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

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Chemical compounds studied in this article
GYY4137-morphilino 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_2S) 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_2S 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^{-1}) 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^{-1} 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_2S-based therapeutic approaches may have value as adjuncts to reperfusion in the treatment of acute myocardial infarction.

List of abbreviations

AAR = area at risk

Akt = protein kinase B

DATS = diallyltrisulfide

eNOS = endothelial nitric oxide synthase

ERK1/2 = Extracellular signal-regulated kinases 1/2 (p42/p44 mitogen activated protein kinase)

GSK-3β = glycogen synthase kinase-3 Beta

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

H_2S = hydrogen sulfide

IPost-C = ischemic postconditioning

mPTP = mitochondrial permeability transition pore

NO = nitric oxide

PBS = phosphate buffered saline

PI3K = Phosphatidylinositol-3-kinase

RISK = reperfusion injury salvage kinase (signalling pathway)

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 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-3β (GSK-3β). Although IPost-C is of limited clinical applicability, a number of pharmacological approaches that mimic IPost-C have been

103 described, including the administration of autacoids and other mediators thought to
104 activate the kinases of the RISK cascade (Burley and Baxter, 2009).
105
106 Hydrogen sulfide (H_2S) has attracted considerable interest as a cardiovascular
107 autacoid. Although produced endogenously within the myocardium and coronary
108 vasculature (Liu et al., 2012, Hackfort and Mishra, 2016), in coronary artery disease,
109 there may be reduced H_2S production (Yong et al., 2008, Han et al., 2015, Islam et
110 al., 2015). The administration of exogenous H_2S donor compounds or increasing
111 endogenous production of H_2S has been well documented to reduce ischemia-
112 reperfusion injury in experimental models (Elrod et al., 2007, Calvert et al., 2009,
113 King et al., 2014). There is also evidence that H_2S is a mediator of IPost-C (Bian et
114 al., 2006, Yong et al., 2008, Huang et al., 2012, Das et al., 2015). However, potential
115 therapeutic extrapolation of this knowledge has been hindered by the limitations of
116 H_2S donor compounds. Much of the experimental literature has reported studies with
117 inorganic sulfide salts (Na_2S and NaSH) which are impure in commercial form and
118 unstable. Despite them being water soluble and inexpensive, a particular issue is
119 that the H_2S release is largely uncontrollable as they dissociate in aqueous medium
120 instantly to generate H_2S at high concentration in a short-lasting burst
121 (Papapetropoulos et al., 2015). In contrast to Na_2S and NaSH, GYY4137 (morpholin-
122 4-ium 4-methoxyphenyl-morpholino-phosphinodithioate) is a donor compound which
123 releases H_2S at a slow steady rate at physiological pH and temperature (Li et al.,
124 2008). Several studies have suggested that GYY4137 effectively delivers H_2S in
125 various physiological systems (Li et al., 2009, Lisjak et al., 2010, Lee et al., 2011,
126 Robinson and Wray, 2012, Liu et al., 2013, Grambow et al., 2014, Meng et al.,
127 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_2S 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

GYY4137 was synthesised by us as previously reported (Li et al., 2008). The purity of GYY4137 was determined by NMR spectroscopy (1H, 31P and 13C). 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® 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 anesthetised by intraperitoneal injection of thiobutabarbital sodium (200 mg kg^{-1}) and maintained by intravenous supplemental dosing (75 mg kg^{-1}) as

