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**AP39, A MITOCHONDRIA-TARGETING HYDROGEN SULFIDE (H₂S) DONOR,
PROTECTS AGAINST MYOCARDIAL REPERFUSION INJURY
INDEPENDENTLY OF SALVAGE KINASE SIGNALLING**

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ABSTRACT

BACKGROUND AND PURPOSE

H₂S protects myocardium against ischaemia-reperfusion injury. This protection may involve the cytosolic reperfusion injury salvage kinase (RISK) pathway, but direct effects on mitochondrial function are possible. Here, we investigated the potential cardioprotective effect of mitochondria-specific H₂S donor, AP39, at reperfusion against ischaemia/reperfusion injury.

EXPERIMENTAL APPROACH

Anaesthetised rats underwent myocardial (30 min ischaemia/120 min reperfusion) with randomisation to receive interventions prior to reperfusion: vehicle, AP39 (0.01, 0.1, 1 μmol kg⁻¹), or the control compounds AP219 or ADT-OH (1 μmol kg⁻¹). LY294002, L-NAME or ODQ were used to interrogate the involvement of RISK pathway. Myocardial samples harvested 5 minutes after reperfusion were analysed for RISK protein phosphorylation and additional experiments were conducted on isolated cardiac mitochondria to examine the direct mitochondrial effects of AP39.

KEY RESULTS

AP39 exerted dose-dependent infarct size limitation. Inhibition of either PI3K/Akt, eNOS or sGC did not affect the infarct limitation of AP39. Western blot analysis confirmed that AP39 did not induce phosphorylation of Akt, eNOS, GSK-3β or ERK1/2. In isolated subsarcolemmal and interfibrillar mitochondria, AP39 significantly attenuated mitochondrial ROS generation without affecting respiratory complexes I or II. Further, AP39 inhibited mitochondrial permeability transition pore (PTP) opening and co-incubation of mitochondria with AP39 and cyclosporine A induced an additive inhibition of PTP.

CONCLUSION AND IMPLICATIONS

AP39 protects against reperfusion injury independently of the cytosolic RISK pathway. Cardioprotection could be mediated by inhibiting PTP via cyclophilin D-independent mechanism. Thus, selective delivery of H₂S to mitochondria may be therapeutically applicable for harnessing the cardioprotective utility of H₂S.

Abbreviations

RISK, reperfusion injury salvage kinase; AP39, (10-oxo-10-(4-(3-thioxo-3H-1, 2-dithiol-5-yl)phenoxy)decyl) triphenylphosphonium bromide); AP219, mitochondria-targeting moiety; ADT-OH, hydrogen sulfide releasing moiety; eNOS, endothelial nitric oxide synthase; GSK-3 β , glycogen synthase kinase-3beta; PTP, permeability transition pore; H₂S, hydrogen sulfide

List of non-approved abbreviations

3-MST = 3-mercaptopyruvate sulphurtransferase

AAR = area at risk

ADT-OH = 5-(4-hydroxyphenyl)-3H-1, 2-dithiole-3-thione

AP39 = 10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide], mitochondria-targeting H₂S donor

CBS = cystathionine beta synthase

CsA = cyclosporine A

CSE = cystathionine gamma lyase

DATS = diallyltrisulfide

eNOS = endothelial nitric oxide synthase

GSK-3 β = glycogen synthase kinase-3 Beta

H₂S = hydrogen sulfide

IFM = interfibrillar mitochondria

IPost-C = ischemic postconditioning

Mito-ROS: mitochondrial reactive oxygen generation

NO = nitric oxide

PBS = phosphate buffered saline

PostC-H₂S = postconditioning with H₂S

PTP = permeability transition pore

RISK = reperfusion injury salvage kinase (signalling pathway)

RNS = reactive nitrogen species

SSM = subsarcolemmal mitochondria

TPP⁺ = triphenylphosphonium

Tables of Links

Targets	Ligands
Enzymes ^a	AP39
<u>PI3K</u>	AP219
<u>Akt (PKB)</u>	ADT-OH
<u>eNOS</u>	<u>LY294002</u>
<u>sGC</u>	<u>L-NAME</u>
<u>GSK-3β</u>	<u>ODQ</u>
<u>ERK1</u>	<u>Cyclosporine A</u>
<u>ERK2</u>	<u>Rhodamine 123</u>
Cyclophilin D	

These tablets list key targets in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016) and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

Introduction

In myocardial ischaemia-reperfusion injury, rapid pH normalisation, Ca^{2+} overload and overwhelming generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) at reperfusion disturb mitochondrial function and result in the opening of the mitochondrial permeability transition pore (PTP) (Hausenloy and Yellon, 2007). PTP opening leads to collapse of mitochondrial membrane potential, swelling of the mitochondria and the leakage of cytochrome c into the cytoplasm. As a result, ATP-production will be impaired, initiating cell apoptosis/necrosis (Pell et al., 2016, Murphy et al., 2016). PTP opening at reperfusion is believed to be the no-return point of reperfusion injury (Hausenloy et al., 2009). Therapeutic targeting of these processes during the first minutes of reperfusion has been investigated intensively in experimental settings as early reperfusion appears to afford a window of opportunity to prevent PTP opening and ultimately reduce lethal cell injury (Ferdinandy et al., 2014).

The roles of endogenous hydrogen sulfide (H_2S), in a wide range of physiological systems, has been extensively explored following the discovery that it is produced by several regulated biochemical pathways in mammalian species (Kimura, 2011). In myocardium, enhanced levels of H_2S , whether by H_2S supplement or increased endogenous production, have been shown to protect the heart against ischaemia-reperfusion injury (Johansen et al., 2006, Elrod et al., 2007, Karwi et al., 2016). The exact cardioprotective mechanism of H_2S yet to be clarified but a number of molecular targets have been identified. These include activation of the reperfusion injury salvage kinase (RISK) pathway (Hausenloy, 2013); enhanced cellular and mitochondrial antioxidant defences; and preservation of mitochondrial integrity (Bos et al., 2015). However, these effects have been inconsistent in many experimental studies for several reasons including variations in animal species and models, different experimental conditions, and inconsistencies in dosing with inorganic sulfide salts (Bos et al., 2015). Inorganic sulfide salts (notably NaHS and Na_2S) have been extensively employed to explore the biological activity of H_2S . Nevertheless, these salts are impure and generate H_2S instantaneously at high (i.e. supraphysiological) concentrations and there is increasing concern that they are unreliable sources of H_2S (Whiteman et al., 2011).

We have examined cardioprotection by a novel mitochondria-targeting H₂S donor, AP39 (10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide) (Le Trionnaire et al., 2014) when given as adjunct to reperfusion and its direct effect on cardiomyocyte mitochondria, namely, subsarcolemmal and interfibrillar mitochondria. The rationale for targeted delivery of H₂S to the mitochondria is based on the evidence that H₂S can attenuate mitochondrial ROS (mito-ROS) generation and preserves mitochondrial integrity. There are recent observations that AP39 can successfully deliver H₂S into the mitochondria when given at reperfusion and that the agent reproducibly protects the mitochondria in particular and the cell in general against ischaemia-reperfusion insults in the brain and kidney (Ikeda et al., 2015, Ahmad et al., 2016). We hypothesised that AP39 protects the heart against ischaemia-reperfusion injury when administered at reperfusion through a cytosolic-independent mechanism. We also hypothesised that AP39 attenuates mito-ROS generation and thereby inhibits PTP opening in the SSM and IFM.

Materials

AP39 and the control compounds, AP219 and ADT-OH, were synthesised by us as previously reported (Tomasova et al., 2014, Szczesny et al., 2014, Le Trionnaire et al., 2014). The purity of the compound was determined by NMR spectroscopy (¹H, ³¹P and ¹³C). The irreversible heme-site soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 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 DMSO were all purchased from Sigma-Aldrich, Gillingham, UK. Western blotting antibodies were all sourced from Cell Signalling, UK.

Methods

Animals and ethical statement

All animals care and procedures for *in vivo* studies complied with UK Home Office Guidelines on the Animals (Scientific Procedures) Act 1986, (published by the Stationery Office, London, UK), project licence (PPL30/3032) and was approved by the Animal Welfare and Ethical Review Body at Cardiff University. Studies involving mitochondria isolation was approved by the Animal Welfare Office of the Justus-Liebig University Giessen. Male Sprague Dawley rats, 300-350 g (9-11 weeks), were obtained for *in vivo*

studies from Harlan, UK. For mitochondria isolation, male Wistar rats, 300-350 g (9-11 weeks), were purchased from Harlan, France. They were housed in polypropylene cages (2-4 rats in each) on wood shaving litter. Animals 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 at all times. Animals studies were reported in accordance with ARRIVE guidelines (Kilkenny et al., 2010, McGrath et al., 2010).

Acute myocardial infarction model

Myocardial infarction was induced as previously reported (Karwi et al., 2016). Briefly, rats were anaesthetised using thiobutabarbital (Inactin[®] 200 mg kg⁻¹, i.p) and anaesthesia was maintained throughout the procedure by supplemental dosing (75 mg kg⁻¹, i.v) as required. The left jugular vein was cannulated for drug administration while the right common carotid artery was cannulated and connected to a pressure transducer (Powerlab data acquisition system, AD instruments, Abingdon, UK) to monitor the heart rate and the blood pressure throughout the experiment. The trachea was intubated and the animal ventilated with room air using a small animal ventilator (Hugo Sachs Elektronik, March, Germany) at a rate of 75 strokes min⁻¹ and tidal volume of 1.0 to 1.25 mL 100 g⁻¹. The chest was opened by midline sternotomy and the heart exposed using a retractor. The pericardium was incised and a 4/0 braided silk suture (Mersilk, Ethicon Ltd, UK) was placed around the left main coronary artery close to its origin to induce regional ischaemia. Electrocardiography (ECG) was monitored using standard lead II electrodes inserted subcutaneously into the limbs and connected to a Powerlab data acquisition system. Rectal temperature was maintained at 37 ± 1 °C using thermal blanket (Harvard Apparatus Ltd, Cambridge, UK). The following inclusion criteria were employed during the stabilisation period of 20 minutes: no ECG or visual signs of ischaemia, steady sinus rhythm without arrhythmia, heart rate ≥ 250 beats per minute, diastolic blood pressure ≥ 50 mmHg.

