of Pak1 (27) to determine the role of Pak1 in IPC. Both WT control and Pak1^{cko} hearts were subjected to IPC protocol as described in the methods section. Figure 5 shows representative ECG recordings from hearts before and after subjected to IPC protocol. As summarized in Table 1, ventricular arrhythmic events including non-sustained and sustained episodes of ventricular tachycardia (VT) and ventricular fibrillation (VF) were more frequently observed in Pak1^{cko} hearts (5 out of 6 mice studied) than control WT hearts 3 out 8 mice studied. Thus, a disruption of Pak1 reduced the cardiac protection of IPC, in other words, a disruption of Pak1 sensitizes the myocardium to ischemia/reperfusion induced ventricular arrhythmias.

4. DISCUSSION

4.1. Transient coronary ischemia induced changes of plasma LCSB levels in human subjects

Our study, for the first time, demonstrates a dramatic increase in LCSB levels following transient cardiac ischemia in humans. The levels of LCSB in both CS and peripheral vein markedly increased within 1 min of transient ischemia mediated by short periods of
coronary vessel occlusion. The levels remained elevated after 5 min and started to
decline after 12 h peripherally.

Sphingolipids are now emerging as important signaling molecules produced by
cardiac tissue during ischaemic stress or as a consequence of inflammation. Evidence
indicating that cardiac tissue may be a putative source of serum sphingolipids comes
from studies showing that cardiac cells produce sphingolipids in response to hypoxia
and reoxygenation (1) as well as the finding that the ischemia caused by coronary
microembolization in a canine model results in significant elevation in myocardial
sphingolipids levels (47). Additionally, Deutschman and co-workers (5) reported that
sphingolipid levels are elevated in patients with coronary artery disease (CAD) where
S1P had a greater predictive value in detecting CAD than traditional risk factors. The
high concentration of LCSB levels seen in the coronary sinus compared to the periphery
suggests that this increase in circulating LCSB are derived from the myocardium, rather
than being released elsewhere as a consequence of myocardial ischemia.

Evidence is rapidly accumulating that suggests a definitive role of sphingolipid
metabolites, including S1P, as important members of the IPC-mediated intracellular
signaling process (14). It has also been shown that the protective effect of IPC could be
mimicked by exogenous S1P, suggesting the possibility that ceramide and sphingosine
formed during preconditioning must be converted to S1P to protect against I/R injury
(30).

4.2. Cardioprotective effects of S1P, SPH and FTY720

Recent studies have provided evidence for a role of S1P signaling in protection against
the stress of cardiac I/R injury (14, 39, 50, 51). S1P, product of sphingosine kinase
(SphK) activation, is recognized as a vital lipid mediator activating a family of five G protein coupled receptors (S1P1-5). These receptors regulate diverse cellular events, including cell survival, growth, motility, differentiation, cytoskeletal reorganization, and calcium mobilization (39). S1P is a phosphorylated derivative of sphingosine, the structural backbone of all sphingolipids, which was initially described as an intermediate in the degradation of long-chain sphingoid bases (21). FTY720 is a sphingolipid drug candidate displaying structural similarity to S1P and has demonstrated a protective effect in the prevention of liver I/R injury in an animal model (20). Importantly, FTY720 is currently under evaluation in a long-term, Phase III clinical trial as an immuno-suppressant agent for the treatment of auto-immune diseases and in organ transplantation (3). Our recent study on rat ex vivo heart model also demonstrated the important role of FTY720 in antagonizing both brady- and tachyarrhythmias induced by I/R injury. The study also determined that FTY720 acts through Pak1/Akt signaling, which identifies this cascade as an important element in this cardiac protection effect. Our data also significantly extend earlier reports providing evidence for a role of S1P signaling in protection against I/R injury and its potential role in pre- and post-conditioning. (18, 39)