169 required to maintain surgical anesthesia throughout the procedure. Body
170 temperature was maintained at 37 ± 1 °C via rectal thermometer attached to a
171 thermo-regulated blanket unit (Harvard Apparatus Ltd, Cambridge, UK). The right
172 common carotid artery was cannulated and connected to a pressure transducer to
173 measure heart rate and blood pressure throughout the procedure (Powerlab data
174 acquisition system, AD instruments, Abingdon, UK). The left jugular vein was
175 cannulated for drug administration. The trachea was cannulated via tracheotomy and
176 the animal ventilated with room air by a small animal volume controlled ventilator
177 (Hugo Sachs Elektronik, March, Germany) at a rate of 75 strokes min^{-1} and tidal
178 volume of 1.0 to 1.25 mL 100 g^{-1}. The electrocardiogram was recorded using
179 standard lead II electrodes inserted subcutaneously into the limbs and connected to
180 a Powerlab data acquisition system. A midline sternotomy was performed and the
181 chest opened using a metal retractor to expose the heart. After pericardiotomy, a 4/0
182 braided silk suture (Mersilk, Ethicon Ltd, UK) was placed around the left main
183 coronary artery close to its origin from the left border of the pulmonary conus. The
184 animal was left to stabilise for 20 minutes during which the two ends of the silk
185 ligature remained loose. For each animal to be included it had to achieve the
186 following hemodynamic parameters during the stabilisation period: heart rate ≥ 250
187 beats per minute, diastolic blood pressure ≥ 50 mmHg, steady sinus rhythm, no
188 signs of ischemia or arrhythmia during the stabilisation period.
189
190 After stabilisation, the ligature was pulled taut through a plastic snare and fastened
191 against the epicardium to induce regional ischemia for 30 minutes. Ischemia was
192 confirmed by a drop in the mean arterial pressure (MAP), a colour change of the left
193 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 ºC 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 triphenyltetrazoilum 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_2S 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 μmol kg^{-1}, respectively) as a slow i.v. bolus (500μL min^{-1}) 10 minutes before reperfusion.
- Group 5: Depleted GYY4137 (n=6). GYY4137 solution (100 mg mL^{-1}) was prepared in saline and left uncovered for 72 hours at room temperature to dissipate all H_2S, then administered at a dose of 266 μmol 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 μmol 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 μmol kg^{-1}, 500 μL min^{-1}) was administered at 10 minutes before reperfusion.
- Group 8: GYY4137 + L-NAME (n=7). An intravenous bolus dose of L-NAME (20 mg kg^{-1}) was administered 15 minutes before reperfusion followed by GYY4137 (266 μmol kg^{-1}, 500 μL min^{-1}) 10 minutes before reperfusion.
- Group 9: L-NAME (n=6). An intravenous bolus dose of L-NAME (20 mg kg^{-1}) was administered 15 minutes before reperfusion.
- Group 10: GYY4137 + LY294002 (n=6). An intravenous bolus dose of LY294002 (0.1 mg kg^{-1} in 5% DMSO) was given 15 minutes before reperfusion followed by GYY4137 (266 μmol kg^{-1}, 500 μL min^{-1}) 10 minutes before reperfusion.
- Group 11: LY294002 (n=6). A bolus dose of LY294002 (0.1 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 ºC for Western blotting of Akt, eNOS, GSK-3β and ERK1/2.

2.6 Western blotting analysis

To investigate the involvement of Akt, eNOS, GSK-3β 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-polyacylamide 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 ºC. The following antibodies were used: Akt (1:1000), phospho- ser^{473}Akt (1:1000), endothelial nitric oxide synthase (eNOS 1:500), phospho- ser^{1177}eNOS (1:500), glycogen-synthase kinase-3 beta (GSK-3β 1:1000), phospho- ser^{9}GSK-3β (1:1000), extracellular signal-regulated kinases ERK 1/2 (1:1000), phospho- Thr^{202}/Tyr^{204} 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 ± SEM. Data were analysed using GraphPad Prism® 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

using one way ANOVA supported by Newman-Keuls *post hoc* test. Differences 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 ± 4.7% (Figure 2B). GYY4137 (266 μmol kg^{-1}) produced significant infarct limitation when given 10 minutes before reperfusion compared to control hearts (27.9 ± 3.8% vs 52.5 ± 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 H_2S donating potential but was otherwise

structurally identical had no effect on infarct size at the same dose (51.9 ± 3.1%), confirming the dependency of GYY4137's infarct-limiting action on H2S 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 μmol kg^{-1}) elicited a significant reduction in %I/AAR compared to control (27.6 ± 2.0% vs 56.8 ± 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 ± 6.3% vs 27.6 ± 2.0%, respectively, $p<0.05$), but did not abolish it (41.1 ± 6.3% vs 56.8 ± 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 ± 4.2% vs 56.8 ± 3.5%, respectively, $p>0.05$). Neither L-NAME nor LY294002 had any effect on infarct size when given alone (55.7 ±3.3% and 51.2 ± 2.7% respectively, both $p>0.05$ vs control).