To induce regional ischaemia in the left ventricle, the left coronary artery was transiently occluded for 30 minutes by pulling the ligature taut through a plastic snare fixed against the epicardium. Ischaemia was confirmed by colour change of the left ventricle, drop in the mean arterial pressure (MAP), ST-segment elevation and arrhythmia developing between 5-13 minutes of ischaemia. The ligature was then released to start reperfusion for 120 minutes.

Successful reperfusion was confirmed by blushing of the previously ischaemic area, reperfusion-induced arrhythmia and increase in the MAP.

Infarct size determination

At the end of 120 minutes reperfusion, the heart was harvested and retrograde perfused with saline through the aorta on a modified Langendorff apparatus. The ligature was re-occluded and the heart perfused with 2% Evans' blue dye to delineate the ischaemic area at risk (AAR), then quickly frozen at -20°C for 24 hours. The heart was transversely sectioned into 5-6 sections of 2 mm thickness and incubated with 1% w/v triphenyltetrazolium chloride (TTC) for 15 minutes. Sections were then fixed with 4% formalin in PBS for 24 hours before being scanned. Sections were scanned using digital scanner and coded using random number generator (<https://www.random.org>) then planimetry was carried out in blind fashion using the image analysis program Image J (version 1.47, NIH, Bethesda, USA). The analysis determined the total ventricular area (TVA, Evans' blue positive), area at risk (AAR, TTC positive) and the infarcted area (I, TTC negative), which were converted to volumes by multiplying these areas by 2 mm section thickness. Infarct size was expressed as a percentage of the area at risk (% I/AAR).

Treatment protocols

Experimental protocols are summarised in Figure 1. Two series of experiments were carried out. The first series characterised the dose-dependent infarct-limiting effect of AP39 along with AP219 (mitochondria-targeting moiety) and ADT-OH (H₂S-releasing moiety) to confirm the selective effect of H₂S delivery into the mitochondria. Doses of AP39, AP219 and ADT-OH used in these experiments derived from *in vitro* and *in vivo* studies undertaken by others (Szczesny et al., 2014, Ikeda et al., 2015, Ahmad et al., 2016).

Animals were randomised to receive one of six interventions (Figure 1A):

- Group 1: Control (n=10). Animals received a bolus dose of (0.05% DMSO, i.v.) 10 min before reperfusion. DMSO was used as a vehicle for AP39, AP219 and ADT-OH.
- Group 2-4: Each group (n=8) received AP39 at (0.01, 0.1 or 1 $\mu\text{mol kg}^{-1}$, respectively) as an i.v. bolus 10 min before reperfusion.
- Group 5: AP219 (n=8). Animals received AP219 (1 $\mu\text{mol kg}^{-1}$) as an i.v. bolus 10 min before reperfusion.

- Group 6: ADT-OH (n=8). Animals received ADT-OH ($1 \mu\text{mol kg}^{-1}$) as an i.v. bolus 10 min before reperfusion.

The optimum dose of AP39 ($1 \mu\text{mol kg}^{-1}$), selected from the first series of experiments, was used in a second series of experiments which investigated the involvement of the RISK pathway components using inhibitors of Akt phosphorylation (LY294002), eNOS (L-NAME) or soluble guanylyl cyclase (ODQ). Animals were randomly assigned to one of the following eight treatment groups (Figure 1B).

- Group 1: Control (n=11). Animals received DMSO 0.05% given as an i.v. bolus 15 min before reperfusion. DMSO was used as vehicle for AP39, LY294002 and ODQ.
- Group 2: AP39 (n=8). Animals received AP39 ($1 \mu\text{mol kg}^{-1}$) as an i.v. bolus 10 min before reperfusion.
- Group 3: AP39 + L-NAME (n=8). L-NAME (20 mg kg^{-1}) was administered 15 min before reperfusion as an i.v. bolus followed by AP39 ($1 \mu\text{mol kg}^{-1}$) 10 min before reperfusion.
- Group 4: L-NAME (n=8). L-NAME (20 mg kg^{-1}) was administered 15 min before reperfusion as an i.v. bolus.
- Group 5: AP39 + LY294002 (n=8). LY294002 (0.3 mg kg^{-1}) was given 15 min before reperfusion as an i.v. bolus followed by AP39 ($1 \mu\text{mol kg}^{-1}$) 10 min before reperfusion.
- Group 6: LY294002 (n=8). LY294002 (0.3 mg kg^{-1}) was administered 15 min before reperfusion as an i.v. bolus.
- Group 7: AP39 + ODQ (n=8). ODQ (1 mg kg^{-1}) was given 15 min before reperfusion as an i.v. bolus followed by AP39 ($1 \mu\text{mol kg}^{-1}$) 10 min before reperfusion.
- Group 8: ODQ (n=8). ODQ (1 mg kg^{-1}) was administered 15 min before reperfusion as an i.v. bolus.

In a parallel series prepared for biochemical analysis of RISK pathway components, animals were randomised to receive either vehicle (0.05% DMSO) or AP39 ($1 \mu\text{mol kg}^{-1}$) 10 minutes before reperfusion (Figure 1C). The heart was excised after 5 min of reperfusion and washed with saline to remove any blood residue. Tissue samples were rapidly harvested from the left ventricle, snap frozen with liquid nitrogen then kept at $-80 \text{ }^\circ\text{C}$. These samples were used to

investigate the effect of AP39 on the phosphorylation of Akt, eNOS, GSK-3 β and ERK1/2 at the commencement of reperfusion using Western blotting.

Isolation of cardiac mitochondria

Isolation of two mitochondrial subpopulations, subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM), was carried out using a modified protocol after Boengler et al. (2009). All the procedures were undertaken at 4 °C to maintain mitochondrial integrity. Each rat was anaesthetised with 4% v/v isoflurane and the heart was quickly excised and washed with Buffer A (100 mM KCl, 50 mM 3-[N-morpholino]-propanesulfonic acid (MOPS), 5 mM MgSO₄, 1 mM ATP, 1 mM EGTA, pH 7.4). The ventricles were isolated and weighed. Ventricles were transferred to Buffer B (Buffer A + 0.04% bovine serum albumin), finely chopped with scissors then gently minced with 6 strokes of a teflon pestle in a glass tube. Homogenate was centrifuged at 800 g for 10 min. The supernatant from the first centrifugation was collected and centrifuged for 10 minutes at 8000 g to isolate the SSM. The sediment was resuspended for 1 minute in Buffer B with protease nargase (8 U g⁻¹) then gently minced with 5 strokes of teflon pestle and glass mortar. The homogenate was centrifuged at 800 g for 10 minutes then the supernatant was collected and centrifuged for 10 minutes at 8000 g to sediment the IFM. SSM and IFM were washed with buffer A and final pellets were resuspended in Buffer A with no ATP. Protein concentration was determined using Lowry assay (BioRad, Hercules, Canada).

Calcium retention capacity (CRC)

Mitochondrial tolerance to calcium overload, a trigger to PTP opening, was investigated in the presence and absence of AP39 using a modified protocol of Chen et al. (2012). Freshly isolated SSM and IFM (0.1mg mL⁻¹) were randomised to be incubated for 4 minutes in 2 mL (in mM: KCl 125, Tris-MOPS 10, KH₂PO₄ 1.2, MgCl₂ 1.2, glutamate 5, malate 2.5). The suspension was supplemented with 8 μ L ADP (10 mM), 10 μ L EGTA (1 mM), 6 μ L CaCl₂ (5 mM) and calcium green-5N (1 μ M, Invitrogen, Carlsbad, Canada). Mitochondria were treated with vehicle (0.003% ethanol) or AP39 (1 μ M) during the incubation period. Cyclosporine A (1 μ M) was used as a positive control as it is a well-known inhibitor of the PTP opening and increases mitochondrial tolerance to Ca²⁺ overload in a cyclophilin D-dependent mechanism. Pulses of Ca²⁺ (5 μ mol) were added at 3 minutes intervals to the

solution with stirring at 25 °C and mitochondrial calcium tolerance was expressed as μmol of Ca^{2+} mg^{-1} of protein. Fluorescence was measured with excitation and emission wavelengths 530 and 530 nm, respectively. Data were coded using random number generator (<https://www.random.org>) and blindly analysed.

Mitochondrial oxygen consumption

The respiration of SSM and IFM was measured using a Clark-type oxygen electrode (Strathkelvin, Glasgow, UK) at 25 °C. The concentrations range of AP39 used for the mitochondrial studies were equivalent to the *in vivo* doses and after assessing the direct effect of AP39 on the mitochondria autofluorescence and membrane potential (data not shown). Basal mitochondrial oxygen consumption was measured in the presence and absence of either the vehicle (0.003% ethanol) or AP39 (0.3, 1, 3, 5 μM). Mitochondria (0.1 mg ml^{-1}) were randomised to receive one of the treatments and were incubated in two chambers simultaneously, one with complex I substrate (5 mM glutamate and 2.5 mM malate) or with complex II substrate (5 mM succinate plus 2 μM of rotenone, to inhibit complex I activity). Respiration was stimulated by addition of 40 μM of ADP and oxygen consumption was reported as nmol of $\text{O}_2/\text{min}/\text{mg}$ of protein. Oxygraph charts were randomly coded (<https://www.random.org>) and blindly analysed.