In the present study, in cultured cardiac myocytes, S1P, SPH and FTY720 all showed protective effects against hypoxic or ischaemic cell injury, by reducing LDH activity; which is consistent with previous findings (7, 11, 50, 51). Several studies have provided evidence for a role of S1P signaling in protection against the stress of ischemia/reperfusion (I/R) injury, in particular its critical role in pre- and post-conditioning mechanisms of rescue of hearts from I/R injury (7, 11, 50, 51). It is highly relevant that FTY720 is currently under evaluation in a long-term, Phase III clinical trial.
for the treatment of auto-immune disease and in organ transplantation (3, 41). FTY720 was found to attenuate liver graft injury by activation of cell-survival Akt signaling (54). We recently also demonstrated the cardioprotective effect of FTY720 in an *ex vivo* rat heart I/R injury model (7).

### 4.3. Mechanism(s) underlying FTY720 cardioprotection in ischemia/hypoxia model

In the present study, our data showed that FTY720 is able to stimulate, via the inhibitory G proteins, Pak1 and Akt auto-phosphorylation and activities, and trigger NO release through eNOS. The NO release via S1P receptors appears to be mediated by PI3K and Akt activation. NO release induced by FTY720 was sensitive to Gi inhibition. We believe this is a strong evidence to demonstrate the mechanistic link between FTY720 and Pak1 and Akt activations. We recently established cause-effect relationship for the observed phosphorylation of Pak-1 and Akt to the cardioprotection (7). In that study, to clarify whether the observed prevention of arrhythmias by FTY720 in I/R model was attributed to the activation of Pak1/Akt, activation of pak1/Akt during I/R model was determined in I/R langendorff hearts. The same hearts used for the functional-arrhythmias studies (made ischemic in the presence or absence of FTY720) were used to check the phosphorylation states of Pak1/Akt. This provided a potential link between the functional effects of FTY720 and intracellular signaling.

Thus, the data presented here provide a new insight into the signaling pathways underlying an FTY720 cardioprotective mechanism. As illustrated in Figure 6 signaling through activation of S1P receptors by S1P and its analogue, FTY720, to Pak1/Akt/eNOS may serve as a key mechanism underlying the S1P/FTY720 cardioprotective effect.
We report here that FTY720 is able to stimulate Pak1 and Akt auto-phosphorylation and activities in cardiomyocytes. Akt is a well-established regulator of myocardial growth and survival, contractile function, and coronary angiogenesis (43). Studies showed that Pak1 is able to activate Akt ((9); whereas Akt can phosphorylate Pak1. Mao et al. recently showed that both Pak1 and Akt can be activated by multiple hypertrophic stimuli and growth factors in a PI3K-dependent manner, suggesting that Pak1 and Akt may lie in the same signaling pathway in cardiomyocytes (31). Using both gain- and loss-of-function approaches in vitro and in vivo, the authors demonstrated that Pak1 is sufficient to activate Akt and is essential for growth factor-induced Akt activity in cardiomyocytes (31). The functional significance of Pak1-Akt signaling is underscored by the observation that the pro-survival effect of Pak1 is diminished by Akt inhibition (31). The Pak1-conferred protection was blocked by the Akt inhibitor X, suggesting that the protective effect of Pak1 is mediated, at least in part, by Akt signaling (31). These findings demonstrate an important role for Pak1-Akt signaling in cardiomyocyte survival. As show in Fig. 5 and table 1, our further experiments on a mouse model of cardiac-specific deletion of Pak1 demonstrates a crucial role of Pak1 in cardiac protection against I/R injury. The recognition of the functional significance of Pak1 in preventing arrhythmogenesis associated with I/R injury may lead to the development of better therapies for treating I/R injury induced ventricular arrhythmias.