The extent of phosphorylation of Akt, eNOS, GSK-3β 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-3β 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^{473}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^{1177} 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^{9} phosphorylation of GSK-3β was also increased 2.2-fold by GYY4137 (Figure 4C). This phosphorylation, leading to inactivation of GSK-3β, was not affected L-NAME. However, pre-treatment with LY294002 prior to GYY4137 abrogated GSK-3β 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-3β activity at early reperfusion by increasing the phosphorylation of its ser^9 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-3β activity.

4.1 Infarct limitation by GYY4137

The results show for the first time the effect of GYY4137, as a slow-releasing H_2S donor, on myocardial infarction in an *in vivo* model. Intracellular levels of H_2S are reported to be decreased during ischaemia-reperfusion as a results of overwhelming ROS generation which limits H_2S 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_2S 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_2S 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_2S donor. Although inorganic H_2S generators (NaSH and Na_2S) 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_2S 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_2S sources, in addition to the different experimental conditions and end-points of interest. Using garlic derivative as an organic source of H_2S, 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_{ATP} channels (Rossoni et al., 2008). Investigators also have examined the possibility of postconditioning the myocardium using NaSH and Na_2S. For example, Elrod et al. (2007), Sodha et al. (2009) and Lambert et al. (2014) all reported that Na_2S 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_2S donors that have been reported are the polysulfide diallyl trisulfide (DATS) and SG-1002, a thiol-activated H_2S donor. Despite generating 10 times less H_2S than Na_2S, 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_2S donor with the possibility of off-target effects. Moreover, H_2S 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_2S level and circulating NO bioavailability (Polhemus et al., 2015). However, none of these studies have shown that the observed effects are due to H_2S 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_2S 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_2S-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-3β, 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_2S.

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_2S at reperfusion could activate ERK1/2 and also inhibit GSK-3β (Lambert et al., 2014, Li et al., 2015b,

Bibli et al., 2015). However, since in our hands GSK-3β 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_2S) and H_2S 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^{1177} 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_2S but the nature of their interactions is uncertain, at least in part because of the large variation in experimental conditions.

SG-1002, H_2S 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_2S 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_2S 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_2S-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_2S 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_2S-delivering systems.

4.4 GYY4137 postconditioning attenuates GSK-3β phosphorylation

GSK-3β has been proposed as one of the key end effectors of some cardioprotective manoeuvres, particularly ischemic conditioning phenomena. It has been demonstrated that GSK-3β 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-3β-dependent opening of mPTP. In line with these results, the present study demonstrated that GYY4137 increased the phosphorylation of GSK-3β at Ser^9 site at reperfusion. This was abolished by LY294002, but not by L-NAME, suggesting that GYY4137 induced inhibition of GSK-3β 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_2S 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_2S donor GYY4137, but not its H_2S-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-3β (see Figure 5, Graphical Abstract). Thus,

stable slow-releasing H_2S 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.

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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 μmol 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

histograms indicate sample size. ** P<0.01 versus Control; † p<0.05 versus GYY4137 266 μmol 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 μmol 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-3β, total GSK-3β 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)