Mitochondrial ROS (mito-ROS) generation

Measurement of mito-ROS generation was carried out as previously described by Soetkamp et al. (2014). Freshly isolated SSM or IFM (50 μg) were suspended in incubation buffer (in mM: Tris-MOPS 10, EGTA 0.02, KCl 125, glutamate 5, malate 2.5, Pi-Tris 1.2, MgCl_2 1.2, pH 7.4). 0.1 U mL^{-1} horseradish peroxidase (HRP, Roche Diagnostic, Grenzach, Germany) and fifty μmol Amplex UltraRed (Invitrogen, Eugene, OR) was added to the suspension directly before the measurement. Cardiomyocyte mitochondria were randomly incubated with: 1) no intervention, (2) vehicle (0.003% ethanol), (3) AP39 (0.3, 1, 3 and 5 μM). A second control group with no intervention was employed at the end of the all measurements to ensure that any observed effects are due to AP39 and not because of the decline in the respiratory capacity. SSM and IFM were also incubated with Rotenone (2 μM) to induce overproduction of mito-ROS generation and used as a positive control. Mito-ROS generation was measured for 4 minutes at room temperature using Cary Eclipse spectrophotometer (Agilent technologies, Santa Clara, Canada) at excitation/emission wavelengths 565/581 nm.

Using code generator (<https://www.random.org>), data were coded and the slope of mito-ROS generation was calculated, as a mean fluorescence per time (a.u.), by an operator blind to the treatments after subtracting the background fluorescence of the incubation buffer.

Western blot analysis

Myocardial samples were homogenised and lysed using a hard tissue lysing kit (Stretton Scientific Ltd, Stretton, UK). Thirty μg of protein was loaded into each well of 10% w/v sodium dodecyl sulfate-polyacrylamide gel and separated electrophoretically at (120 mV). Separated proteins were transferred onto nitrocellulose membrane (Amersham, Germany) and the membrane was blocked for non-specific binding with 5% skimmed milk for 2 hours. The membrane was then probed with the primary antibody overnight at 4 °C. The membrane was then incubated with secondary antibody (goat anti-rabbit HRP, 1:15000, Cell Signalling UK) for 1 hour at room temperature. Super Signal West Dura Extended Duration Substrate (Thermo Scientific, UK) was added on the surface of the membrane to laminate the bands and the bands were visualised on X-ray film. Films were scanned and coded and densitometry was carried out in blind fashion using Image J software (1.48v, National Institutes of Health USA). All protein bands were expressed as the relative density of myocardium sample, harvested after 20 minutes of stabilisation (baseline), and then normalized for correspondent GAPDH bands which served as an internal standard.

Antibodies

The following antibodies were used for western blotting: Akt (1:1000), phospho- ser⁴⁷³Akt (1:1000), endothelial nitric oxide synthase (eNOS 1:500), phospho- ser¹¹⁷⁷eNOS (1:500), glycogen-synthase kinase-3 beta (GSK-3 β 1:1000), phospho- ser⁹GSK-3 β (1:1000), extracellular signal-regulated kinases ERK 1/2 (1:1000), phospho- Thr²⁰²/Tyr²⁰⁴ ERK 1/2 (1:1000) and GAPDH (1:50000).

Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). All data passed the Kolmogorov-Smirnov normality test of distribution. Statistical analysis was performed using GraphPad Prism[®] software (2007, Version 5.01, USA) and data presented as mean \pm SEM. Infarct size data were analysed using one-way ANOVA with Newman-Keuls *post hoc* test and Western blot

analysis was performed using unpaired student's *t* test. Haemodynamic and mitochondrial data were statistically analysed using repeated measures ANOVA supported by Bonferroni's *post hoc* test. $P < 0.05$ was considered statistically significant. Post tests were only carried out if $P < 0.05$ was achieved in the ANOVA.

Results

For the AP39 dose-response study, 52 rats were used, of which two were excluded: one did not have successful reperfusion and one rat did not survive ischaemia-induced ventricular fibrillation. Therefore, data from 50 successfully completed experiments are presented. In the second series, 83 rats were used, of which four were excluded: two did not complete the ischaemia-reperfusion protocol, one did not have successful TTC staining and one did not survive reperfusion-induced arrhythmia. Thus, data from 79 rats, 67 infarct size experiments which were successfully completed and 12 tissue sampling experiments, were reported. For mitochondria functional studies, data from 20 rats are reported.

Pharmacological postconditioning with AP39

Baseline parameters for infarct size studies are shown in (Table 1). There was no difference among the 14 experimental groups in any of the baseline parameters. Risk zone was similar among the experimental groups (50-60% of the total ventricular volume, Figure 2A).

Administration of AP39 10 minutes before reperfusion resulted in a dose-dependent infarct-sparing effect compared with vehicle-treated animals (Figure 2B). The maximum cardioprotection was seen at $1 \mu\text{mol kg}^{-1}$ dose with almost 40% reduction in infarct size compare to vehicle-treated animals. Postconditioning with AP39 ($1 \mu\text{mol kg}^{-1}$) also dose-dependently increased in the post-ischaemic functional recovery (% RPP recovery as a percentage of pre-ischaemia RPP) measured at the end of reperfusion ($67.2 \pm 3.8\%$) compare to the control hearts ($46.2 \pm 3.8\%$, Table 1). The control compounds, namely AP219 and ADT-OH, did not have a significant effect on either RPP recovery or infarct size, confirming that selective delivery of H_2S to the mitochondria mediates AP39's cardioprotection.

Cytosol-independent mechanism of postconditioning with AP39

We next investigated the effect of AP39 on the RISK pathway as a relevant protective cytosolic-signalling pathway using a "signal tracing" technique. Specific pharmacological

inhibitors, namely the PI3K inhibitor LY294002 (Jiang et al., 2007), constitutive NOS inhibitor L-NAME (Fradorf et al., 2010) and sGC inhibitor ODQ (Routhu et al., 2010) were used at doses that have previously been reported to abrogate the activity of their targets in *in vivo* models.

There was no significant difference in either the baseline characteristics or the risk zone (ischaemic bed) among the groups (Figure 3A). None of the pharmacological inhibitors had a significant effect on infarct size when given alone 15 minutes before reperfusion compared to the control group (Figure 3B). Blockade of PI3K activity with LY294002 did not abolish the infarct limitation by AP39. Similarly, neither blockade of NO synthesis by L-NAME nor selective inhibition of its downstream effector, sGC, with ODQ attenuated the protective effect of AP39.

The effect of AP39, used as an adjunct to reperfusion, on the key cytosolic components of the RISK pathway was also evaluated in samples harvested from the left ventricle after 5 minutes of reperfusion (Figure 4). Immunoblotting was carried out using phospho-specific antibodies for Akt, eNOS, GSK-3 β and ERK1/2 to outline their role in the cardioprotection. In line with the infarct size data, Western blot analysis showed that administration of AP39 at reperfusion had no significant effect on the phosphorylation of either Akt, eNOS, GSK-3 β or ERK1/2. This confirms that AP39 mediated its cardioprotection independently of these cytosolic components on the RISK pathway.

Mitochondrial effects of AP39

We examined the effect of specific-delivery of H₂S into the mitochondria on the susceptibility to PTP opening. We used freshly isolated SSM and IFM and treated them with vehicle or AP39 (1 μ M). SSM and IFM were exposed to pulses of Ca²⁺ in the presence and absence of CsA as a positive control (Figure 5). Untreated IFM showed 30% higher calcium tolerance (by 30%) than untreated SSM. AP39 elicited a significant inhibitory effect on the PTP opening in both SSM and IFM, which represents 30% increase in Ca²⁺ overload tolerance, compared to vehicle-treated mitochondria. The inhibitory effect of AP39 on PTP opening was comparable to that observed after CsA in SSM and IFM. Interestingly, AP39 showed 25% additive effect to CsA-induced inhibition of PTP opening when either SSM or

IFM were incubated with both AP39 and CSA before the exposure to Ca^{2+} pulses, compared to CsA alone.

Mitochondrial respiration was measured for both SSM and IFM using substrates for complex I (glutamate and malate) and complex II (succinate, in the presence of rotenone to inhibit complex I). There was no difference in the basal respiration of the two subpopulations of mitochondria. ADP-stimulated respiration was higher in IFM (by 25% and 31% for complex I and II, respectively) compared to SSM. Different concentrations of AP39 (0.3, 1, 3, 5 μM) were tested on SSM or IFM; however, none of the concentrations examined significantly influenced mitochondrial oxygen consumption.

Overwhelming mito-ROS generation at early reperfusion is one of the main determinants of cellular injury. Therefore, we performed *in vitro* experiments to look at the direct effect of AP39 on H_2O_2 generation in the isolated rat left ventricle mitochondria (Figure 7A, B, C, D). In the control groups, ROS generation was significantly lower by 20% in IFM than SSM. AP39 showed a dose-dependent inhibition of ROS generation in both SSM and IFM. AP39 (1 μM) exerted the maximum inhibitory effect (38% in SSM and 61% in IFM) compared to control, vehicle-treated and the second control mitochondria. Interestingly, the inhibitory effect of AP39 on mito-ROS generation was gradually reduced as the concentration was increased. Rotenone, as a positive control, resulted in overproduction of ROS in both SSM and IFM by 65% and 75%, respectively, compare the basal ROS generation level.