Akt is a well-established mediator of cardioprotection in I/R injury, as demonstrated by transfection, gene delivery, and transgenic approaches (8, 32). However, mechanisms of Akt-mediated cardioprotection are still under intense investigation. Akt has been shown to increase eNOS phosphorylation, resulting in activation of eNOS, and
experiments with eNOS knockout mice indicate a role for NO in protection against ischemic damage (17, 33). Described mechanisms for NO-mediated cytoprotection include anti-apoptotic (22), anti-oxidant (45), anti-inflammatory (25), and cyclic guanosine monophosphate (cGMP)-mediated effects (6). In endothelial cells, S1P activates eNOS via an Akt-mediated pathway, and this occurs via the S1P3 receptor (38). Kwon et al (24) showed S1P increases eNOS activity via intracellular Ca\(^{2+}\) mobilization and resulting increases in NO protect endothelial cells from apoptosis by suppression of apoptotic signaling cascades. Importantly, that study revealed that S1P increased NO production from human umbilical vein cells (HUVECs) by enhancing Ca\(^{2+}\)-sensitive eNOS activity without significant increase in the eNOS protein. Two studies have addressed the cardioprotective role of NO during cardiac surgery by supplementation of blood cardioplegia with L-arginine (42) and SPM-5185, a cysteine containing NO donor (35). These agents respectively produced both reduced infarct size and improved post-ischaemic contractile performance.

Cellular subfractionation studies have demonstrated that the cytoplasm of NOS containing neurons is homogeneously stained followed either immunohistochemistry or NADPH diaphorase histochemistry (48). In contrast, NOS-containing vascular endothelial cells exhibit a characteristic pattern of staining in which the reaction product is restricted to a small number of punctate regions within each endothelial cell (36, 37). Other studies have shown that this reaction product is present throughout the cytoplasm and in association with the membranes of vesicles, mitochondria and endoplasmic reticulum (29, 40). Our study has shown that neonatal cardiomyocytes show an identical pattern of staining following fluorescence using the NO sensitive dye DAF-FM, and thus the punctate staining might represent patches of NOS activity. Assuming that these
small vesicles localized by NO fluorescent dye represent NOS activity, our results suggest that active NOS enzyme might be largely associated with the Golgi apparatus and with cytoplasmic vesicles, which thus accounts for the punctate appearance of neonatal cardiomyocytes following fluorescence using the NO sensitive dye DAF-FM. Neonatal cardiomyocytes NOS might probably be the result of a mature molecule in the Golgi apparatus and is incorporated into vesicles derived from the Golgi. Hecker et al. (10) have shown that the concentration of endothelial NOS is highest in the cell fractions associated with the endoplasmic reticulum and the plasma membranes of endothelial cells. Whether neonatal cardiomyocytes patches represent a reserve pool of NO synthase, which may be required when the tissue is under a local stress, remains unclear. However the subcellular punctate patches of NO synthase activity, as seen in our study to be localized to specific area toward cell periphery, may be the most opportune site for a readily available “pool” of NO.

Regulation of NOS activities by protein phosphorylation is complex. They are regulated by phosphorylation at serine/threonine as well as tyrosine residues. Phosphorylation is both stimulatory and inhibitory depending upon the site of phosphorylation (23). For example, phosphorylation at threonine 495 and serine 1177 of eNOS may have opposite effects on the enzymatic activities (23). Phosphorylation of nNOS at threonine 1296 inhibits NO production (46) and activation of PKB activity by cAMP induces eNOS activation (53). On the other hand, phosphorylation of eNOS by Akt at serine 1177 increases the NO production (33). Regulation of NOS by other protein phosphatase, such as PP2A is not quite clear. Therefore, the exact signaling pathways from Pak1 to eNOS require further investigation.
In conclusion, the present study provides the first direct evidence of the behaviour of plasma sphingolipids following transient cardiac ischemia with dramatic and early increases in LCSB in humans. We also demonstrated that S1P, SPH and FTY720 have protective effects against hypoxic/ischaemic cell injury, likely a Pak1/Akt1 signaling cascade and NO release. A further study on a mouse model of cardiac-specific deletion of Pak1 demonstrates a crucial role of Pak1 in cardiac protection against I/R injury.

ACKNOWLEDGEMENT

The project was supported by The Wellcome Trust (ML), The British Heart Foundation (ML, EJC, SES) and National Institutes of Health grants RO1 HL 64035 and PO1 HL 62426 (Project 1) (RJS). We thank Drs Valentine Charlton-Menys and Mohamed A. Shaheen for their assistance and support.