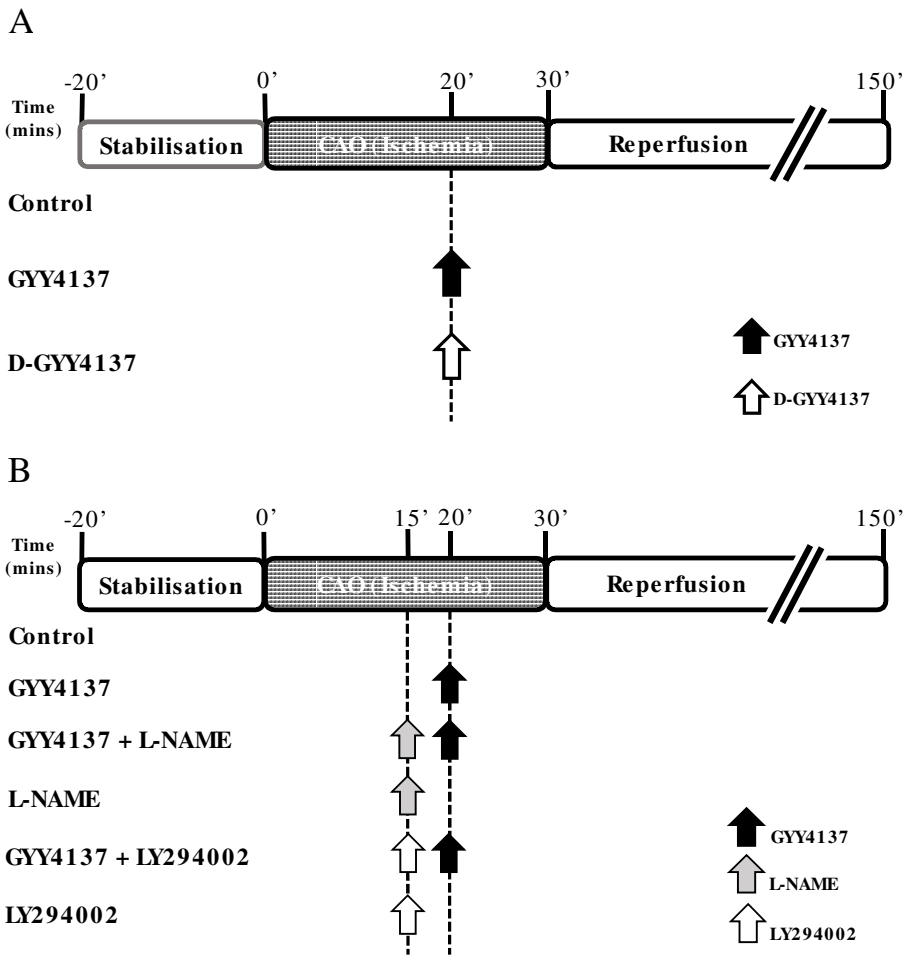
GYY4137, a donor of H_2S, induces marked limitation of myocardial infarct size when given shortly before reperfusion. Based on the present experimental data, we present a mechanistic scheme by which GYY4137 mediates its cardioprotection against reperfusion injury. GYY4137 releases H_2S which triggers a key component

of the reperfusion injury salvage kinase cascade, namely PI3K/Akt activation at reperfusion. Downstream of activated Akt, phosphorylation of eNOS and GSK-3β 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-3β 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.

(Figure 1)



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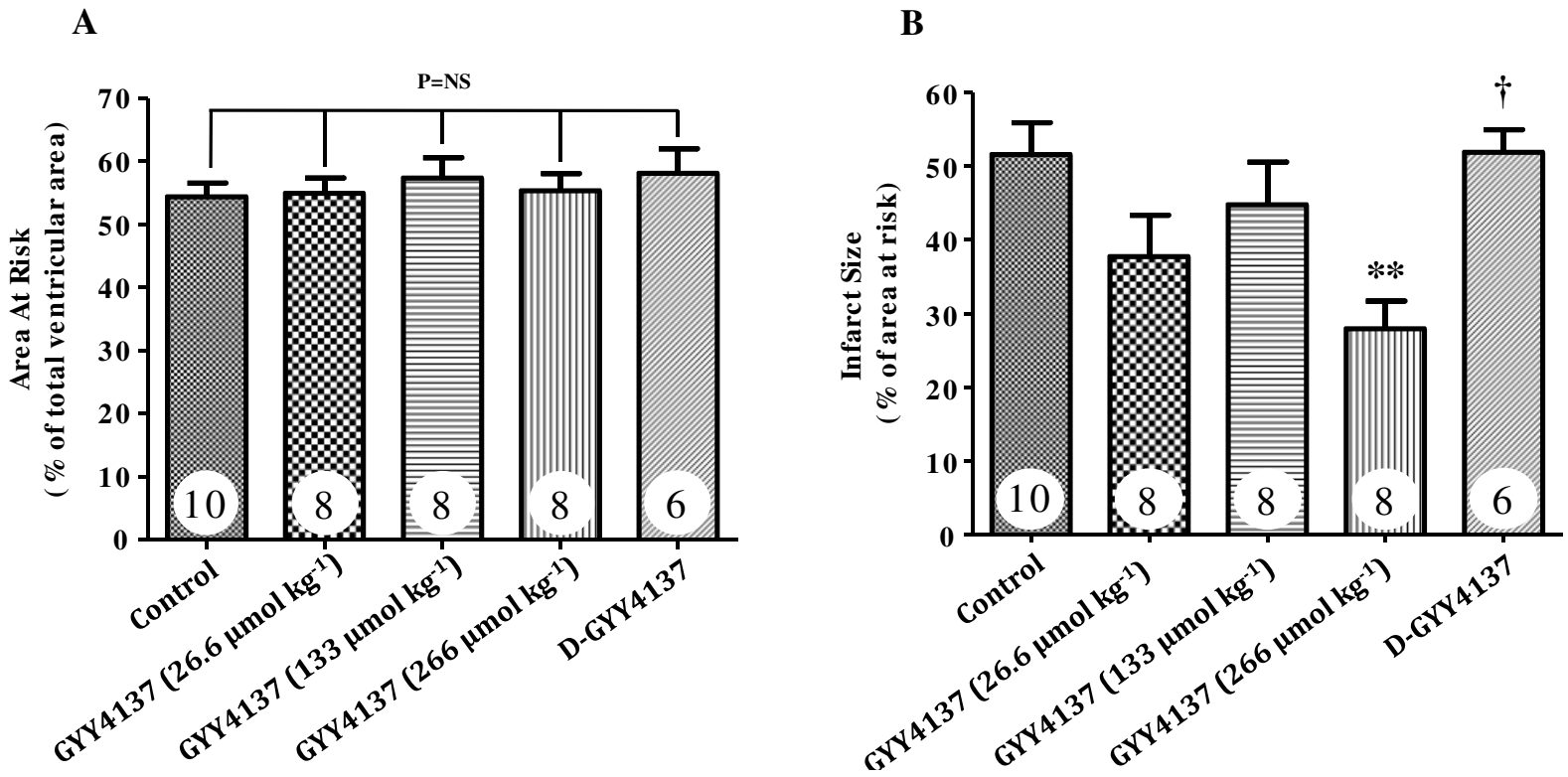
(Figure 2)