Discussion

The lipophilic TPP^+ scaffold is an attractive moiety for investigating mitochondrial function as it selectively accumulates (100-500 fold *versus* cytosol) in the mitochondrial matrix (Murphy and Smith, 2007, Smith et al., 2011). Previous work (Prime et al., 2009) demonstrated infarct limitation using a mitochondria-targeted NO donor (MitoSNO) with an NO-releasing moiety linked to TPP^+ . Work by Krieg's group (Methner et al., 2013) showed that MitoSNO works independently of cytosolic protein kinase G which mediates the cardioprotective effect of non-mitochondrial-targeted NO donors. AP39 represents the first successful attempt to deliver H_2S selectively and at low concentration to the mitochondria (Le Trionnaire et al., 2014, Tomasova et al., 2014, Szczesny et al., 2014, Ikeda et al., 2015, Ahmad et al., 2016).

In the present study, AP39 significantly limited infarct size (Figure 2B) and improved the post-ischaemic functional recovery (Table 1), both in a dose-dependent manner, when administered prior to reperfusion. Consistent with these data, Ahmed et al. (2016) reported that AP39 also exerted attenuation in renal damage, oxidative stress and renal inflammation when applied at reperfusion in an *in vivo* renal ischaemia-reperfusion injury model. We observed that the TPP⁺ scaffold molecule (AP219) and the H₂S-generating moiety (ADT-OH), which were used as controls, had no effect on myocardial injury. This is in agreement with other reports where these controls lacked biological activity when used at nanomolar or micromolar concentrations (Le Trionnaire et al., 2014, Szczesny et al., 2014, Ahmad et al., 2016).

At the time of finalising this manuscript, recent work with AP39 by Papapetropoulos's group (Chatzianastasiou et al., 2016) has appeared. The main focus of Chatzianastasiou's work is "head-to-head" comparison of infarct limitation by different H₂S donors (Na₂S, GYY4137, thiovaline and AP39), and elucidation of the role of NO in mediating protection. Intriguingly, all donors had the same infarct-limiting effect in a mouse model of ischaemia-reperfusion injury. It is noteworthy that the optimum cardioprotective doses of GYY4137 and AP39 used were 26.6 $\mu\text{mole kg}^{-1}$ and 0.25 $\mu\text{mol kg}^{-1}$, respectively. Very recently, we reported that 26.6 $\mu\text{mole kg}^{-1}$ GYY4137 was not cardioprotective in an *in vivo* rat model of ischaemia-reperfusion injury with the optimum cardioprotective dose being 10-fold higher (Karwi et al., 2016). Similarly, in the present study we demonstrated that AP39 exerts an infarct-sparing effect with an optimum cardioprotective dose of 1 $\mu\text{mol kg}^{-1}$, 4-fold higher than the effective dose in mouse reported by Chatzianastasiou et al. (2016). No haemodynamic data are available to compare AP39-induced dose-dependent improvement in post-ischaemic functional recovery with Chatzianastasiou's paper.

Our present study provides important mechanistic insight into AP39's cardioprotective action *in vivo*. We found that selective blockade of PI3K, which is known to mediate the cardioprotective effect of non-mitochondrial H₂S donors (Andreadou et al., 2015, Karwi et al., 2016), did not abolish cardioprotection (Figure 3B). Cross-talk/interaction between H₂S and NO has different patterns depending on organs/tissues, experimental conditions and species (Karwi et al., 2016, Bibli et al., 2015, King et al., 2014). To test if NO/sGC pathway mediates the effect of AP39, we blocked the endogenous NO synthesis pathway and also

inhibited the activity of its end effector (sGC) using L-NAME and ODQ, respectively. We found that cardioprotection by AP39 was still observed in the presence of L-NAME or ODQ, supported by analysis of protein phosphorylation during early reperfusion. We observed that AP39 did not induce phosphorylation of Akt, eNOS, GSK-3 β , a downstream effector of the RISK pathway, or ERK1/2, a parallel arm of the RISK pathway at 5 min reperfusion (Figure 4A, B, C and D). Chatzianastasiou et al. (2016) reported that AP39 did not phosphorylate either eNOS ser¹¹⁷⁶ or VASP ser²³⁹ after 10 minutes of reperfusion and its cardioprotection was not abolished by either L-NAME or DT2, indicating a cGMP/PKG-independent mechanism in the murine model. Their data are complementary to the fuller characterisation of the potential cytosolic signalling targets of AP39 that we present here. Viewed together, our data and those of Chatzianastasiou et al. (2016) provide persuasive evidence that, unlike other H₂S donors, AP39 mediates its cardioprotection by a mechanism that is independent of activation of the cytosolic components of the RISK signalling cascade.

Intra-mitochondrial H₂S is essential for normal function of the citric acid cycle. Levels are disturbed during oxidative stress due to increased H₂S degradation and reduced production (Geng et al., 2004, Doeller et al., 2005, Whiteman et al., 2011, Vandiver and Snyder, 2012). H₂S supplements or overexpression of endogenous synthetic enzymes have been shown to protect against ischaemia-reperfusion injury by mitigating oxidative stress and preserving mitochondrial integrity (Andreadou et al., 2015). Interestingly, Kai et al. (2012) found that NaHS-induced protection was abolished in mitochondria-free cells. Nevertheless, whether H₂S directly interacts with mitochondria or triggers cytosolic signalling pathways that converge on the mitochondria may depend on the intracellular level of H₂S. Therefore, we have characterised for the first time the direct effect of AP39 on the most relevant subpopulations of cardiomyocyte mitochondria, namely subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria. Inhibition of the PTP opening in the first minutes of reperfusion has been extensively reported to protect against reperfusion injury (Halestrap, 2010, Ong et al., 2014). It has been shown that many cardioprotective interventions act to maintain PTP in a closed state (Hausenloy et al., 2009). With this in mind, we investigated the influence of AP39 on the opening of PTP, as a result of Ca²⁺ overload, in cardiac SSM and IFM. We found that AP39 inhibited PTP opening in SSM and IFM (Figure 5) with no significant difference from the inhibitory effect of the positive control, CsA, which can protect myocardium against ischaemia-reperfusion injury (Hausenloy et al., 2012). We observed that AP39 and CsA in combination increased mitochondrial tolerance to Ca²⁺

overload and resulted in an additive effect compared to either compound alone. CsA prevents the opening of PTP by desensitising cyclophilin-D, a component of the multiprotein complex spanning the inner and outer mitochondrial membranes, which is a modulator of PTP located in the mitochondrial matrix (Bernardi and Di Lisa, 2015). Having an additive effect to CsA suggests that AP39 may inhibit PTP opening via a cyclophilin-D independent mechanism. Chatzianastasiou et al. (2016) reported that AP39 (0.3 μM versus 1 μM in our study), exerted an additive effect to CsA in mouse mitochondria isolated from the whole heart. However, isolating mitochondria from whole heart tissue is potentially problematic as a number of cell types contribute to the isolated mitochondrial fraction, for example endothelial cells, fibroblasts and other local resident cells. Even more important, cardiomyocyte mitochondria, namely SSM and IFM, themselves significantly differ in their main characteristics including oxygen consumption, mito-ROS generation and calcium retention capacity (Palmer et al., 1977, Palmer et al., 1986). Our present study confirms for the first time the effect of AP39 in specific cardiomyocyte mitochondria and both IFM and SSM subpopulations.

It has been reported that H_2S can stimulate mitochondrial ATP production by acting as an electron donor for the electron transport chain (Szabo et al., 2014, Modis et al., 2014). Accordingly, we explored the influence of AP39 on mitochondrial respiration through complexes I and II in both SSM and IFM. The respiration control ratio (RCR), an index for the coupling between mitochondrial respiration and oxidative phosphorylation, for isolated mitochondria fractions in this study was around (2.5). Although this result is comparable to our previously data (Boengler et al. 2009), others have reported higher RCR ratios (Chen et al., 2008; Asemu et al., 2013; Gao et al., 2013). This could be due to either measuring oxygen consumption at 30 °C instead of 25 °C, using trypsin instead of nargase to release the interfibrillar mitochondria or stimulating the mitochondria with higher concentration of ADP than what was used in this study. We did not detect any significant effect of AP39 on the oxygen consumption of these complexes in either mitochondrial subpopulation (Figure 6). These data suggest the safety margin of the applied concentration range. More importantly and in line with others, these results show that electron supply by H_2S (at low concentration) to the electron transport chain occurs at the level of coenzyme Q where sulfide quinone reductase (SQR) activity is involved. Following that, electrons will flow forward toward Complex III and Complex IV without affecting either Complexes I or II (Gubern et al., 2007; Szabo et al., 2014). It has been demonstrated that inhibition of Complex I using rotenone did not affect sulfide oxidation while inhibition of Complex III or VI by antimycin

or cyanide, respectively, impeded it (Goubert et al., 2007; Volkel & Grieshaber, 1996; Yong & Searcy, 2001). Investigating the effect of AP39 on mitochondrial respiration at 37 °C also needs further investigation in future work.

