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**PHARMACOLOGICAL POSTCONDITIONING AGAINST  
MYOCARDIAL INFARCTION WITH A SLOW-RELEASING  
HYDROGEN SULFIDE DONOR, GYY4137**

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**Abbreviated running head:** Postconditioning with GYY4137

**Keywords:** postconditioning, hydrogen sulfide, ischemia-reperfusion, myocardial infarction, reperfusion

Chemical compounds studied in this article

GYY4137-morpholino salt (PubChem CID: 469337261), LY294002 (PubChem CID: 3973), N-Nitro-L-arginine methyl ester hydrochloride (PubChem: 39836)

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**ABSTRACT**

Exogenous hydrogen sulfide (H<sub>2</sub>S) protects against myocardial ischemia/reperfusion injury but the mechanism of action is unclear. The present study investigated the effect of GYY4137, a slow-releasing H<sub>2</sub>S donor, on myocardial infarction given specifically at reperfusion and the signalling pathway involved. Thiobutabarbital-anesthetised rats were subjected to 30 minutes of left coronary artery occlusion and 2 hours reperfusion. Infarct size was assessed by tetrazolium staining. In the first study, animals randomly received either no treatment or GYY4137 (26.6, 133 or 266 µmol kg<sup>-1</sup>) by intravenous injection 10 minutes before reperfusion. In a second series, involvement of PI3K and NO signalling were interrogated by concomitant administration of LY294002 or L-NAME respectively and the effects on the phosphorylation of Akt, eNOS, GSK-3β and ERK1/2 during early reperfusion were assessed by immunoblotting. GYY4137 266 µmol kg<sup>-1</sup> significantly limited infarct size by 47% compared to control hearts (P<0.01). In GYY4137-treated hearts, phosphorylation of Akt, eNOS and GSK-3β was increased 2.8, 2.2 and 2.2 fold respectively at early reperfusion. Co-administration of L-NAME and GYY4137 attenuated the cardioprotection afforded by GYY4137, associated with attenuated phosphorylation of eNOS. LY294002 totally abrogated the infarct-limiting effect of GYY4137 and inhibited Akt, eNOS and GSK-3β phosphorylation. These data are the first to demonstrate that GYY4137 protects the heart against lethal reperfusion injury through activation of PI3K/Akt signalling, with partial dependency on NO signalling and inhibition of GSK-3β during early reperfusion. H<sub>2</sub>S-based therapeutic approaches may have value as adjuncts to reperfusion in the treatment of acute myocardial infarction.

61 **List of abbreviations**

62 AAR = area at risk

63 Akt = protein kinase B

64 DATS = diallyltrisulfide

65 eNOS = endothelial nitric oxide synthase

66 ERK1/2 = Extracellular signal-regulated kinases 1/2 (p42/p44 mitogen activated

67 protein kinase)

68 GSK-3 $\beta$  = glycogen synthase kinase-3 Beta

69 GYY4137 = morpholin-4-ium 4-methoxyphenyl-morpholino-phosphinodithioate

70 H<sub>2</sub>S = hydrogen sulfide

71 IPost-C = ischemic postconditioning

72 mPTP = mitochondrial permeability transition pore

73 NO = nitric oxide

74 PBS = phosphate buffered saline

75 PI3K = Phosphatidylinositol-3-kinase

76 RISK = reperfusion injury salvage kinase (signalling pathway)

77

## 1. INTRODUCTION

In acute myocardial infarction, prompt restoration of coronary blood flow with appropriate reperfusion interventions is essential to salvage ischemic myocardium. Paradoxically, sudden reperfusion induces further irreversible cell injury and death beyond that caused by ischemia. Therefore, reperfusion injury contributes to overall clinical outcome since ultimate infarct size will be determined by both ischemic and reperfusion injuries (Ferdinandy et al., 2014). Reperfusion injury is a challenging but important therapeutic target. The molecular pathology of reperfusion injury is complex and is likely to involve overwhelming oxidative/nitrosative stress, sudden intracellular pH normalisation and cytosolic  $\text{Ca}^{2+}$  oscillation, precipitating opening of the mitochondrial permeability transition pore (mPTP) during the early moments of reperfusion which initiates necrosis (Sluijter et al., 2014, Cabrera-Fuentes et al., 2016).

Ischemic postconditioning (IPost-C) is an experimental manoeuvre in which very brief intermittent periods of ischemia are introduced immediately after reperfusion (Zhao et al., 2003). This intervention has been shown to limit infarct size significantly, most likely through the activation of survival signalling mechanisms that reduce opening of the mPTP (Hausenloy and Yellon, 2007). The so-called “reperfusion injury salvage kinase” (RISK) pathway includes as key components phosphatidylinositol-3-kinase (PI3K)/Akt, and endothelial nitric oxide synthase (eNOS). Other kinases have been described as part of the RISK pathway including extracellular regulated kinase (ERK1/2; p42/p44 mitogen activated protein kinase) and glycogen synthase-3 $\beta$  (GSK-3 $\beta$ ). Although IPost-C is of limited clinical applicability, a number of pharmacological approaches that mimic IPost-C have been

described, including the administration of autacoids and other mediators thought to activate the kinases of the RISK cascade (Burley and Baxter, 2009).

Hydrogen sulfide ( $H_2S$ ) has attracted considerable interest as a cardiovascular autacoid. Although produced endogenously within the myocardium and coronary vasculature (Liu et al., 2012, Hackfort and Mishra, 2016), in coronary artery disease, there may be reduced  $H_2S$  production (Yong et al., 2008, Han et al., 2015, Islam et al., 2015). The administration of exogenous  $H_2S$  donor compounds or increasing endogenous production of  $H_2S$  has been well documented to reduce ischemia-reperfusion injury in experimental models (Elrod et al., 2007, Calvert et al., 2009, King et al., 2014). There is also evidence that  $H_2S$  is a mediator of IPost-C (Bian et al., 2006, Yong et al., 2008, Huang et al., 2012, Das et al., 2015). However, potential therapeutic extrapolation of this knowledge has been hindered by the limitations of  $H_2S$  donor compounds. Much of the experimental literature has reported studies with inorganic sulfide salts ( $Na_2S$  and  $NaSH$ ) which are impure in commercial form and unstable. Despite them being water soluble and inexpensive, a particular issue is that the  $H_2S$  release is largely uncontrollable as they dissociate in aqueous medium instantly to generate  $H_2S$  at high concentration in a short-lasting burst (Papapetropoulos et al., 2015). In contrast to  $Na_2S$  and  $NaSH$ , GYY4137 (morpholin-4-ium 4-methoxyphenyl-morpholino-phosphinodithioate) is a donor compound which releases  $H_2S$  at a slow steady rate at physiological pH and temperature (Li et al., 2008). Several studies have suggested that GYY4137 effectively delivers  $H_2S$  in various physiological systems (Li et al., 2009, Lisjak et al., 2010, Lee et al., 2011, Robinson and Wray, 2012, Liu et al., 2013, Grambow et al., 2014, Meng et al., 2015b). Recent work by Meng et al. (2015a) showed that GYY4137 given prior to

myocardial ischemia protected against injury development and improved post-ischemic recovery of function. However, the therapeutically relevant time window for acute myocardial infarction implies administration as a postconditioning mimetic i.e. immediately prior to reperfusion since this is the time at which clinical therapeutic intervention can feasibly be made.

The aim of the present study was to investigate for the first time the injury limiting effects of GYY4137 at early reperfusion *when given specifically as an adjunct to reperfusion* in a rat model of acute myocardial infarction. We hypothesised that GYY4137 was able to limit reperfusion injury when given just prior to reperfusion, thereby limiting ultimate infarct size. We further hypothesised that the protective action was due to H<sub>2</sub>S release and the activation of key components of the RISK signalling cascade at the first minutes of reperfusion associated with postconditioning, namely PI3K/Akt and eNOS.

## 2. MATERIALS AND METHODS

### 2.1 Animals

Male Sprague Dawley rats, 300-350 g, were purchased from Harlan, UK. They were acclimatised in the institutional animal house at constant temperature and humidity on a 12 hour light/dark cycle for at least seven days prior to experimentation, with free access to water and a small animal diet (Teklad global 14% protein rodent maintenance diet) at all times. All handling and procedures were carried out in accordance with UK Home Office Guidelines on the Animals (Scientific Procedures) Act 1986, (published by the Stationery Office, London, UK). The reporting of animal studies was in accordance with ARRIVE guidelines (Kilkenny et al., 2010, McGrath et al., 2010).

### 2.2 Materials

GY4137 was synthesised by us as previously reported (Li et al., 2008). The purity of GY4137 was determined by NMR spectroscopy ( $^1\text{H}$ ,  $^{31}\text{P}$  and  $^{13}\text{C}$ ). It was identical to a commercial sample from SigmaAldrich. The constitutive nitric oxide synthase (NOS) inhibitor L-nitroarginine methyl ester (L-NAME), the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002, thiobutabarbital sodium salt hydrate (Inactin<sup>®</sup> hydrate), Evans blue dye, triphenyltetrazolium chloride (TTC) and dimethylsulfoxide (DMSO) were all purchased from Sigma-Aldrich, Gillingham, UK. Western blotting antibodies were all sourced from Cell Signalling, UK.

### 2.3 Acute myocardial infarction model

Rats were anaesthetised by intraperitoneal injection of thiobutabarbital sodium (200 mg kg<sup>-1</sup>) and maintained by intravenous supplemental dosing (75 mg kg<sup>-1</sup>) as

required to maintain surgical anesthesia throughout the procedure. Body temperature was maintained at  $37 \pm 1$  °C via rectal thermometer attached to a thermo-regulated blanket unit (Harvard Apparatus Ltd, Cambridge, UK). The right common carotid artery was cannulated and connected to a pressure transducer to measure heart rate and blood pressure throughout the procedure (Powerlab data acquisition system, AD instruments, Abingdon, UK). The left jugular vein was cannulated for drug administration. The trachea was cannulated via tracheotomy and the animal ventilated with room air by a small animal volume controlled ventilator (Hugo Sachs Elektronik, March, Germany) at a rate of 75 strokes  $\text{min}^{-1}$  and tidal volume of 1.0 to 1.25 mL  $100 \text{ g}^{-1}$ . The electrocardiogram was recorded using standard lead II electrodes inserted subcutaneously into the limbs and connected to a Powerlab data acquisition system. A midline sternotomy was performed and the chest opened using a metal retractor to expose the heart. After pericardiotomy, a 4/0 braided silk suture (Mersilk, Ethicon Ltd, UK) was placed around the left main coronary artery close to its origin from the left border of the pulmonary conus. The animal was left to stabilise for 20 minutes during which the two ends of the silk ligature remained loose. For each animal to be included it had to achieve the following hemodynamic parameters during the stabilisation period: heart rate  $\geq 250$  beats per minute, diastolic blood pressure  $\geq 50$  mmHg, steady sinus rhythm, no signs of ischemia or arrhythmia during the stabilisation period.