Conflict of interest: none declared.

References

1. Bielawska AE, Shapiro JP, Jiang L, Melkonyan HS, Piot C, Wolfe CL, Tomei LD, Hanun YA, and Umansky SR. Ceramide is involved in triggering of cardiomyocyte apoptosis induced by ischemia and reperfusion. *Am J Pathol* 1997;151: 1257-1263.
2. Bokoch GM. Biology of the P21-activated Kinases. *Ann Rev of Biochem* 2003;72: 743-781.
3. Budde K, Schütz M, Glander P, Peters H, Waiser J, Liefeldt L, Neumayer H-H, and Böhler T. FTY720 (fingolimod) in renal transplantation. *Clin Transplant* 2006;20: 17-24.
4. Cohen MV, Baines CP, and Downey JM. Ischemic Preconditioning: From Adenosine Receptor to KATP Channel. *Ann RevPhysiol* 2000;62: 79-109.
5. Deutschman DH, Carstens JS, Klepper RL, Smith WS, Page MT, Young TR, Gleason LA, Nakajima N, and Sabbadini RA. Predicting obstructive coronary artery disease with serum sphingosine-1-phosphate. *Am Heart J* 2003;146: 62.
6. du Toit EF, McCarthy J, Miyashiro J, Opie LH, and Brunner F. Effect of nitrovasodilators and inhibitors of nitric oxide synthase on ischaemic and reperfusion function of rat isolated hearts. *Br J Pharmacol* 1998;123: 1159-1167.

7. Egom EEA, Ke Y, Musa H, Mohamed TMA, Wang T, Cartwright E, Solaro RJ, and Lei M. FTY720 prevents ischemia/reperfusion injury-associated arrhythmias in an ex vivo rat heart model via activation of Pak1/Akt signaling. *J Mol Cell Cardiol* 2010;48: 406-414.

8. Fujio Y, Nguyen T, Wencker D, Kitsis RN, and Walsh K. Akt Promotes Survival of Cardiomyocytes In Vitro and Protects Against Ischemia-Reperfusion Injury in Mouse Heart. *Circulation* 2000;101: 660-667.

9. Görlich A, BelAiba R, Hess J, and Kietzmann T. Thrombin activates the p21-activated kinase in pulmonary artery smooth muscle cells Role in tissue factor expression. *Thromb Haemost* 2005;93: 1010-1201.

10. Hecker M, Mülsch A, and Busse R. Subcellular Localization and Characterization of Neuronal Nitric Oxide Synthase. *J Neurochem* 1994;62: 1524-1529.

11. Hofmann U, Burkard N, Vogt C, Thoma A, Frantz S, Ertl G, Ritter O, and Bonz A. Protective effects of sphingosine-1-phosphate receptor agonist treatment after myocardial ischaemia-reperfusion. *Cardiovasc Res* 2009;83: 285-293.

12. Huwiler A, Kolter T, Pfeilschiffer J, and Sandhoff K. Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochimica et Biophysica Acta (BBA) - Mol Cell Biol Lip* 2000;1485: 63-99.

13. Itonori S, Takahashi M, Kitamura T, Aoki K, Dulaney JT, and Sugita M. Microwave-mediated analysis for sugar, fatty acid, and sphingoid compositions of glycosphingolipids. *J Lip Res* 2004;45: 574-581.

14. Jin Z-Q, Goetzl EJ, and Karliner JS. Sphingosine Kinase Activation Mediates Ischemic Preconditioning in Murine Heart. *Circulation* 2004;110: 1980-1989.

15. Jin Z-Q, Zhang J, Huang Y, Hoover HE, Vessey DA, and Karliner JS. A sphingosine kinase 1 mutation sensitizes the myocardium to ischemia/reperfusion injury. *Cardiovasc Res* 2007;76: 41-50.

16. Jin Z-Q, Zhang J, Huang Y, Hoover HE, Vessey DA, and Karliner JS. A sphingosine kinase 1 mutation sensitizes the myocardium to ischemia/reperfusion injury. *Cardiovasc Res* 2007;76: 41-50.