The detrimental effect of overwhelming mito-ROS generation, as a result of respiratory chain uncoupling, is one of the hallmarks of ischaemia-reperfusion injury (Brown and Griendling, 2015, Venditti et al., 2001). It is a major contributor to the opening of PTP, initiating cell apoptosis and accelerated necrosis during reperfusion (Hausenloy et al., 2009). Since oxidative stress and the mitochondria play central roles in ischaemia-reperfusion injury, targeting the mitochondria with selective H₂S donors is a plausible therapeutic approach to limit ischaemia-reperfusion injury. Here, we have investigated for the first time the influence of AP39 on mito-ROS generation in both SSM and IFM. We found that AP39 limited mito-ROS level in both subpopulations (Figure 7) which is in line with other studies of mito-ROS reduction with AP39 (Szczeny et al., 2014, Le Trionnaire et al., 2014, Ikeda et al., 2015, Ahmad et al., 2016). We also observed an attenuation in AP39's activity as its concentration was increased, consistent with the findings of others (Szczeny et al., 2014, Ahmad et al., 2016).

Although we have provided novel mechanistic insights into how AP39 could mediate cardioprotection, there are several caveats and current limitations. Assessing the extent of mitochondrial H₂S level increase following AP39 application remains a challenge due to the lack of sensitive probes. Ikeda et al. (2015) reported that AP39 did not influence the expression of CSE, CBS or 3-MST in the brain. This suggests that AP39 probably elevates mitochondrial H₂S level without interfering with the endogenous synthesis of H₂S. The focus of ongoing work is to investigate whether AP39 suppresses oxidative stress by increasing GSH production or by upregulating mito-ROS scavenging pathways or by directly scavenging ROS. The exact mechanism/target whereby AP39 inhibits PTP opening also remains to be determined. It may be interesting to identify what happens to these subpopulations in the animal treated with AP39. Both subpopulations play significant roles in mediating cardioprotection, although it is possible this includes persulfidation (also called S-sulfhydration) of mitochondrial proteins such as ATP synthase (Modis et al., 2016; Wedmann et al., 2016). Very recent work by Murphy's group (Sun, Aponte, Menazza, Gucek, Steenbergen & Murphy, 2016) also proposed that postconditioning with NaHS protected against myocardial infarction via an increase in S-nitrosylation and most of the S-nitrosylated

proteins were mitochondrial proteins. This further emphasises the physiological importance of post-translation modifications of H₂S and its interaction with NO in the mitochondria, a phenomenon that we are now seeking to characterise.

In conclusion, our results confirm that AP39 can protect the heart against myocardial infarction when given at reperfusion in a manner that is independent of classical cytosolic signalling mechanisms. We also report for the first time that AP39 inhibits mito-ROS generation and PTP opening in both SSM and IFM, probably in a cyclophilin D-independent manner without affecting mitochondrial respiration. These findings provide proof-of-concept that direct delivery of H₂S to mitochondria by mitochondria-targeting H₂S donors, of which AP39 is a prototype of several compounds in this class under development, represents a novel and effective adjunctive intervention to mitigate the irreversible myocardial injury associated with reperfusion.

Author contributions

Q GK and GFB conceived and designed the studies and wrote the first draft of the manuscript. QK performed all the *in vivo* studies, biochemical studies and mitochondrial studies. JB, KB and RS designed the mitochondrial studies and assisted QK with performing mitochondrial experiments and analysis of mitochondrial function data. MEW, RT and MW designed, synthesised and characterised AP39 and related compounds. All authors contributed to revision of the manuscript prior to finalisation for submission by QK and GFB.

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Conflicts of interest

MW, MEW and the University of Exeter have intellectual property (patent filings) on AP39, related compounds and their use.

Declaration of transparency and scientific rigour

This declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommendation by funding agencies, publishers and other organizations engaged with supporting research.

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Karwi_Table 1 Summary of the baseline parameters and haemodynamic data throughout ischaemia-reperfusion protocol

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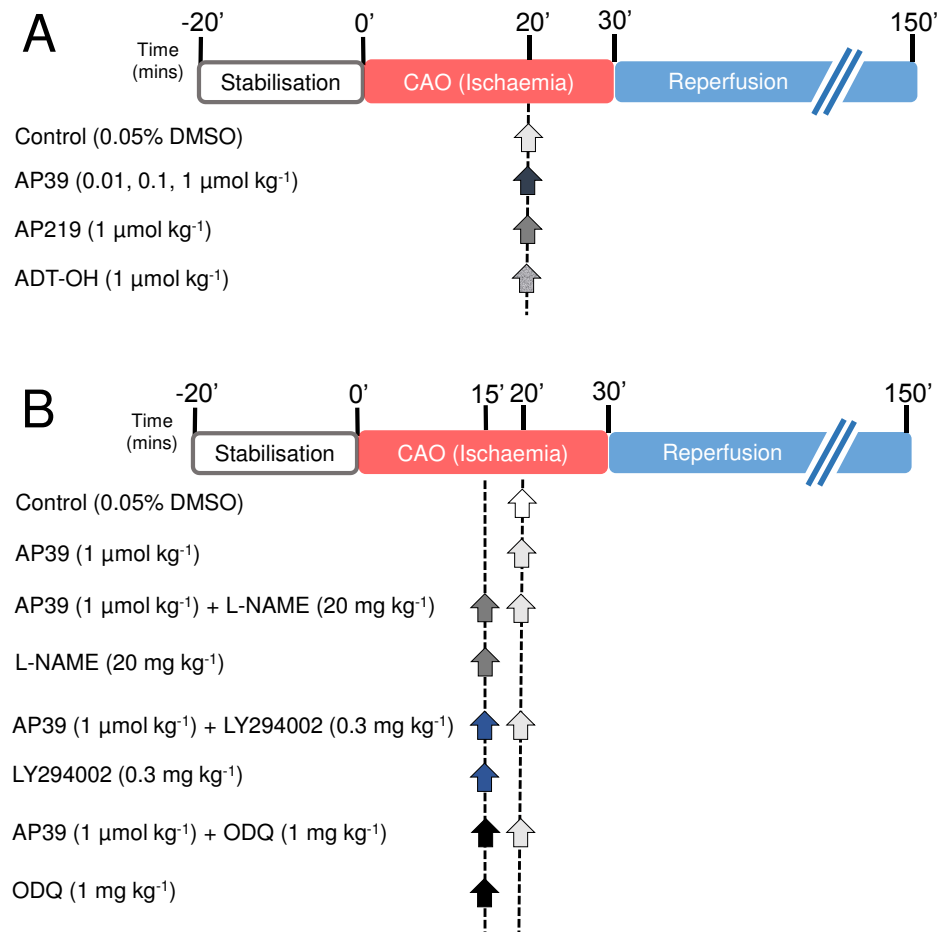
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Karwi_Table 1 Summary of the baseline parameters and haemodynamic data throughout ischaemia-reperfusion protocol

Experimental Protocol	n	BW (g)	Baseline			20 min Ischaemia			120 min Reperfusion		
			HR (BPM)	RPP (mmHg $\text{min}^{-1} \cdot 10^3$)	MAP (mmHg)	HR (BPM)	RPP (mmHg $\text{min}^{-1} \cdot 10^3$)	MAP (mmHg)	HR (BPM)	RPP (mmHg $\text{min}^{-1} \cdot 10^3$)	MAP (mmHg)
Series 1											
Control (0.05% DMSO)	10	342 ± 5	342 ± 10	36.2 ± 2.1	90 ± 6	311 ± 14	26.0 ± 1.9	70 ± 3	257 ± 9	16.7 ± 2.2	55 ± 5
AP39 0.01 $\mu\text{mol kg}^{-1}$	8	347 ± 7	351 ± 8	38.4 ± 2.8	92 ± 4	302 ± 10	27.1 ± 2.4	70 ± 5	245 ± 12	17.7 ± 1.8	58 ± 3
AP39 0.1 $\mu\text{mol kg}^{-1}$	8	339 ± 5	349 ± 12	40.0 ± 2.0	89 ± 6	299 ± 14	28.7 ± 1.2	68 ± 7	278 ± 8	20.0 ± 1.3	54 ± 5
AP39 1 $\mu\text{mol kg}^{-1}$	8	355 ± 5	356 ± 9	38.5 ± 1.5	85 ± 5	303 ± 10	29.4 ± 2.3	67 ± 3	332 ± 10	25.8 ± 1.2*	49 ± 4
AP219 1 $\mu\text{mol kg}^{-1}$	8	356 ± 7	341 ± 10	37.8 ± 2.0	87 ± 7	315 ± 9	27.6 ± 1.6	71 ± 5	250 ± 9	16.2 ± 1.5	51 ± 6
ADT-OH 1 $\mu\text{mol kg}^{-1}$	8	342 ± 6	355 ± 12	39.4 ± 2.5	90 ± 4	309 ± 11	29.1 ± 2.3	68 ± 4	243 ± 11	17.5 ± 1.8	54 ± 5
Series 2											
Control (0.05% DMSO)	11	361 ± 5	352 ± 12	35.5 ± 2.0	87 ± 6	302 ± 12	26.1 ± 1.7	65 ± 4	255 ± 12	15.2 ± 1.8	53 ± 5
AP39 1 $\mu\text{mol kg}^{-1}$	8	356 ± 7	346 ± 9	39.6 ± 3.1	92 ± 4	289 ± 10	31.1 ± 2.0	68 ± 5	320 ± 9	28.2 ± 1.5*	54 ± 6
AP39 1 $\mu\text{mol kg}^{-1}$ + L-NAME 20 mg kg^{-1}	8	365 ± 6	345 ± 11	36.0 ± 1.9	90 ± 7	285 ± 15	29.5 ± 1.8	67 ± 8	314 ± 13	22.4 ± 2.4*	53 ± 5
L-NAME 20 mg kg^{-1}	8	371 ± 9	343 ± 10	39.3 ± 1.6	89 ± 5	281 ± 12	28.6 ± 2.4	74 ± 5	230 ± 8	14.4 ± 2.1	56 ± 4
AP39 1 $\mu\text{mol kg}^{-1}$ + LY294002 0.3 mg kg^{-1}	8	359 ± 10	350 ± 7	37.4 ± 1.5	86 ± 6	291 ± 10	32.0 ± 2.2	70 ± 6	306 ± 10	24.5 ± 1.6*	52 ± 5
LY294002 0.3 mg kg^{-1}	8	367 ± 9	351 ± 11	42.3 ± 2.5	91 ± 4	305 ± 14	30.2 ± 1.7	69 ± 6	235 ± 12	16.4 ± 2.0	60 ± 7
AP39 1 $\mu\text{mol kg}^{-1}$ + ODQ 1 mg kg^{-1}	8	365 ± 7	350 ± 11	39.6 ± 1.5	93 ± 5	291 ± 11	31.5 ± 1.6	71 ± 4	329 ± 9	26.3 ± 1.9*	55 ± 5
ODQ 1 mg kg^{-1}	8	370 ± 8	342 ± 9	40.3 ± 2.5	88 ± 7	300 ± 10	28.6 ± 1.4	66 ± 5	228 ± 15	15.6 ± 2.0	63 ± 4