After stabilisation, the ligature was pulled taut through a plastic snare and fastened against the epicardium to induce regional ischemia for 30 minutes. Ischemia was confirmed by a drop in the mean arterial pressure (MAP), a colour change of the left ventricle (from red to pale), and ECG changes (ST-segment elevation). After 30

minutes, the snare was released to allow reperfusion for 120 minutes. Successful reperfusion was confirmed by hyperemic colour change of the ischemic tissue bed, occurrence of reperfusion-induced arrhythmia during the first minute after reperfusion, and an increase in the MAP.

## **2.4 Infarct size determination**

After 120 min reperfusion, the heart was excised and perfused via the aorta with saline on a modified Langendorff apparatus. After re-occluding the coronary ligature, the heart was perfused with 2% Evans' blue dye to identify the ischemic zone (area at risk, AAR). The heart was then frozen at -20 °C for 5-24 hours. The frozen heart was transversely sliced at 2 mm thickness into 5-6 sections from apex to base and the sections incubated with triphenyltetrazolium chloride (TTC) 1% w/v in phosphate buffered saline (PBS; pH 7.4) at 37 °C for 15 minutes. TTC is reduced to a red formazan pigment in viable tissue while necrotic tissue is unstained. Stained sections were fixed in 4% formalin in PBS for 24 hours before being scanned. Planimetry was conducted using the image analysis program Image J (version 1.47, NIH, Bethesda, USA). Sections were coded so that image analysis was undertaken in a blinded fashion to obviate bias. Planimetric analysis determined the total ventricular area, the AAR (Evans blue negative), and the infarcted area (TTC negative). These areas were then converted into volumes by multiplying each total area by 2mm section thickness and the infarct size was reported as a percentage of the area at risk volume (% I/AAR).

## **2.5 Treatment protocols**

Treatment protocols are illustrated in Figure 1. Two separate series of experiments were undertaken. The first series examined the dose-dependent effects of GYY4137

on infarct size and the involvement of H<sub>2</sub>S in mediating any responses. The dose range employed in these studies was derived from previous studies in the rat heart *ex vivo* by our group (Suveren et al., 2012) and *in vivo* studies conducted by others (Li et al., 2008, Meng et al., 2015a). Animals were randomly assigned to one of five groups (Figure 1A):

- Group 1: Control (n=9). Animals were subjected to coronary occlusion and reperfusion with saline given as a slow i.v. bolus 10 minutes before reperfusion.
- Group 2-4: Each group (n=8) received GYY4137 at 26.6, 133 or 266  $\mu\text{mol kg}^{-1}$ , respectively) as a slow i.v. bolus (500 $\mu\text{L min}^{-1}$ ) 10 minutes before reperfusion.
- Group 5: Depleted GYY4137 (n=6). GYY4137 solution (100 mg mL<sup>-1</sup>) was prepared in saline and left uncovered for 72 hours at room temperature to dissipate all H<sub>2</sub>S, then administered at a dose of 266  $\mu\text{mol kg}^{-1}$  i.v. 10 minutes before reperfusion.

The second series of experiments explored the involvement of RISK pathway components in the cardioprotective effect of GYY4137. The optimum dose of GYY4137 (266  $\mu\text{mol kg}^{-1}$ ) was selected from the first series and animals were randomised into six treatment groups (Figure 1B).

- Group 6: Control (n=7). Animals were subjected to coronary occlusion and reperfusion with saline or DMSO 5% given as a slow i.v. bolus 15 minutes before reperfusion. DMSO was used as vehicle for LY294002. Since DMSO exerted no effect on cardiodynamics or infarct size, saline and DMSO treated animals are reported collectively.

- Group 7: GYY4137 (n=7). A slow bolus dose of GYY4137 ( $266 \mu\text{mol kg}^{-1}$ ,  $500 \mu\text{L min}^{-1}$ ) was administered at 10 minutes before reperfusion.
- Group 8: GYY4137 + L-NAME (n=7). An intravenous bolus dose of L-NAME ( $20 \text{ mg kg}^{-1}$ ) was administered 15 minutes before reperfusion followed by GYY4137 ( $266 \mu\text{mol kg}^{-1}$ ,  $500 \mu\text{L min}^{-1}$ ) 10 minutes before reperfusion.
- Group 9: L-NAME (n=6). An intravenous bolus dose of L-NAME ( $20 \text{ mg kg}^{-1}$ ) was administered 15 minutes before reperfusion.
- Group 10: GYY4137 + LY294002 (n=6). An intravenous bolus dose of LY294002 ( $0.1 \text{ mg kg}^{-1}$  in 5% DMSO) was given 15 minutes before reperfusion followed by GYY4137 ( $266 \mu\text{mol kg}^{-1}$ ,  $500 \mu\text{L min}^{-1}$ ) 10 minutes before reperfusion.
- Group 11: LY294002 (n=6). A bolus dose of LY294002 ( $0.1 \text{ mg kg}^{-1}$  in 5% DMSO) was administered intravenously 15 minutes before reperfusion.

In a parallel series of experiments, rats were subjected to the same interventions as in groups 6 to 11 to prepare samples for biochemical analysis. After 5 minutes of reperfusion, the experiment was terminated and myocardial biopsies were harvested from the left ventricle, rapidly frozen in liquid nitrogen then kept at  $-80^\circ\text{C}$  for Western blotting of Akt, eNOS, GSK-3 $\beta$  and ERK1/2.

## 2.6 Western blotting analysis

To investigate the involvement of Akt, eNOS, GSK-3 $\beta$  and ERK1/2, protein immunoblotting was carried out to analyse protein phosphorylation at 5 minutes of reperfusion. Myocardial biopsies were homogenised and lysed using a hard tissue lysing kit (Stretton Scientific Ltd, Stretton, UK). Equal amounts of protein were

loaded onto 10% w/v sodium dodecyl sulfate-polyacrylamide gel, separated electrophoretically (120 mV) and transferred onto nitrocellulose membrane (Amersham, Germany). The membrane was then blocked with 5% skimmed milk for 2 hours and probed with the primary antibody overnight at 4 °C. The following antibodies were used: Akt (1:1000), phospho- ser<sup>473</sup>Akt (1:1000), endothelial nitric oxide synthase (eNOS 1:500), phospho- ser<sup>1177</sup>eNOS (1:500), glycogen-synthase kinase-3 beta (GSK-3 $\beta$  1:1000), phospho- ser<sup>9</sup>GSK-3 $\beta$  (1:1000), extracellular signal-regulated kinases ERK 1/2 (1:1000), phospho- Thr<sup>202</sup>/Tyr<sup>204</sup> ERK 1/2 (1:1000) and GAPDH (1:50000). The immunoblots were probed with secondary antibody (goat anti-rabbit HRP, 1:15000, Cell Signalling UK) for 1 hour then probed with Super Signal West Dura Extended Duration Substrate (Thermo Scientific) to visualise the bands on X-ray film. The film was scanned and densitometry was conducted in a blinded fashion using Image J software (1.48v, National Institutes of Health USA). Phosphorylated and total protein bands were normalised to corresponding GAPDH bands and to baseline samples, harvested after 20 minutes of stabilisation, loaded at either side of each gel.

## 2.7 Statistical analysis

All data are reported as arithmetic mean  $\pm$  SEM. Data were analysed using GraphPad Prism<sup>®</sup> software (2007, Version 5.01, USA). Cardiodynamics including rate-pressure product (RPP, heart rate \* systolic blood pressure) and mean arterial pressure (MAP, diastolic pressure + 1/3[systolic pressure - diastolic pressure]) were statistically analysed using repeated measures ANOVA supported by Bonferroni's *post hoc* test. Baseline data including body weight, RPP, and MAP passed the Kolmogorov-Smirnov normality test of distribution. Infarct size data were analysed

295 using one way ANOVA supported by Newman-Keuls *post hoc* test. Differences  
296 between groups were considered significant if  $p < 0.05$ .

### 3. RESULTS

In series 1, 42 rats were used, of which two were excluded from final analysis, one due to failure of TTC staining and one rat which did not survive the ischemia-reperfusion protocol. Thus data for 40 successfully completed experiments are reported. In series 2, 66 rats were employed, of which three did not complete the ischemia-reperfusion protocol. Thus, data from a total of 63 completed experiments are reported in series 2: these comprised 39 completed infarct size experiments and 24 preparations for Western blot analysis.

#### 3.1 Hemodynamic parameters

Baseline hemodynamics for series 1 and 2 are summarised in Table 1. There was no significant difference in any of the parameters among the experimental groups. Cardiodynamics (MAP and RPP) measurements before ischemia, during ischemia and at the end of reperfusion are also presented in Table 1. GYY4137 had no detectable effect on cardiodynamics during the ischemia-reperfusion protocol.

#### 3.2 Infarct size following GYY4137 postconditioning

Series 1 examined the response to three doses of GYY4137 on infarct size (Figure 2). AAR constituted approximately 40-60% of the total ventricular volume with no significant differences among the treatment groups (Figure 2A). Control infarct size (%I/AAR) was  $52.5 \pm 4.7\%$  (Figure 2B). GYY4137 ( $266 \mu\text{mol kg}^{-1}$ ) produced significant infarct limitation when given 10 minutes before reperfusion compared to control hearts ( $27.9 \pm 3.8\%$  vs  $52.5 \pm 4.7\%$ ,  $p < 0.01$ ). This represents a 47% relative reduction in infarct size. In contrast, depleted-GYY4137 (produced as described in Alexander *et al.*, 2015) which lacked  $\text{H}_2\text{S}$  donating potential but was otherwise

structurally identical had no effect on infarct size at the same dose ( $51.9 \pm 3.1\%$ ), confirming the dependency of GYY4137's infarct-limiting action on H<sub>2</sub>S release.