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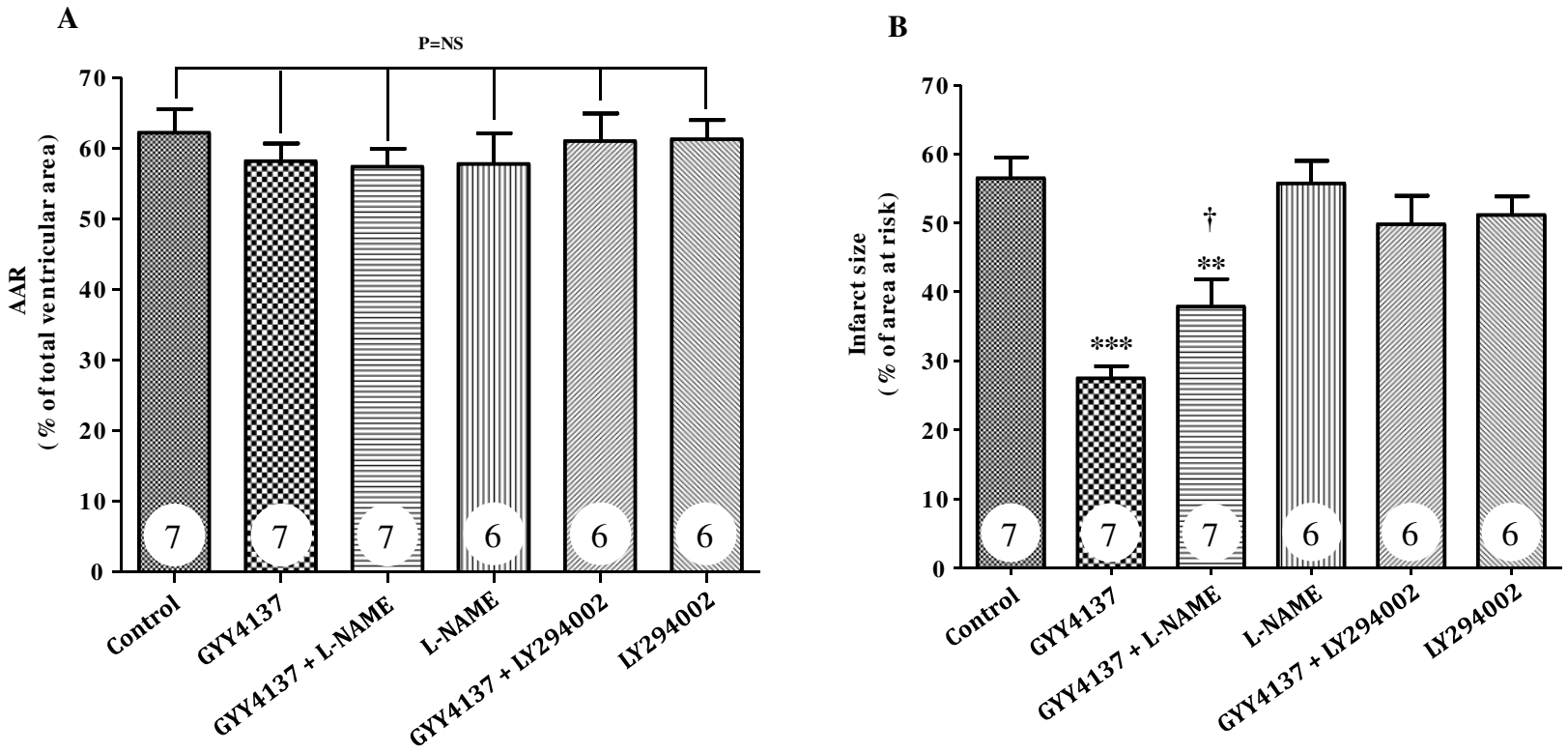
(Figure 3)