Animals body weight and haemodynamic parameters for infarct size studies at the end of stabilisation period (baseline), after 20 minutes of ischaemia and at the end of reperfusion. *n* number of animals per group, *BW* body weight, *HR* heart rate (beat per minutes *BPM*), *RPP* rate pressure product, *MAP* mean arterial pressure. There was no significant difference among experimental group in the baselines or the application of the pharmacological inhibitors on the haemodynamics. Values expressed as mean \pm SEM. (Two-way ANOVA followed by Bonferroni *post hoc* test), * $p < 0.05$ vs control value at the same time point

Karwi_Figure 1



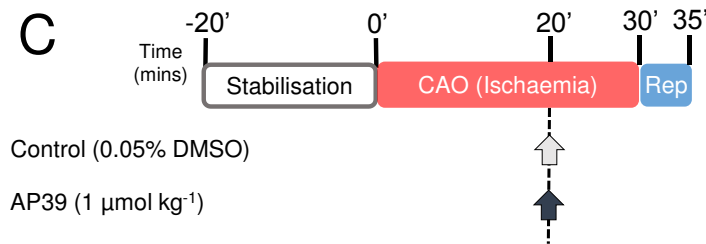


Figure 1 Experimental protocols: animals were underwent 30 minutes of ischaemia followed by 2 hours of reperfusion. Infarct size was determined using Evans' blue/TTC staining technique. Infarction was reported as a percentage of the area at risk (I/AAR %). **A** AP39 dose effect on infarct size: Animals were randomly assigned to be treated with either vehicle or AP39 or the controls (AP219 or ADT-OH) at 10 minutes before reperfusion. **B** mechanistic study: rats were randomised to receive the pharmacological inhibitors, namely LY294002, L-NAME and ODQ, at 15 minutes before reperfusion with or without AP39 applied at 10 minutes before reperfusion. Control group only received the vehicle (0.05% DMSO) 10 minutes before reperfusion. Arrows indicate the time of pharmacological interventions

Karwi_Figure 2

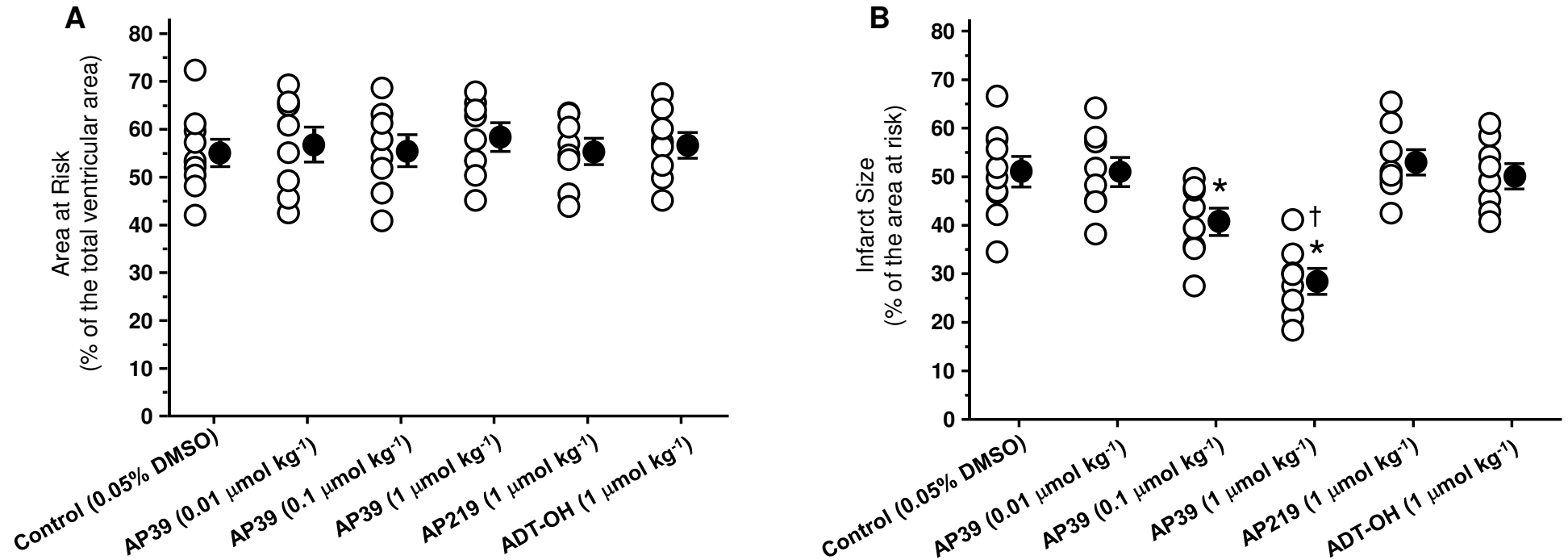


Figure 2 Infarct-limiting effect of AP39 at reperfusion: **A** area at risk reported as a percentage of the total ventricular volume. **B** infarct size presented as a percentage of the area at risk. Data were analysed using one-way ANOVA with Neuman Keuls *post hoc* test and presented as mean \pm SEM, $n = 8$ for all groups except the control group (0.05% DMSO) where $n = 10$. The mean of infarct size for each group is represented by a filled circle (with error bars) next to the individual values (open circles). * $p < 0.05$ vs control, † $p < 0.05$ versus AP39 0.1 $\mu\text{mol kg}^{-1}$

Karwi_Figure 3

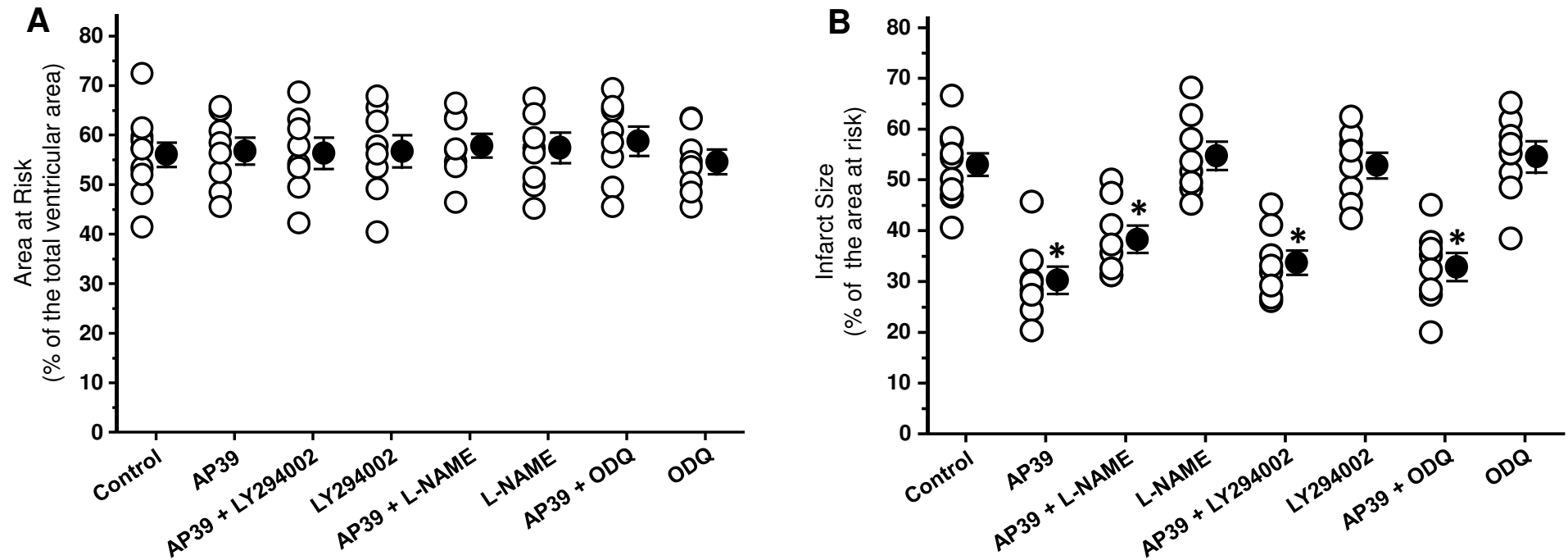


Figure 3 Effect of pharmacological inhibitors of the RISK pathway on infarct-limitation by AP39: **A** risk zone measurements of experimental groups expressed as a percentage of the total ventricular area. **B** myocardial infarction data are expressed as a percentage of the risk zone. Individual animal data in each group are represented by empty circles while the mean of infarct size is presented by a full circle. Data were analysed via one-way ANOVA followed by Newman Keuls *post hoc* test and reported as mean \pm SEM, $n = 8$ for all groups except the control group where $n = 11$. * $p < 0.05$ versus control.