### 3.3 Involvement of PI3K/Akt and eNOS in GYY4137 postconditioning

The second series of experiments was undertaken to examine components of the RISK signalling pathway in the protective effect of GYY4137 (Figure 3). There was no significant difference in the AAR among the experimental groups (Figure 3A). GYY4137 ( $266 \mu\text{mol kg}^{-1}$ ) elicited a significant reduction in %I/AAR compared to control ( $27.6 \pm 2.0\%$  vs  $56.8 \pm 3.5\%$ , respectively,  $p < 0.001$ , Figure 3B).

Pharmacological inhibition of eNOS with L-NAME prior to GYY4137 almost halved the cardioprotective effect of GYY4137 ( $41.1 \pm 6.3\%$  vs  $27.6 \pm 2.0\%$ , respectively,  $p < 0.05$ ), but did not abolish it ( $41.1 \pm 6.3\%$  vs  $56.8 \pm 3.5\%$ , respectively,  $p < 0.01$ , Figure 3B). Concomitant administration of LY294002 to inhibit PI3K activity completely abrogated the cardioprotective effect of GYY4137 ( $49.8 \pm 4.2\%$  vs  $56.8 \pm 3.5\%$ , respectively,  $p > 0.05$ ). Neither L-NAME nor LY294002 had any effect on infarct size when given alone ( $55.7 \pm 3.3\%$  and  $51.2 \pm 2.7\%$  respectively, both  $p > 0.05$  vs control).

The extent of phosphorylation of Akt, eNOS, GSK-3 $\beta$  and ERK1/2 in early reperfusion was investigated with phospho-specific antibodies to determine the possible roles in cardioprotection by GYY4137. Immunoreactivity measurements were performed using myocardial tissue samples harvested from the left ventricle 5 minutes after reperfusion and are presented in Figure 4A-D. There was no significant difference in protein expression to GAPDH of Akt, eNOS, GSK-3 $\beta$  or ERK1/2 among any of the experimental groups. There was a significant 2.8-fold

increase ( $p < 0.001$  vs. control) in phospho-ser<sup>473</sup>Akt at reperfusion following GYY4137 treatment (Figure 4A). Prior administration of L-NAME did not limit this increase in Akt phosphorylation. However, administration of LY294002 alone or prior to GYY4137 abolished Akt phosphorylation (Figure 5A). Postconditioning with GYY4137 also increased eNOS phosphorylation at the activating ser<sup>1177</sup> site by 2.2-fold in early reperfusion ( $p < 0.01$  vs. control; Figure 4B). This activation was abrogated by prior administration of either L-NAME or LY294002. Ser<sup>9</sup> phosphorylation of GSK-3 $\beta$  was also increased 2.2-fold by GYY4137 (Figure 4C). This phosphorylation, leading to inactivation of GSK-3 $\beta$ , was not affected L-NAME. However, pre-treatment with LY294002 prior to GYY4137 abrogated GSK-3 $\beta$  phosphorylation. GYY4137 had no significant effect on the phosphorylation of ERK1/2 at early reperfusion (Figure 4D).

## 4. DISCUSSION

The principal observations of this study can be summarised as follows:

1. GYY4137 limited myocardial infarction *in vivo* when given specifically prior to reperfusion indicating potent attenuation of lethal reperfusion injury in a postconditioning-like manner.
2. The infarct-limiting effect of GYY4137 at early reperfusion was mediated through activation of the PI3K/Akt survival cascade.
3. There was a partial dependency of GYY4137's protective effect on increased eNOS activation.
4. GYY4137 inhibited GSK-3 $\beta$  activity at early reperfusion by increasing the phosphorylation of its ser<sup>9</sup> site downstream of PI3K/Akt signalling.

These findings support the hypothesis that administration of GYY4137 at reperfusion can protect the heart against reperfusion injury by activating the key components of the RISK cascade (PI3K/Akt/NO) and inhibition of GSK-3 $\beta$  activity.

### 4.1 Infarct limitation by GYY4137

The results show for the first time the effect of GYY4137, as a slow-releasing H<sub>2</sub>S donor, on myocardial infarction in an *in vivo* model. Intracellular levels of H<sub>2</sub>S are reported to be decreased during ischaemia-reperfusion as a results of overwhelming ROS generation which limits H<sub>2</sub>S synthesis and increases its degradation (Vandiver and Snyder, 2012). GYY4137 elicited significant infarct limitation when administered prior to reperfusion. Depleted GYY4137 (Alexander et al., 2015) was employed as a control to ensure that any detectable effect was due to H<sub>2</sub>S released and not by the parent molecule or by-products formed from GYY4137 decomposition. Depleted GYY4137 had no effect on infarct size and this is consistent with previous studies

where loss of H<sub>2</sub>S from GYY4137 was shown to be associated with loss of biological activity (Li et al., 2009, Whiteman et al., 2010, Fox et al., 2012, Jamroz-Wisniewska et al., 2014, Alexander et al., 2015).

This is the first study of pharmacological postconditioning against reperfusion injury *in vivo* using GYY4137 as a stable H<sub>2</sub>S donor. Although inorganic H<sub>2</sub>S generators (NaSH and Na<sub>2</sub>S) have been used in different experimental species, the specific targeting of reperfusion injury by GYY4137 in this study is novel. Several studies have investigated the effect of H<sub>2</sub>S against myocardial ischemia-reperfusion when commercially available sulfide salts were perfused or given pre-ischemia. For example, Johansen et al. (2006) were the first to show that NaSH limited infarct size in a rat isolated heart preparation, while Pan et al. (2009), Sivarajah et al. (2009), Zhuo et al. (2009) and Yao et al. (2012) all showed that NaSH limited infarct size in an *in vivo* rat model through diverse mechanisms. Part of this variation is arguably due to the unstable nature of these H<sub>2</sub>S sources, in addition to the different experimental conditions and end-points of interest. Using garlic derivative as an organic source of H<sub>2</sub>S, Zhang et al. (2001) and Chuah et al (2007) reported that allitridum and S-allylcysteine respectively also elicited cardioprotection against myocardial infarction when given before ischemia. Preconditioning the heart with the thiol derivative S-diclofenac was also protective partially through the opening of mitochondrial K<sub>ATP</sub> channels (Rossoni et al., 2008). Investigators also have examined the possibility of postconditioning the myocardium using NaSH and Na<sub>2</sub>S. For example, Elrod et al. (2007), Sodha et al. (2009) and Lambert et al. (2014) all reported that Na<sub>2</sub>S protected mouse heart against myocardial infarction *in vivo* when given at reperfusion. Bibli et al. (2015) showed that a bolus dose of NaSH 10 minutes before reperfusion then continuous infusion of NaSH till the end of reperfusion was required to significantly exert cardioprotection in rabbit. In

comparison with these results, in this study we showed that a single bolus dose of GYY4137 at reperfusion had a significant cardioprotective effect against myocardial infarction in the rat. To our knowledge, the only other long-lasting H<sub>2</sub>S donors that have been reported are the polysulfide diallyl trisulfide (DATS) and SG-1002, a thiol-activated H<sub>2</sub>S donor. Despite generating 10 times less H<sub>2</sub>S than Na<sub>2</sub>S, DATS was shown to improve mitochondrial respiration and stimulate eNOS at reperfusion in an *in vivo* mouse model of ischemia-reperfusion injury. However, DATS is a polysulfide compound, and thus cannot be considered a pure H<sub>2</sub>S donor with the possibility of off-target effects. Moreover, H<sub>2</sub>S release from GYY4137 is reported to last longer compare to DATS (Li et al., 2008, Predmore et al., 2012). In the setting of pressure-overload-induced heart failure, SG-1002-treated hearts were protected during transverse aortic constriction via triggering VEGF/Akt/eNOS/NO/cGMP pathway. Recently, SG-1002 has successfully passed Phase I clinical study in patient with heart failure (ClinicalTrials.gov #NCT01989208 and #NCT02278276), by increasing blood H<sub>2</sub>S level and circulating NO bioavailability (Polhemus et al., 2015). However, none of these studies have shown that the observed effects are due to H<sub>2</sub>S release due to the lack of negative control (like depleted GYY4137, for example). Therefore, there is persuasive experimental evidence that a stable level of H<sub>2</sub>S release confers effective cardioprotection against ischemia-reperfusion injury. The present study confirms for the first time that administration of GYY4137 prior to reperfusion (postconditioning), rather than prior to coronary artery occlusion (preconditioning), exerts a marked cardioprotective effect due to H<sub>2</sub>S-releasing capacity.

#### **4.2 GYY4137 postconditioning activates PI3K/Akt signalling**

The second series of experiments aimed to explore the signalling mechanisms underpinning the protective effect of GYY4137. The involvement during early

reperfusion of specific kinase mechanisms, notably activation of PI3K/Akt and/or ERK1/2, activation of eNOS and inhibition of GSK-3 $\beta$ , has attracted considerable attention in relation to cardiac conditioning phenomena, especially postconditioning. Elucidation of the RISK pathway has confirmed that it is a key modulator of protection against reperfusion injury in many species, although not all. Here, we explored the effects of pharmacological inhibition of two key components, PI3K/Akt and eNOS, confirmed by assessment of the phosphorylation status of these proteins. We found that the PI3K inhibitor LY294002 abrogated the infarct-limiting effect of GYY4137 which indicated the involvement of PI3K/Akt survival pathway in cardioprotection established by GYY4137. This was supported by the observation that GYY4137 increased Akt phosphorylation in left ventricular myocardium during early reperfusion, an effect abolished by LY294002. Li et al. (2015a) showed that NaSH at reperfusion limited cell death by activating PI3K/Akt pathway in aging rat heart and cardiomyocytes. However, Lambert et al. (2014) demonstrated that in diabetic rats NaSH-induced postconditioning might signal through the other arm of the RISK pathway, namely ERK1/2. LY294002 alone had no significant effect on either the infarct size or Akt phosphorylation compared to control which is consistent with the findings of other investigators (Wang et al., 2013, Barsukevich et al., 2015). This suggests that the PI3K/Akt pathway is almost inactive at basal physiological levels of H<sub>2</sub>S.