17. Jones SP, Girod WG, Granger DN, Palazzo AJ, and Lefer DJ. Reperfusion injury is not affected by blockade of P-selectin in the diabetic mouse heart. *Am J Physiol - Heart Circ Physiol* 1999;277: H763-H769.

18. Karliner JS. Sphingosine Kinase and Sphingosine 1-Phosphate in Cardioprotection. *J Cardiovasc Pharmacol* 2009;53: 189-197.

19. Karliner JS, Honbo N, Summers K, Gray MO, and Goetzl EJ. The Lysophospholipids Sphingosine-1-Phosphate and Lysosphatidic Acid Enhance Survival during Hypoxia in Neonatal Rat Cardiac Myocytes. *J Mol Cell Cardiol* 2001;33: 1713-1717.

20. Kaudel CP, Frink M, van Griensven M, Schmiddem U, Probst C, Bergmann S, Krettek C, Klemmpnauer J, and Winkler M. FTY720 Application Following Isolated Warm Liver Ischemia Improves Long-Term Survival and Organ Protection in a Mouse Model. *Transplant Proceed* 2007;39: 493-498.

21. Kennedy S, Kane KA, Pyne NJ, and Pyne S. Targeting sphingosine-1-phosphate signalling for cardioprotection. *Curr Opin Pharmacol* 2009;9: 194-201.
22. Kim Y-M, Bombeck CA, and Billiar TR. Nitric Oxide as a Bifunctional Regulator of Apoptosis. *Circ Res* 1999;84: 253-256.

23. Kupatt C, Dessy C, Hinkel R, Raake P, Daneau G, Bouzin C, Boekstegers P, and Feron O. Heat Shock Protein 90 Transfection Reduces Ischemia-Reperfusion-Induced Myocardial Dysfunction via Reciprocal Endothelial NO Synthase Serine 1177 Phosphorylation and Threonine 495 Dephosphorylation. *Arterioscler Thromb Vasc Biol* 2004;24: 1435-1441.

24. Kwon Y-G, Min J-K, Kim K-M, Lee D-J, Billiar TR, and Kim Y-M. Sphingosine 1-Phosphate Protects Human Umbilical Vein Endothelial Cells from Serum-deprived Apoptosis by Nitric Oxide Production. *J Biol Chem* 2001;276: 10627-10633.

25. Lamas S, Pérez-Sala D, and Moncada S. Nitric oxide: from discovery to the clinic. *Trends Pharmacol Sci* 1998;19: 436-438.

26. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, and Sack MN. Identification of a Novel Role for Sphingolipid Signaling in TNF [alpha] and Ischemic Preconditioning Mediated Cardioprotection. *J Mol Cell Cardiol* 2002;34: 509-518.

27. Liu W, Min Z, Naumann R, Ke Y, Ulm S, Jin JW, Taglieri D, M, Prehar S, Gui J, Xiao Y, Neyses L, Solaro RJ, Cartwright E, Lei M, and Wang X. PAK1 is a novel signal transducer attenuating cardiac hypertrophy. *under review* 2011.

28. Lobner D. Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis? *J Neurosci Meth* 2000;96: 147-152.

29. Loesch A, Belai A, and Burnstock G. An ultrastructural study of NADPH-diaphorase and nitric oxide synthase in the perivascular nerves and vascular endothelium of the rat basilar artery. *J Neurocytol* 1994;23: 49-59.

30. Maceyka M, Milstien S, and Spiegel S. Shooting the Messenger: Oxidative Stress Regulates Sphingosine-1-Phosphate. *Circ Res* 2007;100: 7-9.

31. Mao K, Kobayashi S, Jaffer ZM, Huang Y, Volden P, Chernoff J, and Liang Q. Regulation of Akt/PKB activity by P21-activated kinase in cardiomyocytes. *J Mol Cell Cardiol* 2008;44: 429-434.