Karwi_Figure 4

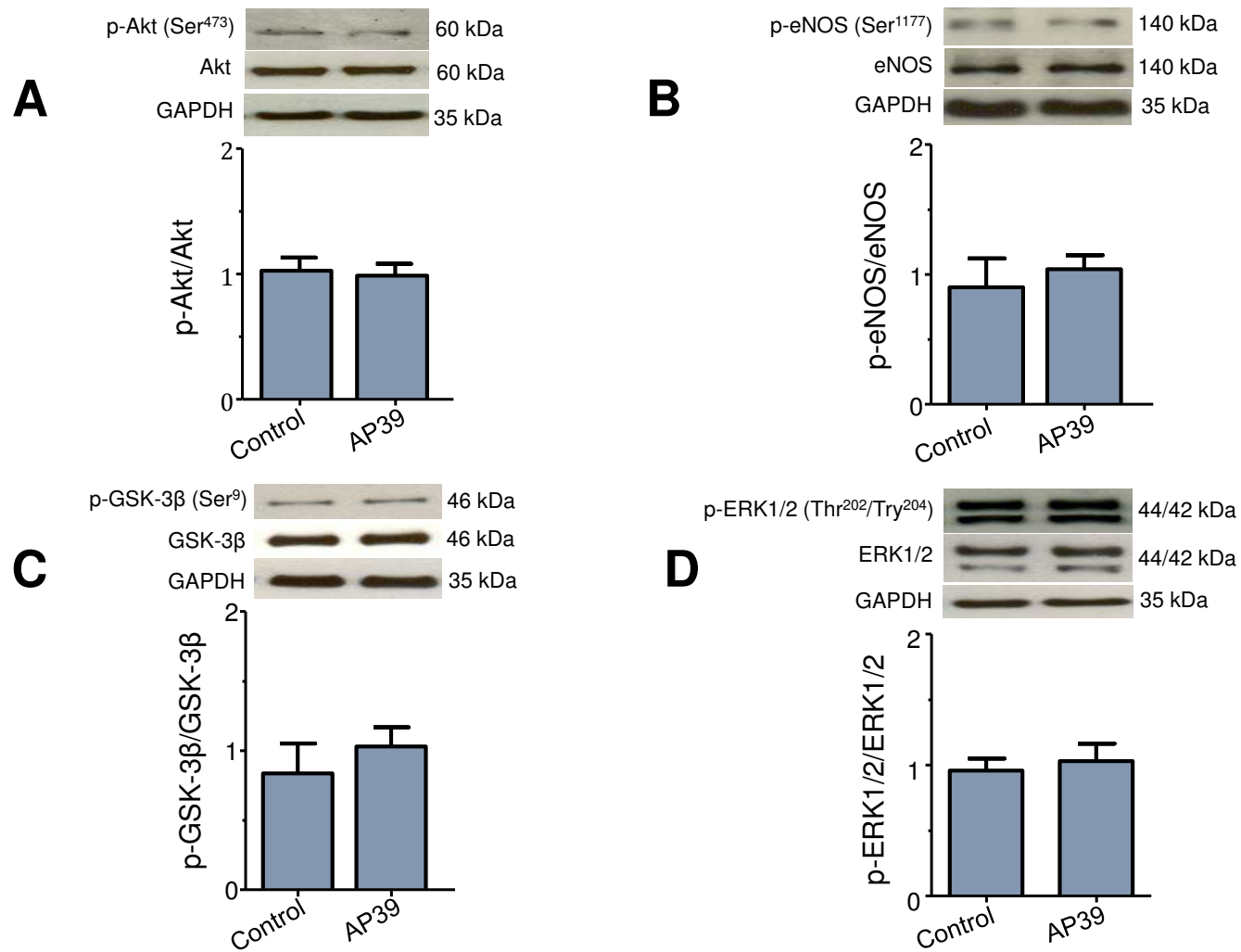


Figure 4 Effect of AP39 on RISK pathway proteins at early reperfusion: representative Western blots and densitometry analysis of **A** pAkt^{S473}, total Akt and GAPDH **B** p-eNOS^{S1177}, total eNOS, GAPDH **C** p-GSK-3 β ^{S9}, GSK-3 β and GAPDH **D** p-ERK1/2^{Thr202/Tyr204}, ERK1/2 and GAPDH. Specific antibodies were used to assess the effect of AP39 on the phosphorylation of the RISK components in myocardial biopsies harvested from the left ventricle at early reperfusion. Histograms show the relative ratio of phosphorylated protein to the total level of protein. GAPDH was used as an internal standard for all quantifications. Data were analysed using student's *t* test and presented as mean \pm SEM, *n*=6 per group

Karwi_Figure 5

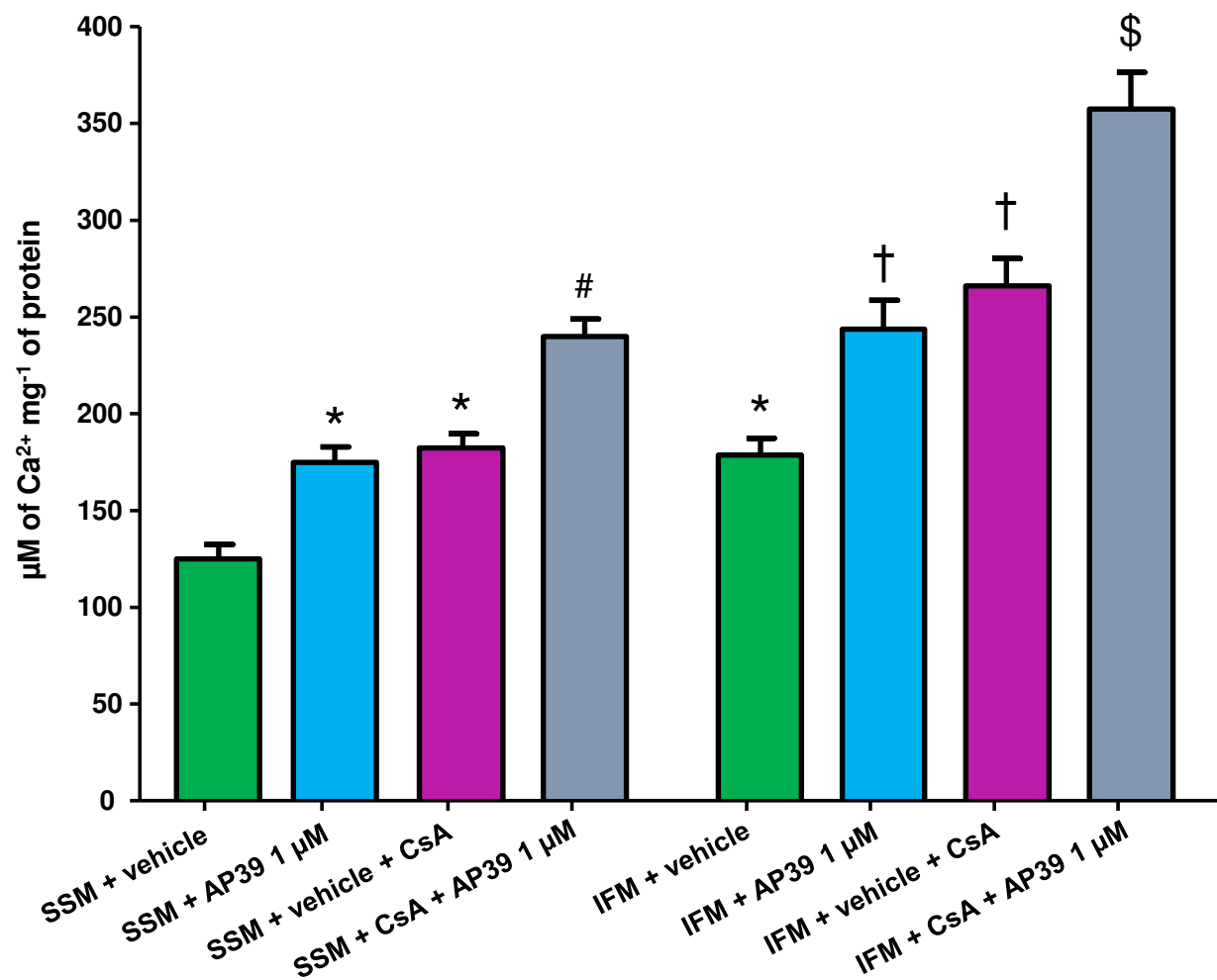
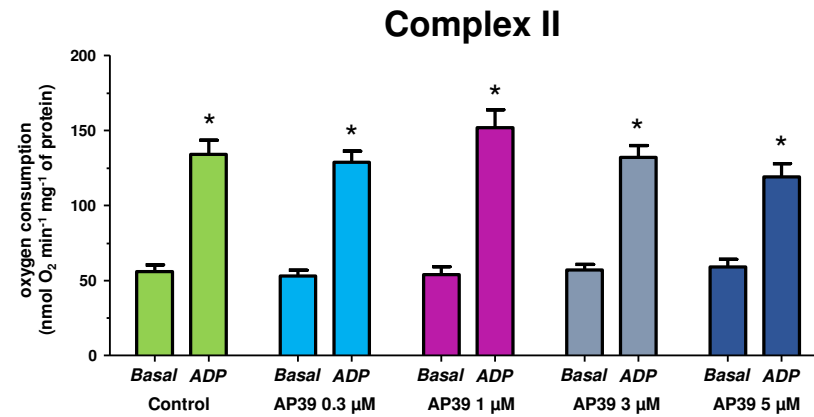
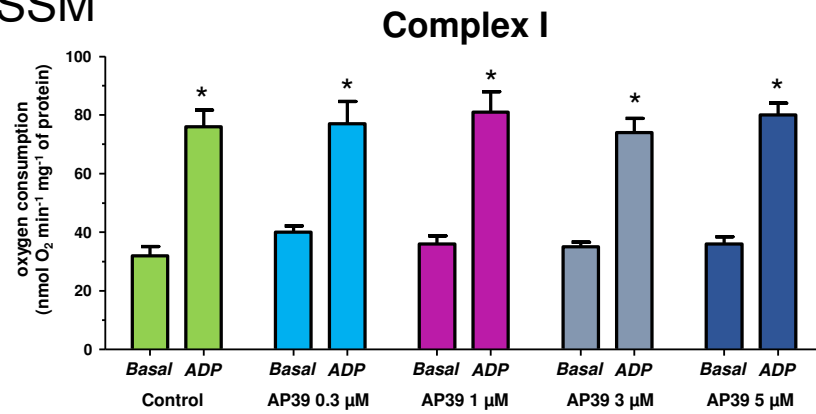