We also investigated the involvement of ERK1/2 in cardioprotection established by GYY4137. In contrast to Akt phosphorylation, we observed no significant increase in ERK1/2 phosphorylation at early reperfusion following postconditioning with GYY4137. It has been reported by others that a bolus dose of Na<sub>2</sub>S at reperfusion could activate ERK1/2 and also inhibit GSK-3 $\beta$  (Lambert et al., 2014, Li et al., 2015b,

Bibli et al., 2015). However, since in our hands GSK-3 $\beta$  phosphorylation (leading to enzyme inhibition) by GYY4137 was abrogated by LY294002, this suggests it is downstream of PI3K/Akt, rather than ERK1/2. It again emphasises the physiological differences between bolus sulfide (with NaSH or Na<sub>2</sub>S) and H<sub>2</sub>S generated in a more physiological manner (with GYY4137).

#### 4.3 Dependency of GYY4137-postconditioning on NO

Inhibition of NO synthesis using L-NAME had no effect on the infarct size *per se* which is consistent with other investigators (Fradorf et al., 2010, Imani et al., 2011). This observation implies that NO does not afford any cardioprotection against myocardial infarction at basal physiological levels. GYY4137 treatment induced an increase in the phosphorylation of eNOS at its activating site, ser<sup>1177</sup> suggesting that NO bioavailability is increased following GYY4137 treatment. L-NAME prior to GYY4137 administration limited the phosphorylation of eNOS and partially attenuated infarct limitation but did not completely abolish the protective effect. These data suggest that enhancing NO bioavailability synergises the cardioprotection of GYY4137 against reperfusion injury but blocking eNOS phosphorylation only partially limits the cardioprotection of GYY4137, suggesting the involvement of parallel NO-independent pathway(s). There has been considerable interest in cross-regulation of NO and H<sub>2</sub>S but the nature of their interactions is uncertain, at least in part because of the large variation in experimental conditions.

SG-1002, H<sub>2</sub>S donor, was protective and increased NO bioavailability in an *in vivo* model of heart failure (Kondo et al., 2013). An increase in NO metabolites following DATS treatment was also observed by Lefer and co-workers (2012) in mouse heart. King et al. (2014) found that H<sub>2</sub>S did not limit infarction in eNOS phospho-mutant

(S1179A) or eNOS knockout mice. Considered together, these studies suggest that an increase in one of the gaseous mediators can eventually lead to an increase in the other but the picture is obscured by variations across species, pathological models and tissue types. The NO-dependency of H<sub>2</sub>S has recently been studied by Bibli et al. (2015) in an *in vivo* model of myocardial infarction using two species, rabbit and mouse. Pharmacologically limiting NO availability with L-NAME did not limit the protection of NaSH in rabbits, while genetic mutation or pharmacological blockade of eNOS totally abolished H<sub>2</sub>S-induced protection in mice. Dependency of NaSH-induced cardioprotection on NO in mice was previously reported by Sojitra et al. (2012). Together and in line with our data, it seems plausible that NO involvement in the infarct-limiting effect of H<sub>2</sub>S could be tissue and/or species-dependent. Further detailed work needs to be carried out for better understanding of the molecular pharmacology of these molecules and to enhance the clinical implementation of H<sub>2</sub>S-delivering systems.

#### **4.4 GYY4137 postconditioning attenuates GSK-3 $\beta$ phosphorylation**

GSK-3 $\beta$  has been proposed as one of the key end effectors of some cardioprotective manoeuvres, particularly ischemic conditioning phenomena. It has been demonstrated that GSK-3 $\beta$  promotes the opening of mPTP during reperfusion, an event thought to be a major determinant of cell death (Cabrera-Fuentes et al., 2016). In isolated cardiomyocytes, Yao et al. (2010) and Li et al. (2015b) found that NaSH protected against hypoxia/reoxygenation induced cell death by inhibiting GSK-3 $\beta$ -dependent opening of mPTP. In line with these results, the present study demonstrated that GYY4137 increased the phosphorylation of GSK-3 $\beta$  at Ser<sup>9</sup> site at reperfusion. This was abolished by LY294002, but not by L-NAME, suggesting that GYY4137 induced inhibition of GSK-3 $\beta$  is downstream of PI3K/Akt. There is

evidence that the increase in Akt phosphorylation (Hausenloy et al., 2009) and NO bioavailability (Burley et al., 2007) at early reperfusion may also inhibit the opening of mPTP. Considering these data together, it seems plausible that postconditioning with GYY4137 is associated with a reduced susceptibility of mPTP opening, although this remains to be determined by specific measurements of mPTP opening.

#### **4.5 Study limitations**

There are still questions which this study did not address and they could be interesting topics for further investigations. This study found that GYY4137 activates the RISK pathway at early minutes of reperfusion to limit the infarct size where infarction was quantified after 2 hours of reperfusion. Nevertheless, whether GYY4137 could exert a comparable cardioprotection via similar or different mechanism(s) with longer reperfusion protocol, where there could be no-flow phenomena or late apoptosis, needs to be investigated. Although spent-GYY4137 did not exert any cardioprotection, the direct effect of GYY4137 administration on the level of H<sub>2</sub>S in the heart and circulation needs to be measured. Similarly, measuring the proposed elevation in NO bioavailability as a result of activating eNOS at reperfusion by GYY4137 administration could also underpin the conclusion.

#### **4.6 Conclusion**

In summary, we have demonstrated that the slow-releasing H<sub>2</sub>S donor GYY4137, but not its H<sub>2</sub>S-depleted control, protected the heart against lethal reperfusion injury when administered as an adjunct treatment prior to reperfusion. This cardioprotective action is dependent on activation of PI3K/Akt signalling pathway at early reperfusion, which in turn, increases NO bioavailability by increasing eNOS phosphorylation, and increases the phosphorylation of GSK-3 $\beta$  (see Figure 5, Graphical Abstract). Thus,

stable slow-releasing H<sub>2</sub>S donor compounds may be promising candidates for the development of adjunct therapies to reperfusion for the treatment of acute myocardial infarction.

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### **Conflicts of interest**

None.

## References

- ALEXANDER, B. E., COLES, S. J., FOX, B. C., KHAN, T. F., MALISZEWSKI, J., PERRY, A., PITAK, M. B., WHITEMAN, M. & WOOD, M. E. 2015. Investigating the generation of hydrogen sulfide from the phosphoramidodithioate slow-release donor GYY4137. *Medicinal Chemical Communications*, 6, 1649-1655.
- BARSUKEVICH, V., BASALAY, M., SANCHEZ, J., MROCHEK, A., WHITTLE, J., ACKLAND, G. L., GOURINE, A. V. & GOURINE, A. 2015. Distinct cardioprotective mechanisms of immediate, early and delayed ischaemic postconditioning. *Basic Res Cardiol*, 110, 452.
- BIAN, J. S., YONG, Q. C., PAN, T. T., FENG, Z. N., ALI, M. Y., ZHOU, S. & MOORE, P. K. 2006. Role of hydrogen sulfide in the cardioprotection caused by ischemic preconditioning in the rat heart and cardiac myocytes. *J Pharmacol Exp Ther*, 316, 670-8.
- BIBLI, S. I., ANDREADOU, I., CHATZIANASTASIOU, A., TZIMAS, C., SANOUDOU, D., KRANIAS, E., BROUCKAERT, P., COLETTA, C., SZABO, C., KREMASTINOS, D. T., ILIODROMITIS, E. K. & PAPAPETROPOULOS, A. 2015. Cardioprotection by H<sub>2</sub>S engages a cGMP-dependent protein kinase G/phospholamban pathway. *Cardiovasc Res*, 106, 432-42.
- BURLEY, D. S. & BAXTER, G. F. 2009. Pharmacological targets revealed by myocardial postconditioning. *Curr Opin Pharmacol*, 9, 177-88.
- BURLEY, D. S., FERDINANDY, P. & BAXTER, G. F. 2007. Cyclic GMP and protein kinase-G in myocardial ischaemia-reperfusion: opportunities and obstacles for survival signaling. *Br J Pharmacol*, 152, 855-69.
- CABRERA-FUENTES, H. A., ALBA-ALBA, C., ARAGONES, J., BERNHAGEN, J., BOISVERT, W. A., BOTKER, H. E., CESARMAN-MAUS, G., FLEMING, I., GARCIA-DORADO, D., LECOUR, S., LIEHN, E., MARBER, M. S., MARINA, N., MAYR, M., PEREZ-MENDEZ, O., MIURA, T., RUIZ-MEANA, M., SALINAS-ESTEFANON, E. M., ONG, S. B., SCHNITTNER, H. J., SANCHEZ-VEGA, J. T., SUMOZA-TOLEDO, A., VOGEL, C. W., YARULLINA, D., YELLON, D. M., PREISSNER, K. T. & HAUSENLOY, D. J. 2016. Meeting report from the 2nd International Symposium on New Frontiers in Cardiovascular Research. Protecting the cardiovascular system from ischemia: between bench and bedside. *Basic Res Cardiol*, 111, 7.
- CALVERT, J. W., JHA, S., GUNDEWAR, S., ELROD, J. W., RAMACHANDRAN, A., PATTILLO, C. B., KEVIL, C. G. & LEFER, D. J. 2009. Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. *Circ Res*, 105, 365-74.
- CHUAH, S. C., MOORE, P. K. & ZHU, Y. Z. 2007. S-allylcysteine mediates cardioprotection in an acute myocardial infarction rat model via a hydrogen sulfide-mediated pathway. *Am J Physiol Heart Circ Physiol*, 293, H2693-701.
- DAS, A., SAMIDURAI, A., HOKE, N. N., KUKREJA, R. C. & SALLOUM, F. N. 2015. Hydrogen sulfide mediates the cardioprotective effects of gene therapy with PKG-1 $\alpha$ . *Basic Res Cardiol*, 110, 42.
- ELROD, J. W., CALVERT, J. W., MORRISON, J., DOELLER, J. E., KRAUS, D. W., TAO, L., JIAO, X., SCALIA, R., KISS, L., SZABO, C., KIMURA, H., CHOW, C. W. & LEFER, D. J. 2007. Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. *Proc Natl Acad Sci U S A*, 104, 15560-5.
- FERDINANDY, P., HAUSENLOY, D. J., HEUSCH, G., BAXTER, G. F. & SCHULZ, R. 2014. Interaction of risk factors, comorbidities, and comedications with

- ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. *Pharmacol Rev*, 66, 1142-74.
- FOX, B., SCHANTZ, J. T., HAIGH, R., WOOD, M. E., MOORE, P. K., VINER, N., SPENCER, J. P., WINYARD, P. G. & WHITEMAN, M. 2012. Inducible hydrogen sulfide synthesis in chondrocytes and mesenchymal progenitor cells: is H<sub>2</sub>S a novel cytoprotective mediator in the inflamed joint? *J Cell Mol Med*, 16, 896-910.
- FRADORF, J., HUHN, R., WEBER, N. C., EBEL, D., WINGERT, N., PRECKEL, B., TOMA, O., SCHLACK, W. & HOLLMANN, M. W. 2010. Sevoflurane-induced preconditioning: impact of protocol and aprotinin administration on infarct size and endothelial nitric-oxide synthase phosphorylation in the rat heart in vivo. *Anesthesiology*, 113, 1289-98.
- GRAMBOW, E., MUELLER-GRAF, F., DELYAGINA, E., FRANK, M., KUHLA, A. & VOLLMAR, B. 2014. Effect of the hydrogen sulfide donor GYY4137 on platelet activation and microvascular thrombus formation in mice. *Platelets*, 25, 166-74.
- HACKFORT, B. T. & MISHRA, P. K. 2016. Emerging role of hydrogen sulfide-microRNA cross-talk in cardiovascular diseases. *Am J Physiol Heart Circ Physiol*, ajpheart 00660 2015.
- HAN, S. J., KIM, J. I., PARK, J. W. & PARK, K. M. 2015. Hydrogen sulfide accelerates the recovery of kidney tubules after renal ischemia/reperfusion injury. *Nephrol Dial Transplant*, 30, 1497-506.
- HAUSENLOY, D. J., ONG, S. B. & YELLON, D. M. 2009. The mitochondrial permeability transition pore as a target for preconditioning and postconditioning. *Basic Res Cardiol*, 104, 189-202.
- HAUSENLOY, D. J. & YELLON, D. M. 2007. Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. *Heart Fail Rev*, 12, 217-34.
- HUANG, Y. E., TANG, Z. H., XIE, W., SHEN, X. T., LIU, M. H., PENG, X. P., ZHAO, Z. Z., NIE, D. B., LIU, L. S. & JIANG, Z. S. 2012. Endogenous hydrogen sulfide mediates the cardioprotection induced by ischemic postconditioning in the early reperfusion phase. *Exp Ther Med*, 4, 1117-1123.
- IMANI, A., FAGHIHI, M., SADR, S. S., NIARAKI, S. S. & ALIZADEH, A. M. 2011. Noradrenaline protects in vivo rat heart against infarction and ventricular arrhythmias via nitric oxide and reactive oxygen species. *J Surg Res*, 169, 9-15.
- ISLAM, K. N., POLHEMUS, D. J., DONNARUMMA, E., BREWSTER, L. P. & LEFER, D. J. 2015. Hydrogen Sulfide Levels and Nuclear Factor-Erythroid 2-Related Factor 2 (NRF2) Activity Are Attenuated in the Setting of Critical Limb Ischemia (CLI). *J Am Heart Assoc*, 4.
- JAMROZ-WISNIEWSKA, A., GERTLER, A., SOLOMON, G., WOOD, M. E., WHITEMAN, M. & BELTOWSKI, J. 2014. Leptin-induced endothelium-dependent vasorelaxation of peripheral arteries in lean and obese rats: role of nitric oxide and hydrogen sulfide. *PLoS One*, 9, e86744.
- JOHANSEN, D., YTREHUS, K. & BAXTER, G. F. 2006. Exogenous hydrogen sulfide (H<sub>2</sub>S) protects against regional myocardial ischemia-reperfusion injury--Evidence for a role of K ATP channels. *Basic Res Cardiol*, 101, 53-60.
- KILKENNY, C., BROWNE, W., CUTHILL, I. C., EMERSON, M., ALTMAN, D. G. & GROUP, N. C. R. R. G. W. 2010. Animal research: reporting in vivo experiments: the ARRIVE guidelines. *Br J Pharmacol*, 160, 1577-9.

- KING, A. L., POLHEMUS, D. J., BHUSHAN, S., OTSUKA, H., KONDO, K., NICHOLSON, C. K., BRADLEY, J. M., ISLAM, K. N., CALVERT, J. W., TAO, Y. X., DUGAS, T. R., KELLEY, E. E., ELROD, J. W., HUANG, P. L., WANG, R. & LEFER, D. J. 2014. Hydrogen sulfide cytoprotective signaling is endothelial nitric oxide synthase-nitric oxide dependent. *Proc Natl Acad Sci U S A*, 111, 3182-7.
- KONDO, K., BHUSHAN, S., KING, A. L., PRABHU, S. D., HAMID, T., KOENIG, S., MUROHARA, T., PREDMORE, B. L., GOJON, G., SR., GOJON, G., JR., WANG, R., KARUSULA, N., NICHOLSON, C. K., CALVERT, J. W. & LEFER, D. J. 2013. H(2)S protects against pressure overload-induced heart failure via upregulation of endothelial nitric oxide synthase. *Circulation*, 127, 1116-27.
- LAMBERT, J. P., NICHOLSON, C. K., AMIN, H., AMIN, S. & CALVERT, J. W. 2014. Hydrogen sulfide provides cardioprotection against myocardial/ischemia reperfusion injury in the diabetic state through the activation of the RISK pathway. *Med Gas Res*, 4, 20.
- LEE, Z. W., ZHOU, J., CHEN, C. S., ZHAO, Y., TAN, C. H., LI, L., MOORE, P. K. & DENG, L. W. 2011. The slow-releasing hydrogen sulfide donor, GYY4137, exhibits novel anti-cancer effects in vitro and in vivo. *PLoS One*, 6, e21077.
- LI, H., WANG, Y., WEI, C., BAI, S., ZHAO, Y., LI, H., WU, B., WANG, R., WU, L. & XU, C. 2015a. Mediation of exogenous hydrogen sulfide in recovery of ischemic post-conditioning-induced cardioprotection via down-regulating oxidative stress and up-regulating PI3K/Akt/GSK-3 $\beta$  pathway in isolated aging rat hearts. *Cell Biosci*, 5, 11.
- LI, H., ZHANG, C., SUN, W., LI, L., WU, B., BAI, S., LI, H., ZHONG, X., WANG, R., WU, L. & XU, C. 2015b. Exogenous hydrogen sulfide restores cardioprotection of ischemic post-conditioning via inhibition of mPTP opening in the aging cardiomyocytes. *Cell Biosci*, 5, 43.
- LI, L., SALTO-TELLEZ, M., TAN, C. H., WHITEMAN, M. & MOORE, P. K. 2009. GYY4137, a novel hydrogen sulfide-releasing molecule, protects against endotoxic shock in the rat. *Free Radic Biol Med*, 47, 103-13.
- LI, L., WHITEMAN, M., GUAN, Y. Y., NEO, K. L., CHENG, Y., LEE, S. W., ZHAO, Y., BASKAR, R., TAN, C. H. & MOORE, P. K. 2008. Characterization of a novel, water-soluble hydrogen sulfide-releasing molecule (GYY4137): new insights into the biology of hydrogen sulfide. *Circulation*, 117, 2351-60.
- LISJAK, M., SRIVASTAVA, N., TEKLIĆ, T., CIVALE, L., LEWANDOWSKI, K., WILSON, I., WOOD, M. E., WHITEMAN, M. & HANCOCK, J. T. 2010. A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation. *Plant Physiol Biochem*, 48, 931-5.
- LIU, Y. H., LU, M., HU, L. F., WONG, P. T., WEBB, G. D. & BIAN, J. S. 2012. Hydrogen sulfide in the mammalian cardiovascular system. *Antioxid Redox Signal*, 17, 141-85.
- LIU, Z., HAN, Y., LI, L., LU, H., MENG, G., LI, X., SHIRHAN, M., PEH, M. T., XIE, L., ZHOU, S., WANG, X., CHEN, Q., DAI, W., TAN, C. H., PAN, S., MOORE, P. K. & JI, Y. 2013. The hydrogen sulfide donor, GYY4137, exhibits anti-atherosclerotic activity in high fat fed apolipoprotein E(-/-) mice. *Br J Pharmacol*, 169, 1795-809.
- MCGRATH, J. C., DRUMMOND, G. B., MCLACHLAN, E. M., KILKENNY, C. & WAINWRIGHT, C. L. 2010. Guidelines for reporting experiments involving animals: the ARRIVE guidelines. *Br J Pharmacol*, 160, 1573-6.