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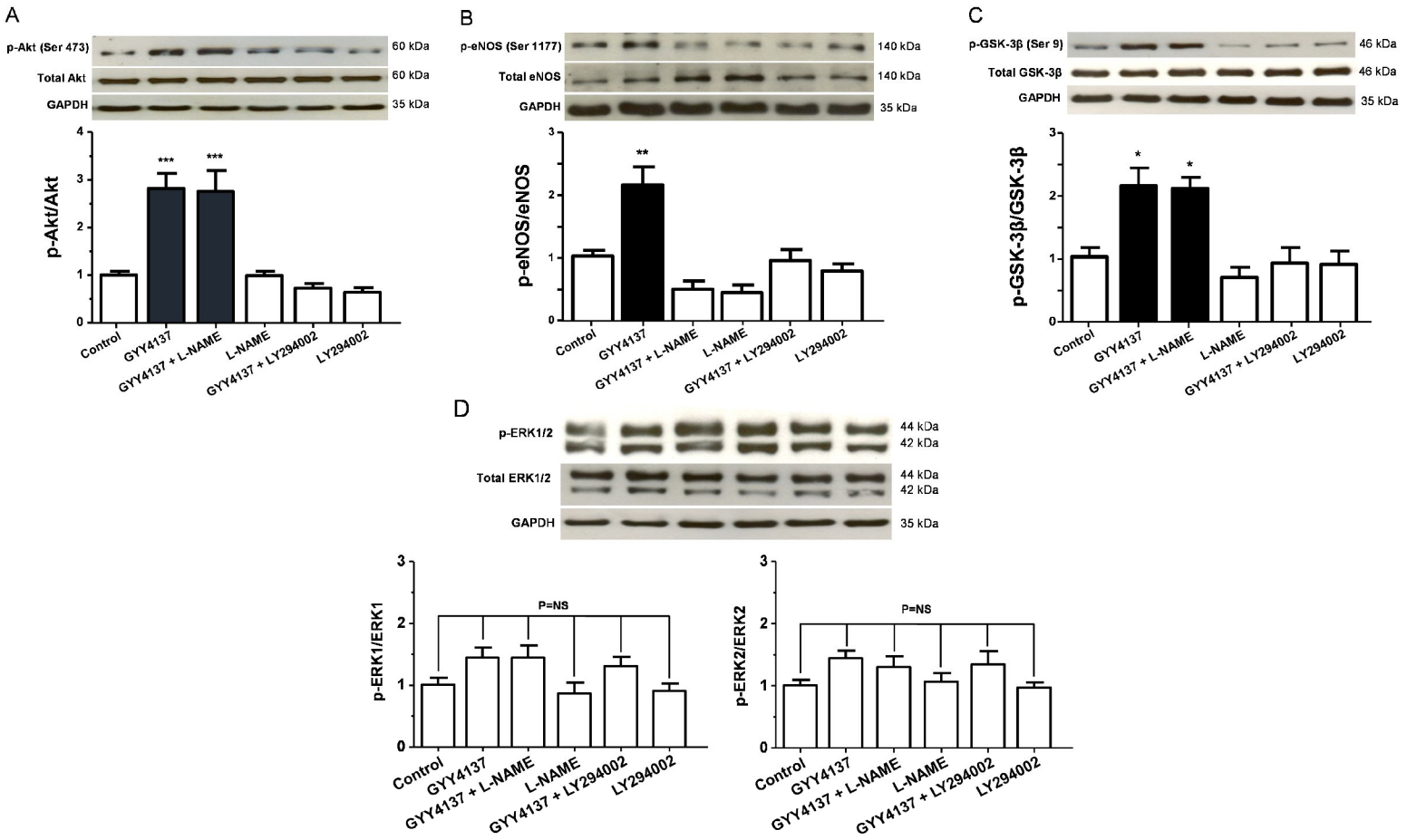
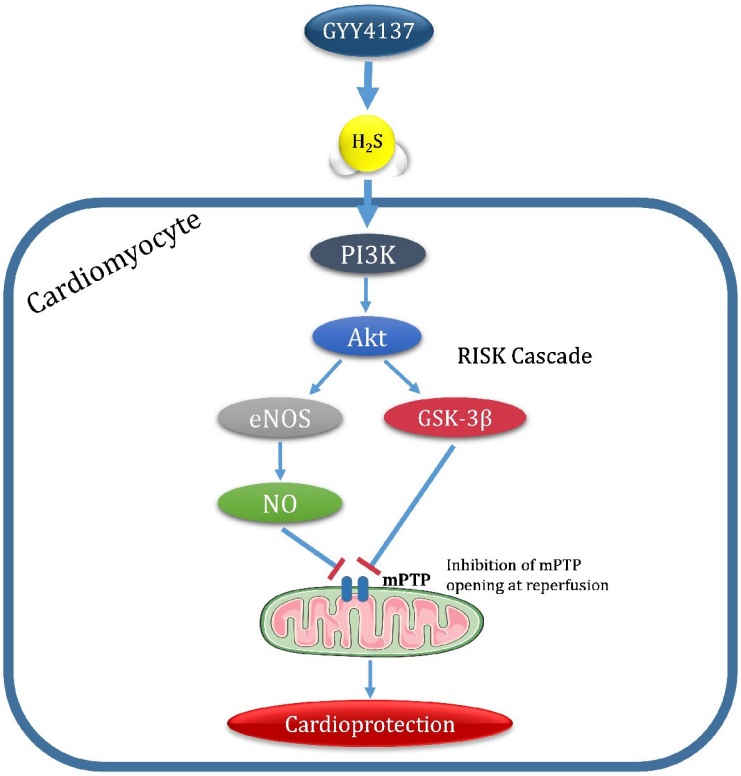
(Figure 4)