Figure 5 Effect of AP39 on mitochondrial permeability transition pore (PTP) opening: SSM and IFM were incubated individually with vehicle (0.003% ethanol) or different concentrations of AP39 and subjected to pulses of 5 μ M of CaCl₂ per 3 minutes at 25 °C until the opening of PTP in the presence and absence of cyclosporine A (CsA). Data expressed as mean \pm SEM, n=10, * p<0.05 vs SSM + vehicle, # p<0.05 vs SSM + vehicle + CSA, † p<0.05 vs IFM + vehicle, † p<0.05 vs IFM + vehicle, \$ p<0.05 vs IFM + vehicle + CSA (two-way ANOVA followed by Bonferroni *post hoc* test, n=10)

Karwi_Figure 6

SSM



IFM

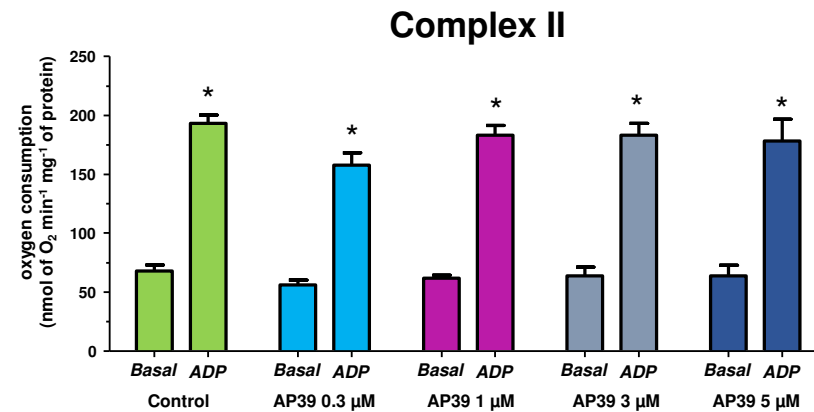
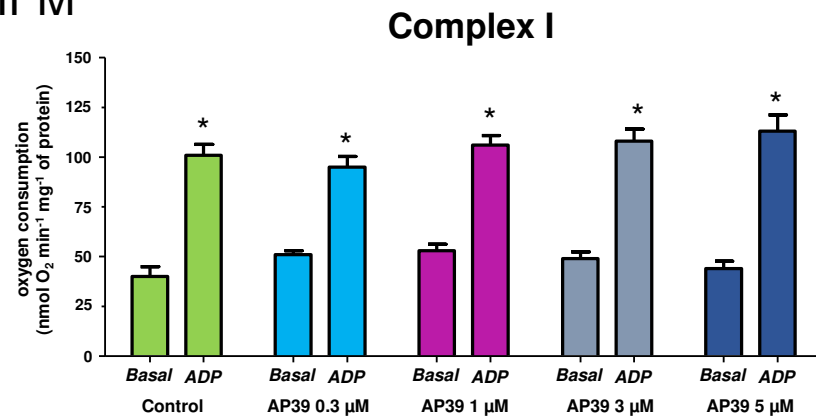


Figure 6 Effect of AP39 on mitochondrial respiration: respiration of complexes I and II were measured at basal level and after ADP-stimulation in the presence and absence of the vehicle or different concentrations of AP39 in subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria. Data were analysed via two-way ANOVA with Bonferroni *post hoc* test and reported as mean \pm SEM, $n=10$, * $p<0.05$ vs basal respiration

Karwi_Figure 7

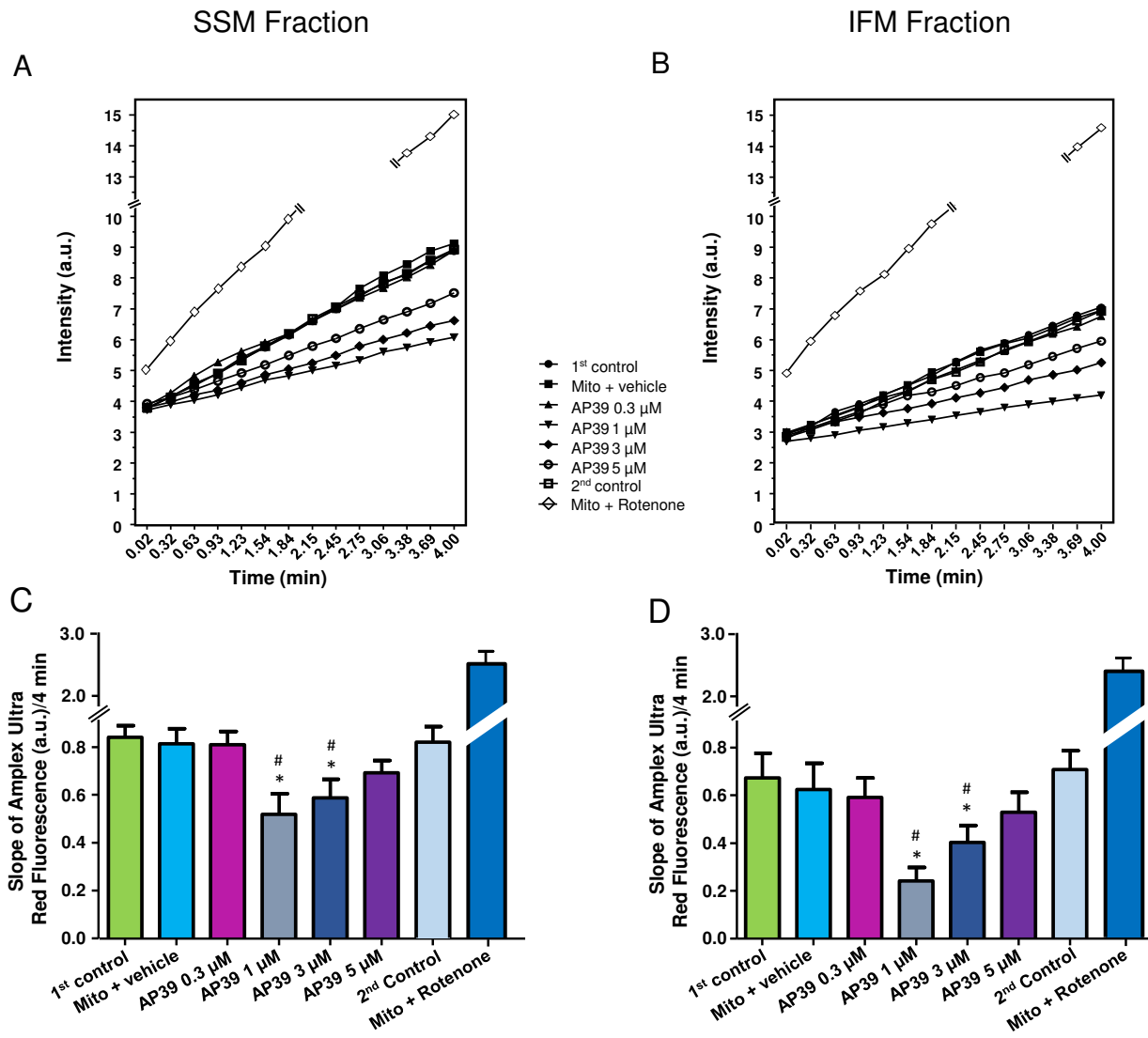


Figure 7 Effect of AP39 on mitochondrial-ROS generation: mitochondria were incubated with either vehicle (0.05% DMSO) or different concentration of AP39. **A** and **C** are representative charts for the ROS generation of SSM and IFM, respectively, and error bars were removed for clarity. The slope of ROS generation was measured continuously for 4 minutes with the fluorescence indicator Amplex Ultrared both in **B** subsarcolemmal (SSM) and **D** interfibrillar (IFM) mitochondria. Data were expressed as mean \pm SEM, $n=10$, * $p<0.05$ vs 1st control, # $p<0.05$ vs 2nd control (two-way ANOVA with Bonferroni *post hoc* test, $n=10$)