- MENG, G., WANG, J., XIAO, Y., BAI, W., XIE, L., SHAN, L., MOORE, P. K. & JI, Y. 2015a. GYY4137 protects against myocardial ischemia and reperfusion injury by attenuating oxidative stress and apoptosis in rats. *J Biomed Res*, 29, 203-13.
- MENG, G., ZHU, J., XIAO, Y., HUANG, Z., ZHANG, Y., TANG, X., XIE, L., CHEN, Y., SHAO, Y., FERRO, A., WANG, R., MOORE, P. K. & JI, Y. 2015b. Hydrogen Sulfide Donor GYY4137 Protects against Myocardial Fibrosis. *Oxid Med Cell Longev*, 2015, 691070.
- PAN, T. T., CHEN, Y. Q. & BIAN, J. S. 2009. All in the timing: a comparison between the cardioprotection induced by H<sub>2</sub>S preconditioning and post-infarction treatment. *Eur J Pharmacol*, 616, 160-5.
- PAPAPETROPOULOS, A., WHITEMAN, M. & CIRINO, G. 2015. Pharmacological tools for hydrogen sulphide research: a brief, introductory guide for beginners. *Br J Pharmacol*, 172, 1633-7.
- POLHEMUS, D. J., LI, Z., PATTILLO, C. B., GOJON, G., SR., GOJON, G., JR., GIORDANO, T. & KRUM, H. 2015. A novel hydrogen sulfide prodrug, SG1002, promotes hydrogen sulfide and nitric oxide bioavailability in heart failure patients. *Cardiovasc Ther*, 33, 216-26.
- PREDMORE, B. L., KONDO, K., BHUSHAN, S., ZLATOPOLSKY, M. A., KING, A. L., ARAGON, J. P., GRINSFELDER, D. B., CONDIT, M. E. & LEFER, D. J. 2012. The polysulfide diallyl trisulfide protects the ischemic myocardium by preservation of endogenous hydrogen sulfide and increasing nitric oxide bioavailability. *Am J Physiol Heart Circ Physiol*, 302, H2410-8.
- ROBINSON, H. & WRAY, S. 2012. A new slow releasing, H<sub>2</sub>S generating compound, GYY4137 relaxes spontaneous and oxytocin-stimulated contractions of human and rat pregnant myometrium. *PLoS One*, 7, e46278.
- ROSSONI, G., SPARATORE, A., TAZZARI, V., MANFREDI, B., DEL SOLDATO, P. & BERTI, F. 2008. The hydrogen sulphide-releasing derivative of diclofenac protects against ischaemia-reperfusion injury in the isolated rabbit heart. *Br J Pharmacol*, 153, 100-9.
- SIVARAJAH, A., COLLINO, M., YASIN, M., BENETTI, E., GALLICCHIO, M., MAZZON, E., CUZZOCREA, S., FANTOZZI, R. & THIEMERMANN, C. 2009. Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial I/R. *Shock*, 31, 267-74.
- SLUIJTER, J. P., CONDORELLI, G., DAVIDSON, S. M., ENGEL, F. B., FERDINANDY, P., HAUSENLOY, D. J., LECOUR, S., MADONNA, R., OVIZE, M., RUIZ-MEANA, M., SCHULZ, R., VAN LAAKE, L. W. & NUCLEUS OF THE EUROPEAN SOCIETY OF CARDIOLOGY WORKING GROUP CELLULAR BIOLOGY OF THE, H. 2014. Novel therapeutic strategies for cardioprotection. *Pharmacol Ther*, 144, 60-70.
- SODHA, N. R., CLEMENTS, R. T., FENG, J., LIU, Y., BIANCHI, C., HORVATH, E. M., SZABO, C., STAHL, G. L. & SELLKE, F. W. 2009. Hydrogen sulfide therapy attenuates the inflammatory response in a porcine model of myocardial ischemia/reperfusion injury. *J Thorac Cardiovasc Surg*, 138, 977-84.
- SOJITRA, B., BULANI, Y., PUTCHA, U. K., KANWAL, A., GUPTA, P., KUNCHHA, M. & BANERJEE, S. K. 2012. Nitric oxide synthase inhibition abrogates hydrogen sulfide-induced cardioprotection in mice. *Mol Cell Biochem*, 360, 61-9.

- SUVEREN, E., WHITEMAN, M. & BAXTER, G. F. 2012. The cardioprotective effect of GYY4137, a novel H<sub>2</sub>S donor, in ischaemia reperfusion injury. *Cardiovascular Research*, 93, S111-S111.
- VANDIVER, M. & SNYDER, S. H. 2012. Hydrogen sulfide: a gasotransmitter of clinical relevance. *J Mol Med (Berl)*, 90, 255-63.
- WANG, J., YANG, H., HU, X., FU, W., XIE, J., ZHOU, X., XU, W. & JIANG, H. 2013. Dobutamine-mediated heme oxygenase-1 induction via PI3K and p38 MAPK inhibits high mobility group box 1 protein release and attenuates rat myocardial ischemia/reperfusion injury in vivo. *J Surg Res*, 183, 509-16.
- WHITEMAN, M., LI, L., ROSE, P., TAN, C. H., PARKINSON, D. B. & MOORE, P. K. 2010. The effect of hydrogen sulfide donors on lipopolysaccharide-induced formation of inflammatory mediators in macrophages. *Antioxid Redox Signal*, 12, 1147-54.
- YAO, L. L., HUANG, X. W., WANG, Y. G., CAO, Y. X., ZHANG, C. C. & ZHU, Y. C. 2010. Hydrogen sulfide protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by preventing GSK-3 $\beta$ -dependent opening of mPTP. *Am J Physiol Heart Circ Physiol*, 298, H1310-9.
- YAO, X., TAN, G., HE, C., GAO, Y., PAN, S., JIANG, H., ZHANG, Y. & SUN, X. 2012. Hydrogen sulfide protects cardiomyocytes from myocardial ischemia-reperfusion injury by enhancing phosphorylation of apoptosis repressor with caspase recruitment domain. *Tohoku J Exp Med*, 226, 275-85.
- YONG, Q. C., LEE, S. W., FOO, C. S., NEO, K. L., CHEN, X. & BIAN, J. S. 2008. Endogenous hydrogen sulphide mediates the cardioprotection induced by ischemic postconditioning. *Am J Physiol Heart Circ Physiol*, 295, H1330-H1340.
- ZHANG, W. J., SHI, Z. X., WANG, B. B., CUI, Y. J., GUO, J. Z. & LI, B. 2001. Allitridum mimics effect of ischemic preconditioning by activation of protein kinase C. *Acta Pharmacol Sin*, 22, 132-6.
- ZHAO, Z. Q., CORVERA, J. S., HALKOS, M. E., KERENDI, F., WANG, N. P., GUYTON, R. A. & VINTEN-JOHANSEN, J. 2003. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol*, 285, H579-88.
- ZHUO, Y., CHEN, P. F., ZHANG, A. Z., ZHONG, H., CHEN, C. Q. & ZHU, Y. Z. 2009. Cardioprotective effect of hydrogen sulfide in ischemic reperfusion experimental rats and its influence on expression of survivin gene. *Biol Pharm Bull*, 32, 1406-10.

**FIGURE LEGENDS**

**Figure 1.** Treatment protocols. **A. *Series 1 infarct studies.*** After surgical preparation, rats were stabilised for 20 minutes then subjected to 30 minutes of left coronary artery occlusion (CAO) followed by 120 minutes of reperfusion. Control rats did not receive any further intervention, while treatment groups received one of three GYY4137 doses or depleted GYY4137 (D-GYY4137) 10 minutes before reperfusion. Hearts were excised at the end of reperfusion for infarct size determination, n = 6-10. Arrows indicate the time of pharmacological interventions.

**B. *Series 2 infarct studies.*** Following stabilisation, rats were subjected to 30 minutes of left coronary artery occlusion and 120 minutes of reperfusion. Animals were randomised into six groups. Control heart did not receive any further intervention. GYY4137 was administered 10 minutes before reperfusion. LY294002 and L-NAME were administered 15 minutes before reperfusion. Hearts were excised at the end of reperfusion for infarct size determination, n = 6-7. Parallel groups, n=4, were prepared identically but hearts were excised 5 minutes after reperfusion for analysis by immunoblotting. Arrows indicate the time of pharmacological interventions.

**Figure 2.** Infarct size data: GYY4137 dose-response study (Series 1). Area at risk was determined Evans' blue exclusion and infarction was assessed by TTC staining. GYY4137 was administered at 26.6, 133, or 266  $\mu\text{mol kg}^{-1}$  10 minutes before reperfusion. **A.** area at risk as a percentage of the total ventricular volume. **B.** myocardial infarction expressed as a percentage of the area at risk. Numbers in

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histograms indicate sample size. \*\*  $P < 0.01$  versus Control; †  $p < 0.05$  versus GYY4137  $266 \mu\text{mol kg}^{-1}$  (one way ANOVA with Newman Keuls *post hoc* test).

**Figure 3.** Infarct size data: GYY4137 with pharmacological inhibitors (Series 2). Area at risk was determined Evans' blue exclusion and infarction was assessed by TTC staining. GYY4137 was administered at  $266 \mu\text{mol kg}^{-1}$  10 minutes before reperfusion. LY294002 or L-NAME were given 15 minutes before reperfusion. **A.** area at risk expressed as a percentage of the total ventricular volume. **B.** infarct size expressed as a percentage of the area at risk. Numbers in histograms indicate sample size. \*\*  $p < 0.01$  versus Control; \*\*\*  $p < 0.001$  versus control; †  $p < 0.01$  versus GYY4137 (one way ANOVA with Newman Keuls *post hoc* test).

**Figure 4.** Western blot analysis of left ventricular myocardium harvested from the area at risk 5 minutes after reperfusion. Histograms show densitometric ratios of phosphorylated to total protein. GAPDH was used as loading control for all determinations. **A.** p-Akt, total Akt and GAPDH. **B.** p-eNOS, total eNOS and GAPDH. **C.** p-GSK-3 $\beta$ , total GSK-3 $\beta$  and GAPDH. **D.** p-ERK1/2, total ERK1/2 and GAPDH. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  versus control. In all groups,  $n=4$ .