32. Miao W, Luo Z, Kitsis RN, and Walsh K. Intracoronary, Adenovirus-mediated Akt Gene Transfer in Heart Limits Infarct Size Following Ischemia-reperfusion Injury in Vivo. *J Mol Cell Cardiol* 2000;32: 2397-2402.

33. Michell BJ, Griffiths JE, Mitchelhill KI, Rodriguez-Crespo I, Tiganis T, Bozinovski S, de Montellano PRO, Kemp BE, and Pearson RB. The Akt kinase signals directly to endothelial nitric oxide synthase. *Curr Biol* 1999;9: 845.

34. Mohamed TMA, Oceandy D, Prehar S, Alatwi N, Hegab Z, Baudoin FM, Pickard A, Zaki AO, Nadif R, Cartwright EJ, and Neyses L. Specific Role of Neuronal Nitric-oxide Synthase when Tethered to the Plasma Membrane Calcium Pump in Regulating the ß2-Adrenergic Signal in the Myocardium. *J Biol Chem* 2009;284: 12091-12098.

35. Nakanishi K, Zhao Z-Q, Vinten-Johansen J, Hudspheth DA, McGee DS, and Hammon JW. Blood cardioplegia enhanced with nitric oxide donor SPM-5185 outperforms postischemic endothelial and ventricular dysfunction. *J Thoracic Cardiovasc Surg* 1995;109: 1146-1154.

36. Nichols K, Krantis A, and Staines W. Histochemical localization of nitric oxide-synthesizing neurons and vascular sites in the guinea-pig intestine. *Neuroscience* 1992;51: 791-799.
37. Nichols K, Staines W, Rubin S, and Krantis A. Distribution of nitric oxide synthase activity in arterioles and venules of rat and human intestine. *Am J Physiol - Gastroint Liver Physiol* 1994;267: G270-G275.

38. Nofer J-R, van der Giet M, TÄ¶lle M, Wolinska I, von Wnuck Lipinski K, Baba HA, Tietge UJ, GÅ¶decke A, Ishii I, Kleuser B, SchÅ¶fers M, Fobker M, Zidek W, Assmann G, Chun J, and Levkau B. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest* 2004;113: 569-581.

39. Peters SLM, and Alewijnse AE. Sphingosine-1-phosphate signaling in the cardiovascular system. *Curr Opin Pharmacol* 2007;7: 186-192.

40. Pollock JS, Nakane M, Buttery LD, Martinez A, Springall D, Polak JM, Forstermann U, and Murad F. Characterization and localization of endothelial nitric oxide synthase using specific monoclonal antibodies. *Am J Physiol - Cell Physiol* 1993;265: C1379-C1387.

41. Salvadori M, Budde K, Charpentier B, Klemmnauser J, Nashan B, Pallardo LM, Eris J, Schena FP, Eisenberger U, Rostaing L, Hmissi A, Aradhye S, and Group FTYS. FTY720 versus MMF with Cyclosporine in de novo Renal Transplantation: A 1-Year, Randomized Controlled Trial in Europe and Australasia. *Am J Transplant* 2006;6: 2912-2921.

42. Sato H, Zhao Z-Q, McGee DS, Williams MW, Hammon JW, and Vinten-Johansen J. Supplemental l-arginine during cardioplegic arrest and reperfusion avoids regional posts ischemic injury. *J Thoracic Cardiovasc Surg* 1995;110: 302-314.

43. Shiojima I, and K W. Regulation of cardiac growth and coronary angiogenesis by the Akt/PKB signaling pathway. *Genes & Dev* 2006;20: 3347-3365.

44. Siakotos AN, Kulkarni S, and S. P. The quantitative analysis of sphingolipids by determination of long chain base as the trinitrobenzene sulfonic acid derivative. *Lipids* 1971;6: 254-259.

45. Siow RCM, Sato H, and Mann GE. Heme oxygenase*carbon monoxide signalling pathway in atherosclerosis: anti-atherogenic actions of bilirubin and carbon monoxide? *Cardiovasc Res* 1999;41: 385-394.

