

## **Hydrogen sulfide preserves eNOS function by inhibiting PYK2: implications for cardiomyocyte survival and cardioprotection**

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**List of abbreviations**

ANOVA (analysis of variance); AOAA (aminooxyacetic acid); BSA (bovine serum albumin); cGMP(cyclic guanosine-3',5'-monophosphate); CSE (cystathionine-γ lyase); DHE (dihydroethidium); DMEM (dulbecco's modified Eagle Medium); dn(dominant negative); DTT (dithiothritol); EDTA (ethylenediaminetetraacetic acid); EGTA (ethylene glycol tetraacetic acid); eNOS (endothelial nitric oxide synthase); GFP (green fluorescent protein); GTP (guanosine 5'-triphosphate); H<sub>2</sub>S (hydrogen sulfide); IBMX (isobutyl methyl xanthine); I/R (ischemia-reperfusion); KO (knockout); LAD (left anterior descending coronary artery); MDA (malondialdehyde); MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); NO (nitric oxide); PYK2 (proline-rich kinase 2); ROS (reactive oxygen species); Scrsi (scrambled siRNA); Sol (solvent)

## Abstract

Hydrogen sulfide ( $\text{H}_2\text{S}$ ) exhibits beneficial effects in the cardiovascular system, many of which depend on nitric oxide (NO). Proline-rich tyrosine kinase 2 (PYK2), a redox-sensitive tyrosine kinase, directly phosphorylates and inhibits eNOS. Herein, we investigated the ability of  $\text{H}_2\text{S}$  to relieve PYK2-mediated eNOS inhibition and evaluated the importance of the  $\text{H}_2\text{S}$ /PYK2/eNOS axis on cardiomyocyte injury *in vitro* and *in vivo*. Exposure of H9c2 cardiomyocytes to  $\text{H}_2\text{O}_2$  or pharmacological inhibition of  $\text{H}_2\text{S}$  production increased PYK2 (Y402) and eNOS (Y656) phosphorylation. These effects were blocked by treatment with  $\text{Na}_2\text{S}$  or by overexpression of cystathionine  $\gamma$ -lyase (CSE). In addition, PYK2 overexpression reduced eNOS activity in a  $\text{H}_2\text{S}$ -reversible manner. The viability of cardiomyocytes exposed to  $\text{H}_2\text{O}_2$  was reduced and declined further following inhibition of  $\text{H}_2\text{S}$  production. PYK2 downregulation, L-cysteine supplementation or CSE overexpression alleviated the effects of  $\text{H}_2\text{O}_2$  on H9c2 survival. Moreover,  $\text{H}_2\text{S}$  promoted PYK2 sulfhydration and inhibited its activity. *In vivo*,  $\text{H}_2\text{S}$  administration reduced reactive oxygen species levels, as well as PYK2 (Y402) and eNOS (Y656) phosphorylation. Pharmacological blockade of PYK2 or inhibition of PYK2 activation by  $\text{Na}_2\text{S}$  reduced myocardial infarct size in mice. Co-administration of a PYK2 inhibitor and  $\text{Na}_2\text{S}$  did not result in additive effects on infarct size. We conclude that  $\text{H}_2\text{S}$  relieves the inhibitory effect of PYK2 on eNOS, allowing the latter to produce greater amounts of NO affording cardioprotection. Our results unravel the existence of a novel  $\text{H}_2\text{S}$ -NO interaction and identify PYK2 as a crucial target for the protective effects of  $\text{H}_2\text{S}$  under conditions of oxidative stress.

## Introduction

Hydrogen sulfide ( $H_2S$ ) has emerged as an important gaseous signaling molecule in mammalian cells, regulating a multitude of basic biological processes, including bioenergetics, proliferation, apoptosis and necrosis (Li et al., 2011;Modis et al., 2014;Mustafa et al., 2009;Szabo and Papapetropoulos, 2011;Wang, 2012). Endogenous  $H_2S$  is produced by three enzymes namely cystathionine gamma lyase (CSE), cystathionine beta synthase and 3-mercaptopyruvate sulfurtransferase (Kimura, 2011;Kabil and Banerjee, 2014;Papapetropoulos et al., 2015). Although all three enzymes are expressed in the cardiovascular system, existing data suggests that CSE plays a major role in cardiovascular physiology (Wang, 2012;Polhemus and Lefer, 2014;Katsouda et al., 2016). CSE has been shown to exert angiogenic (Papapetropoulos et al., 2009), hypotensive (Yang et al., 2008), cardioprotective (Bibli et al., 2015a;Elrod et al., 2007), as well as anti-oxidant and anti-inflammatory effects in the myocardium and the vessel wall (Kimura, 2011;Calvert et al., 2009;Shibuya et al., 2013;Szabo et al., 2011;Salloum, 2015;Kanagy et al., 2017). Reduced generation or increased breakdown of  $H_2S$  leads to lower level of this gasotransmitter and is associated with several cardiovascular pathologies and conditions, such as endothelial dysfunction, atherosclerosis, hypertension, heart failure and preeclampsia (Polhemus and Lefer, 2014;Greaney et al., 2017;Kanagy et al., 2017;Wang et al., 2015). Studies from several laboratories have proven that endogenously produced and exogenously administered  $H_2S$  limits ischemia-reperfusion injury and reduces infarct size in isolated hearts and *in vivo* (Polhemus and Lefer, 2014;Pan et al., 2006;Bibli et al., 2015a;Elrod et al., 2007;Calvert et al., 2009;Szabo et al., 2011;King et al., 2014;Polhemus et al., 2014;Das et al., 2015;Johansen et al., 2006).