### **Figure 5 (Graphical abstract)**

GYY4137, a donor of  $\text{H}_2\text{S}$ , induces marked limitation of myocardial infarct size when given shortly before reperfusion. Based on the present experimental data, we present a mechanistic scheme by which GYY4137 mediates its cardioprotection against reperfusion injury. GYY4137 releases  $\text{H}_2\text{S}$  which triggers a key component

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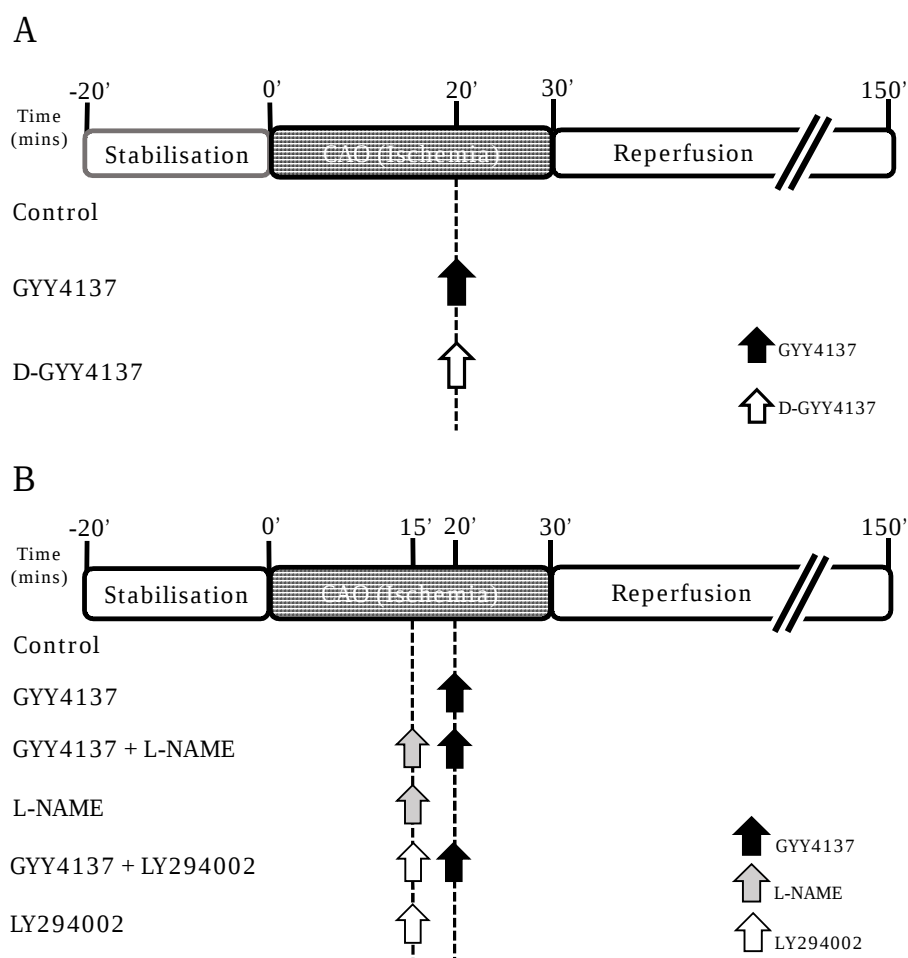
of the reperfusion injury salvage kinase cascade, namely PI3K/Akt activation at reperfusion. Downstream of activated Akt, phosphorylation of eNOS and GSK-3 $\beta$  are induced by GYY4137 treatment. Although not yet determined, it seems plausible that GYY4137 eventually inhibits the opening of mPTP at early reperfusion as a result of the increase in NO level and inhibition GSK-3 $\beta$  activity, resulting in reduced cardiomyocyte susceptibility to lethal reperfusion injury.

**Table 1.**

Baseline and cardiodynamics for series 1 and 2 at the end of stabilisation period, after 20 minutes of ischaemia and at the end of reperfusion.

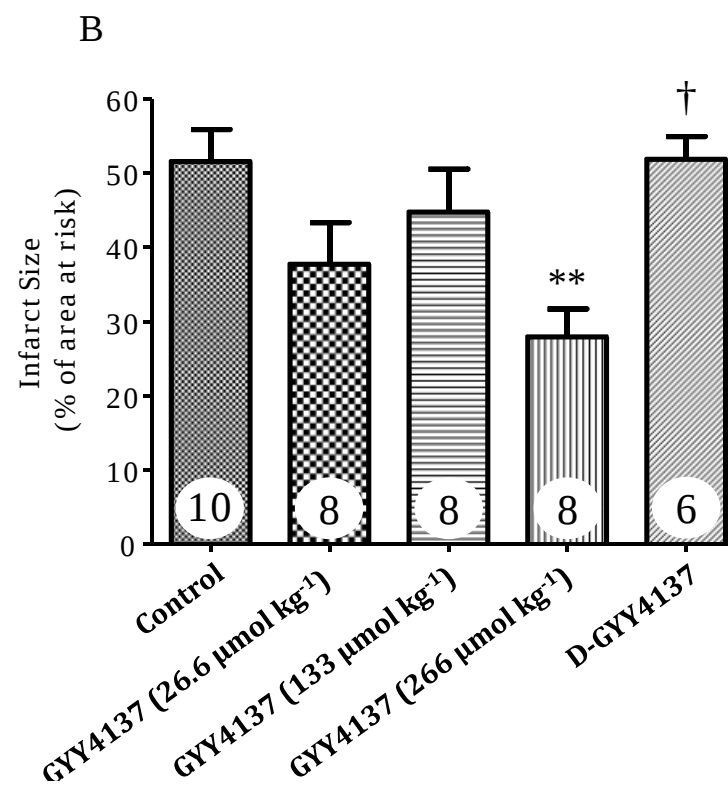
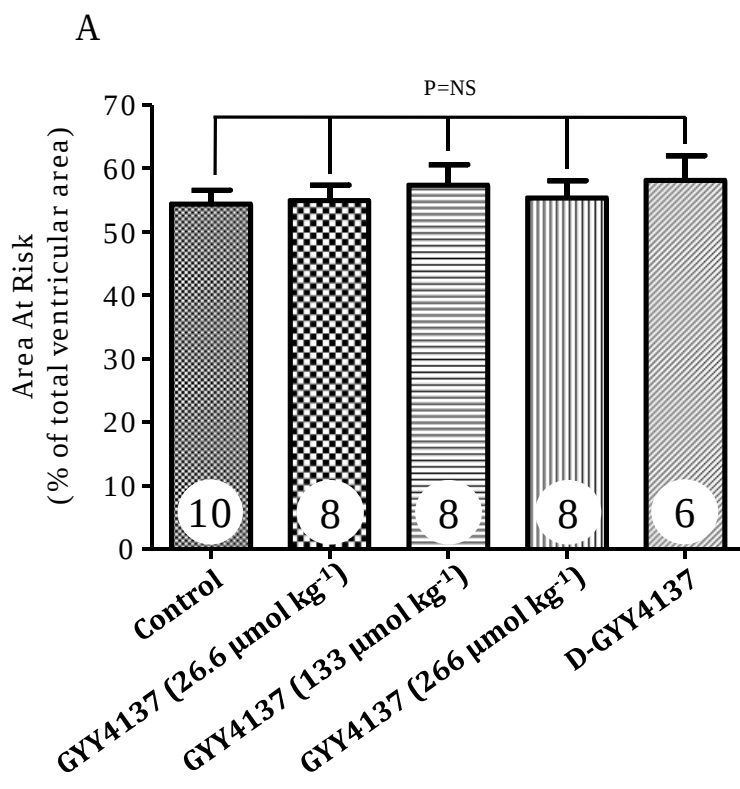
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(Figure 1)



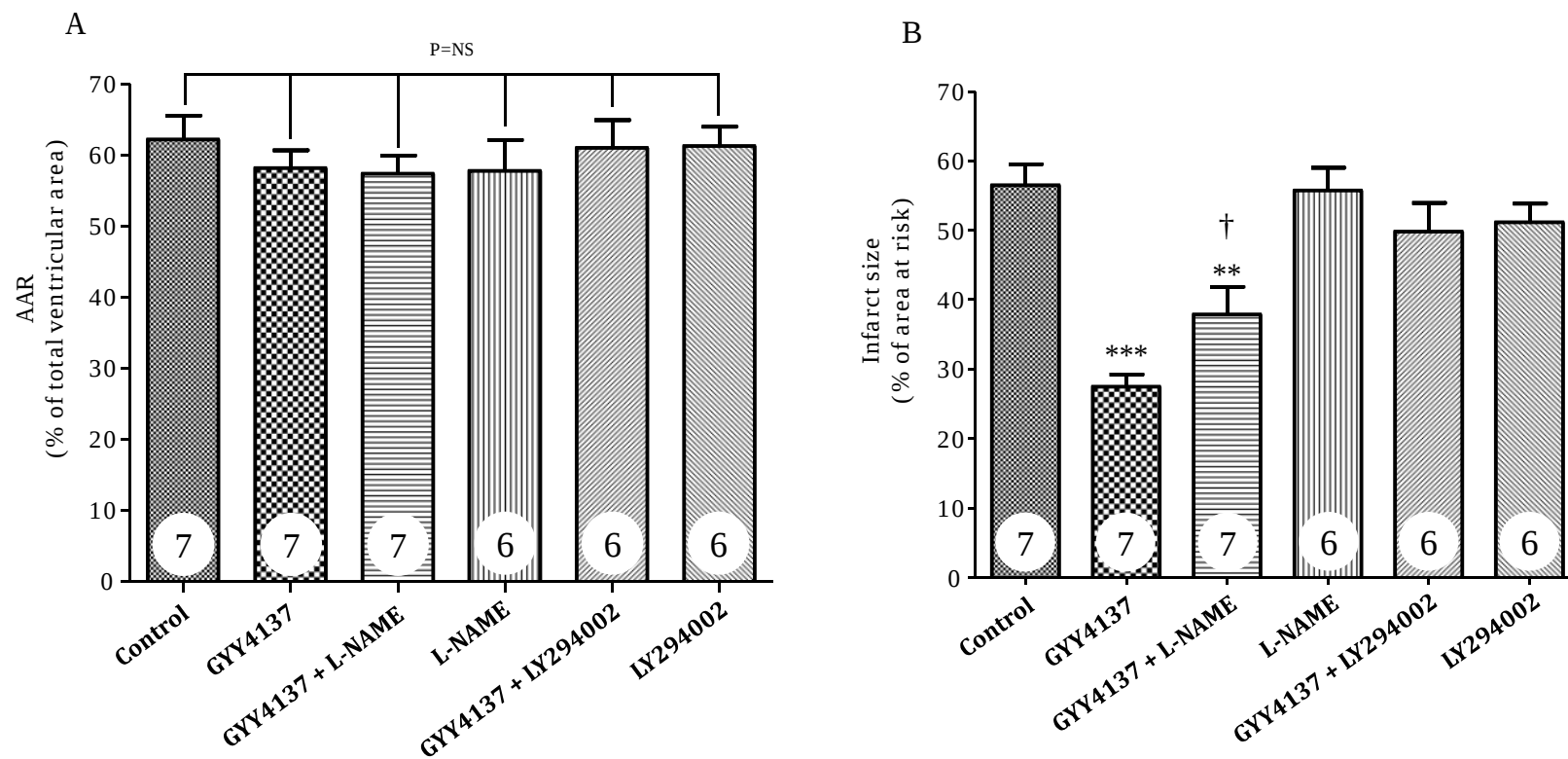
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(Figure 2)