(Figure 5)



Experimental Protocol	*n*	BW (g)	Baseline		20 min Ischaemia		120 min Reperfusion	
			RPP (mmHg min^{-1}*10^3)	MAP (mmHg)	RPP (mmHg min^{-1}*10^3)	MAP (mmHg)	RPP (mmHg min^{-1}*10^3)	MAP (mmHg)
Series 1								
Control	10	355 ± 6	37.5 ± 2.0	88 ± 4	27.9 ± 1.8	68 ± 4	24.0 ± 1.4	53 ± 3
GYY4137 26.6 µmol kg^{-1}	8	360 ± 9	40.3 ± 3.0	90 ± 6	29.1 ± 2.1	71 ± 5	26.3 ± 2.0	59 ± 4
GYY4137 133 µmol kg^{-1}	8	346 ± 7	41.4 ± 1.8	94 ± 6	29.5 ± 2.2	70 ± 6	24.3 ± 1.5	53 ± 4
GYY4137 266 µmol kg^{-1}	8	368 ± 6	39.1 ± 1.7	83 ± 4	28.6 ± 2.0	65 ± 5	20.9 ± 1.6	45 ± 3
D-GYY4137	6	368 ± 6	38.4 ± 2.3	85 ± 5	26.3 ± 2.1	69 ± 4	22.7 ± 1.3	48 ± 4
Series 2								
Control	7	384 ± 7	36.3 ± 2.2	85 ± 5	24.8 ± 2.2	61 ± 6	21.2 ± 2.0	48 ± 4
GYY4137	7	379 ± 8	41.3 ± 3.0	96 ± 6	26.8 ± 1.6	66 ± 6	21.6 ± 1.1	47 ± 2
GYY4137 + 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
GYY4137 + 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), $p > 0.05$.