46. Song T, Hatano N, Kume K, Sugimoto K, Yamaguchi F, Tokuda M, and Watanabe Y. Inhibition of neuronal nitric-oxide synthase by phosphorylation at Threonine1296 in NG108-15 neuronal cells. *FEBS Letters* 2005;579: 5658-5662.

47. Thielmann M, Dorge H, Martin C, Belosjorow S, Schwanke U, van de Sand A, Konietzka I, Buchert A, Kruger A, Schulz R, and Heusch G. Myocardial Dysfunction With Coronary Microembolization: Signal Transduction Through a Sequence of Nitric Oxide, Tumor Necrosis Factor-{alpha}, and Sphingosine. *Circ Res* 2002;90: 807-813.

48. Valschanoff J, Weinberg R, Rustioni A, and Schmidt H. Nitric oxide synthase and GABA colocalize in lamina II of rat spinal cord. *Neurosci Lett* 1992;148: 6-10.

49. Vengellur A, and LaPres J. The Role of Hypoxia Inducible Factor 1{alpha} in Cobalt Chloride Induced Cell Death in Mouse Embryonic Fibroblasts. *Toxicol Sci* 2004;82: 638-646.

50. Vessey DA, Li L, Honbo N, and Karliner JS. Sphingosine 1-phosphate is an important endogenous cardioprotectant released by ischemic pre- and postconditioning. *Am J Physiol Heart Circ Physiol* 2009;297: H1429-1435.
51. Vessey DA, Li L, Kelley M, and Karliner JS. Combined sphingosine, S1P and ischemic postconditioning rescue the heart after protracted ischemia. *BBRC* 2008;375: 425-429.

52. Zhang DX, Fryer RM, Hsu AK, Zou A-P, Gross GJ, Campbell WB, and Li P-L. Production and metabolism of ceramide in normal and ischemic-reperfused myocardium of rats. *Basic Res Cardiol* 2001;96: 267-274.

53. Zhang X-P, and Hintze TH. cAMP signal transduction induces eNOS activation by promoting PKB phosphorylation. *Am J Physiol - Heart and Circu Physiol* 2006;290: H2376-H2384.

54. Zhao Y, Man K, Lo CM, Ng KT, Li XL, Sun CK, Lee TK, Dai XW, and Fan ST. Attenuation of Small-for-Size Liver Graft Injury by FTY720: Significance of Cell-survival Akt Signaling Pathway. *Am J Transplant* 2004;4: 1399-1407.

**Table 1. VT/VF occurrence during reperfusion after IPC**

| Genotype  | During reperfusion |  |  |
|-----------|--------------------|---|---|
|           | Transient VT/VF    | Sustain VT/VF | Total VT/VF occurrence |
| WT        | 3/8 (37.5%)        | 0/8 (0%)     | 3/8 (37.5%)             |
| PAK1<sup>CKO</sup> | 2/6 (33.3%) | 3/6 (50%) | 5/6 (83.3%) |


**Figure legends**

**Figure 1.** Changes in LCBS concentrations in CS blood (A) and comparative concentrations in peripheral blood (B) at different time course points following balloon inflation. Values are mean ± S.E.

**Figure 2.** Effects of S1P, FTY720 and SPH on CoCl2-induced hypoxia (A) and on ischaemic solution-induced ischemia (B). Protective effect of pre-treatment with 100 µM CoCl2 or with ischaemic solution was studied in the presence or absence of S1P (25 nM), FTY720 (25 nM) and SPH (25 nM) (10 µM). *P < 0.01 versus control. § p < 0.01 versus ischemia or CoCl2.