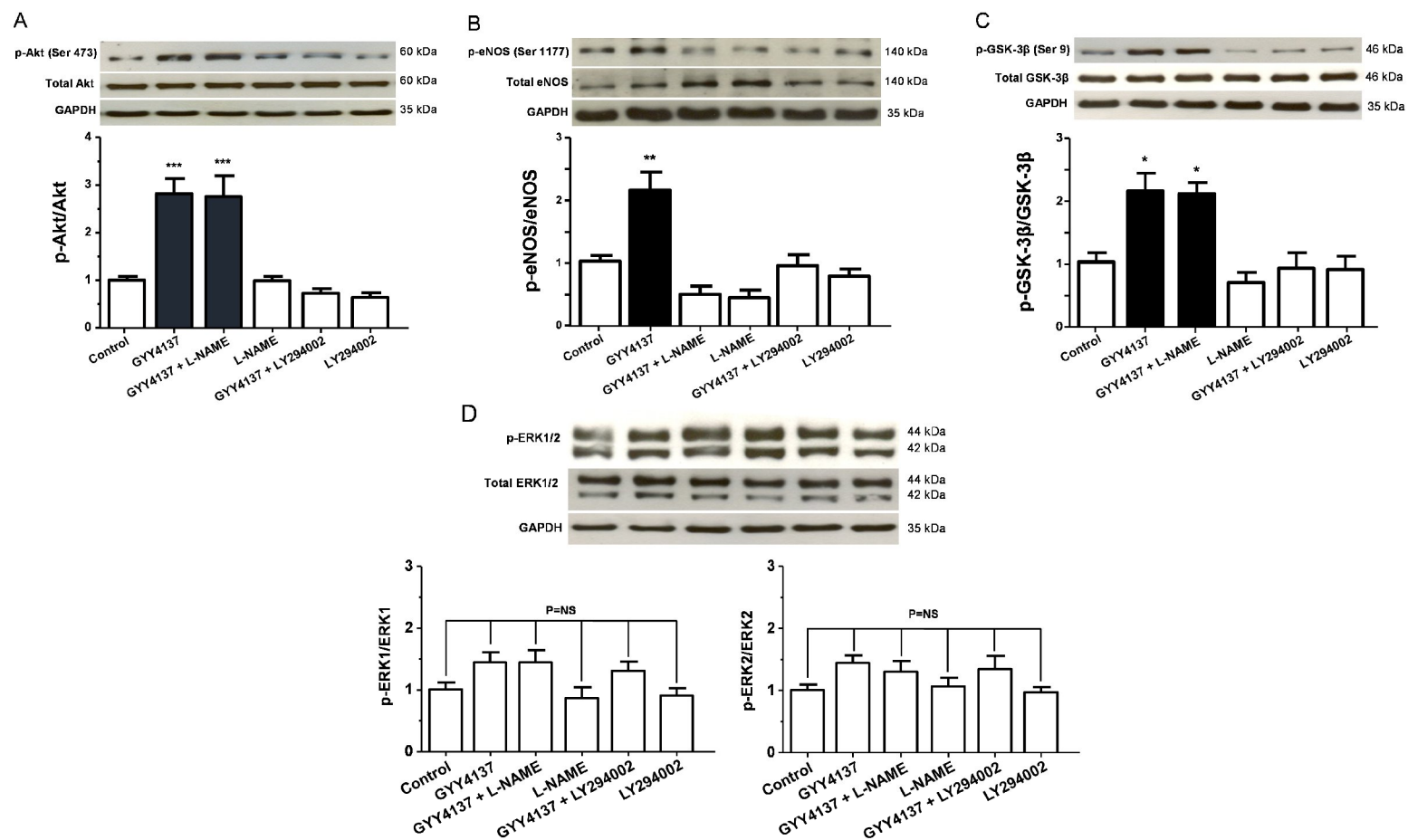
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(Figure 3)



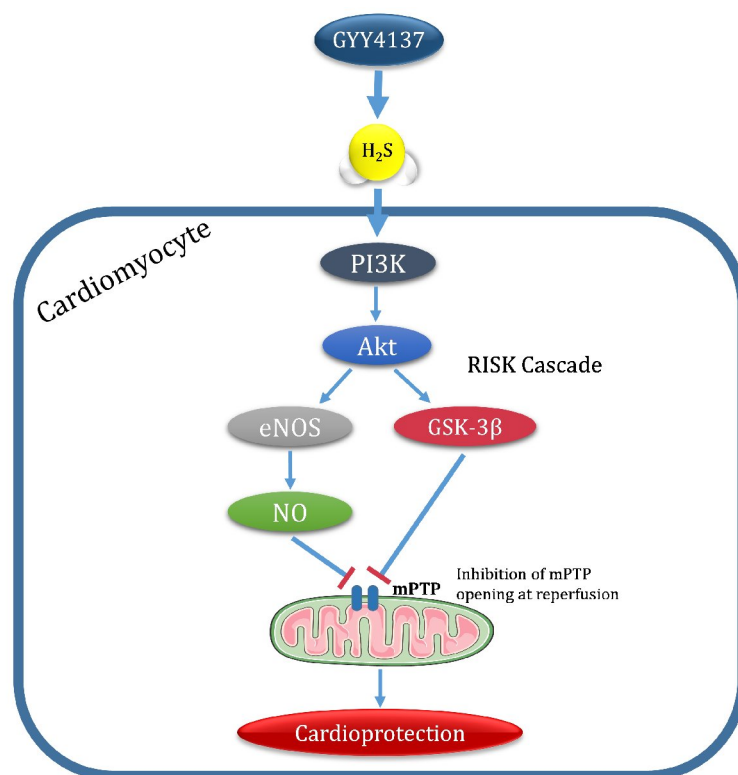
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(Figure 4)



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(Figure 5)



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| Experimental Protocol             | <i>n</i> | BW (g)   | Baseline                                           |               | 20 min Ischaemia                                   |               | 120 min Reperfusion                                |               |
|-----------------------------------|----------|----------|----------------------------------------------------|---------------|----------------------------------------------------|---------------|----------------------------------------------------|---------------|
|                                   |          |          | RPP<br>(mmHg min <sup>-1</sup> × 10 <sup>3</sup> ) | MAP<br>(mmHg) | RPP<br>(mmHg min <sup>-1</sup> × 10 <sup>3</sup> ) | MAP<br>(mmHg) | RPP<br>(mmHg min <sup>-1</sup> × 10 <sup>3</sup> ) | MAP<br>(mmHg) |
| <b>Series 1</b>                   |          |          |                                                    |               |                                                    |               |                                                    |               |
| Control                           | 10       | 355 ± 6  | 37.5 ± 2.0                                         | 88 ± 4        | 27.9 ± 1.8                                         | 68 ± 4        | 24.0 ± 1.4                                         | 53 ± 3        |
| GY4137 26.6 μmol kg <sup>-1</sup> | 8        | 360 ± 9  | 40.3 ± 3.0                                         | 90 ± 6        | 29.1 ± 2.1                                         | 71 ± 5        | 26.3 ± 2.0                                         | 59 ± 4        |
| GY4137 133 μmol kg <sup>-1</sup>  | 8        | 346 ± 7  | 41.4 ± 1.8                                         | 94 ± 6        | 29.5 ± 2.2                                         | 70 ± 6        | 24.3 ± 1.5                                         | 53 ± 4        |
| GY4137 266 μmol kg <sup>-1</sup>  | 8        | 368 ± 6  | 39.1 ± 1.7                                         | 83 ± 4        | 28.6 ± 2.0                                         | 65 ± 5        | 20.9 ± 1.6                                         | 45 ± 3        |
| D-GY4137                          | 6        | 368 ± 6  | 38.4 ± 2.3                                         | 85 ± 5        | 26.3 ± 2.1                                         | 69 ± 4        | 22.7 ± 1.3                                         | 48 ± 4        |
| <b>Series 2</b>                   |          |          |                                                    |               |                                                    |               |                                                    |               |
| Control                           | 7        | 384 ± 7  | 36.3 ± 2.2                                         | 85 ± 5        | 24.8 ± 2.2                                         | 61 ± 6        | 21.2 ± 2.0                                         | 48 ± 4        |
| GY4137                            | 7        | 379 ± 8  | 41.3 ± 3.0                                         | 96 ± 6        | 26.8 ± 1.6                                         | 66 ± 6        | 21.6 ± 1.1                                         | 47 ± 2        |
| GY4137 + L-NAME                   | 7        | 387 ± 7  | 39.3 ± 2.5                                         | 88 ± 5        | 29.5 ± 2.4                                         | 69 ± 5        | 19.4 ± 2.4                                         | 50 ± 6        |
| L-NAME                            | 6        | 381 ± 9  | 39.3 ± 1.6                                         | 97 ± 3        | 30.4 ± 2.1                                         | 76 ± 7        | 22.4 ± 5.1                                         | 57 ± 9        |
| GY4137 + LY294002                 | 6        | 362 ± 8  | 39.3 ± 1.8                                         | 88 ± 4        | 30.2 ± 1.8                                         | 71 ± 5        | 24.2 ± 0.6                                         | 53 ± 1        |
| LY294002                          | 6        | 367 ± 11 | 44.5 ± 2.8                                         | 98 ± 4        | 29.6 ± 1.2                                         | 72 ± 4        | 25.0 ± 0.7                                         | 54 ± 2        |

**Table 1.**

RPP=rate pressure product, MAP=mean arterial pressure. Data are reported as Mean ± SEM. There was no significant difference among the experimental groups (One way ANOVA + Newman Keuls post-hoc), *p* > 0.05.