**Figure 3.** FTY720 induces Pak1 and Akt activation. A-B: Representative blots are shown for activation of phospho-Akt and phospho-Pak1 in isolated neonatal rat myocytes treated with FTY720. Myocytes were exposed for 15 min to 25 nM FTY720. C-D: Representative blots for Akt and phospho-Akt proteins in control or 25 nM FTY720 plus 100 ng/ml PTX and for Pak1 and phospho-Pak1 proteins in control or FTY720 plus PTX... Myocytes were treated with FTY720/PTX for 15 min and then assayed for phosphorylation of Akt, Pak1 and by Western blotting. E-F: Akt and phospho-Akt proteins, Pak1 and phospho-Pak1 (Thr 423) were quantified by Western blotting from 4 experiments. Values are mean ± S.E. (n =4 for each group). *, p < 0.05 versus vehicle. G-H: Phosphorylation was quantified by densitometry and normalized to vehicle (Veh) for for Akt and phospho-Akt proteins (G) and Pak1 and phospho-Pak1 proteins (H) from 4 experiments. Values are mean ± S.E. (n =4 for each group). *, p < 0.05 versus vehicle. †, p < 0.05 versus FTY720.
Figure 4. Intracellular NO bioavailability. Intracellular NO levels were assessed in neonatal rat cardiomyocytes by staining with NO-sensitive dye (DAF-FM). A-B, effect of 25 nM FTY720 on nitric oxide (NO) production (A); effect of 25 nM SEW2871 (a specific S1P1 receptor agonist) on NO production (B). Effect of 25 nM FTY720 on NO production in the presence of L-NAME (eNOS, iNOS and nNOS inhibitor) (C), Effect of 25 nM FTY720 on NO production in the presence of SMLT (nNOS inhibitor) (D). Effect of 25 nM FTY720 on NO production in the presence of L-NIL (iNOS inhibitor) (E), Effect of 25 nM FTY720 on NO production in the presence of L-NIO (Potent inhibitor of eNOS) (F). effect of 25 nM FTY720 on NO production in the presence of LY294002 (Potent PI3K inhibitor) (G), effect of 25 nM FTY720 on A6730 (Akt1/2 kinase inhibitor) (H). (n= 64 cells from 3 independent cell preparations in each group, *P<0.05).

Figure 5. Representative ECG recordings from hearts before and after subjected to IPC protocol. Ventricular arrhythmic events including non-sustained and sustained episodes of ventricular tachycardia and ventricular fibrillation were more frequently observed in Pak1cko hearts than control WT hearts. A-C: ECG recordings before IPC. CD: ECG recordings during reperfusion.

Figure 6. The hypothesized mechanisms for sphingolipids in cardioprotective signaling in response to ischemia or hypoxia. First, I/R activates sphingosine kinase 1 (SK1) through a PKCε-dependent mechanism, and induces the enzymatic processing or “shedding” of membrane-bound TNFα (pro-TNF) by metallomatrix proteases (also known as TACE, or TNFα converting enzyme) in the extracellular matrix of the cardiomyocyte. TNFα is released which acts in a paracrine fashion on cardiomyocyte TNFα receptors (TNFRI) that, in turn, activates the sphingomyelin signal transduction
system. The principal signaling molecule produced by the TNFα trigger is SPH, the majority of which crosses the sarcolemma membrane and is released into the extracellular fluid compartment. It is also known that blood platelets and other blood components that possess SK1 convert SPH to S1P. S1P is cardioprotective through both intracellular and putative ‘inside-out’ pathways, the latter involving S1P1 and S1P3 receptors. SPH is also cardioprotective through a PKG/PKA-dependent pathway. Therefore, in contrast to S1P, the cardioprotective effects of sphingosine may not be mediated through S1P-specific G protein coupled receptor.
Figure 1

A. Coronary Sinus blood

B. Peripheral vein blood

n=7; p<0.001

n=24; p<0.001
Figure 2

A

n=3 independent experiments; p<0.01

Total LDH Activity in sample (mU/ml)

Control  CoC2  CoC2 + 25 nM SIP  CoC2 + 25 nM FTY720  CoC2 + 25 nM SfH

B

n=3; p<0.01

Total LDH Activity in sample (mU/ml)

Control  Ischemia  Ischemia + SIP  Ischemia + FTY720  Ischemia + SfH
Figure 3
Figure 3
Figure 4
Figure 5
ISCHEMIA-REPERFUSION (PCI)

TACE

TNF

PKA   PKG

SPH release

“Platelet”

S1P1

S1P3

Figure 6