To exert its biological responses,  $H_2S$  utilizes a variety of signaling pathways by regulating the activity of kinases, phosphatases, transcription factors and ion channels (Wang, 2012;Polhemus and Lefer, 2014;Kanagy et al., 2017;Szabo, 2007;Paul and Snyder, 2012).

Many of these actions are attributed to a post-translational modification of cysteine residues in a modification referred to as sulfhydration or persulfidation (Paul and Snyder, 2012). In addition, some of the biological effects exerted by H<sub>2</sub>S require nitric oxide (NO) production. Angiogenesis, vasodilation and cardioprotection are reduced or blunted when endothelial NO synthase (eNOS) is inhibited (Bibli et al., 2015a;King et al., 2014;Coletta et al., 2012). At the molecular level, the H<sub>2</sub>S/NO interaction involves increased eNOS phosphorylation at the activator site S1177 (Papapetropoulos et al., 2009;Bibli et al., 2015a;King et al., 2014;Coletta et al., 2012;Kondo et al., 2013;Minamishima et al., 2009;Altaany et al., 2013;Chatzianastasiou et al., 2016;Karwi et al., 2017) and reduced phosphorylation at the T495 inhibitory site (Bibli et al., 2015b;Coletta et al., 2012;Polhemus et al., 2013). Moreover, H<sub>2</sub>S promotes eNOS dimerization and coupling through the sulfhydration of C443 (Altaany et al., 2014). The above mentioned posttranslational modifications of eNOS enhance NO production and/or bioavailability after exposure to H<sub>2</sub>S, as evidenced by increases in cGMP accumulation or NO metabolite levels (King et al., 2014;Coletta et al., 2012;Kondo et al., 2013;Szabo, 2017;Predmore et al., 2012). With respect to cardioprotection the importance of S1176 phosphorylation *in vivo* was demonstrated using S1176A knock-in mice in which H<sub>2</sub>S donor administration was ineffective in limiting infarct size. (King et al., 2014)

The proline rich tyrosine kinase 2 (PYK2) is a redox sensitive kinase (Lev et al., 1995;Tokiwa et al., 1996;Chappell et al., 2008;Tai et al., 2002;Loot et al., 2009) which has been linked to cardiac remodeling (Takeishi, 2014), hypertrophic responses (Hirotsu et al., 2004), dilated cardiomyopathy (Koshman et al., 2014) and ischemia reperfusion injury (Fisslthaler et al., 2008). Recently we demonstrated that PYK2, directly phosphorylates eNOS on Y657 (human eNOS sequence, corresponds to murine Y656), rendering it inactive (Loot et al., 2009;Fisslthaler et al., 2008). PYK2 is activated in the early minutes of myocardial reperfusion following ischemia, resulting in increased phosphorylation of eNOS on the Y656 inhibitory residue and reduced NO output; this mechanism was found to define myocardial infarct size and PYK2 was proposed to serve as a novel therapeutic target for

cardioprotection (Bibli et al., 2017). Since H<sub>2</sub>S is known to possess anti-oxidant properties, we hypothesized that it could block oxidative stress-induced PYK2 activation in early reperfusion, limiting eNOS inhibition and reducing myocardial infarct size. Data from the current study indicate that H<sub>2</sub>S restrains the activation of PYK2, providing a novel mechanism of positive interaction between H<sub>2</sub>S and NO in cardiomyocytes.

## Materials and Methods

### *Chemicals and reagents*

All chemicals and reagents including AOAA, L-cysteine, Na<sub>2</sub>S, PF-431396, Triton X100, NaCl, NaF, EDTA, EGTA, PMSF, protease and phosphatase inhibitors, glycerolphosphatase, MTT were purchased from Sigma-Aldrich (Taufkirchen, Germany), while DMSO, H<sub>2</sub>O<sub>2</sub>, Tris and SDS were purchased from AppliChem (Bioline Scientific, Athens, Greece). Lipofectamine™ RNAiMAX was obtained from Invitrogen (Antisel, Athens, Greece); DMEM, sodium pyruvate, antibiotics, Opti-MEM, FBS were obtained from Gibco (Antisel, Athens, Greece); the cGMP kit was obtained from Enzo (Zafeiropoulos SA, Athens, Greece); ECL was obtained from ThermoScientific (Bioanalytica, Athens, Greece). The pPYK2, PYK2, eNOS,  $\beta$ -tubulin, nitrotyrosine and secondary Ab were purchased from Cell Signaling Technologies (Bioline, Athens, Greece); anti-CSE was from Proteintech Europe (Manchestere, UK); the peNOS(Y657) was generated by Eurogentec (Köln, Germany). SSP4 was purchased from Dojindo EU (Munich, Germany). The Amplex® Red Assay Kit was purchased from Invitrogen (Thermo Fischer Scientific, Darmstadt, Germany). The ADP-Glo™ Kinase Assay kit was obtained from Promega (Mannheim, Germany)

### *Cell culture*

The rat embryonic-heart derived H9c2 cell line was obtained from ATCC (CRL-1446™) (ATCC, LGC Standards, Middlesex, UK). H9c2 cells were cultured in Dulbecco's modified essential medium DMEM containing 25 mM D-glucose, 1 mM sodium pyruvate, and supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% streptomycin (100  $\mu$ g/ml), and 1% penicillin (100 U/ml) at pH 7.4 in a 5% CO<sub>2</sub> incubator at 37°C. Cells were maintained in a subconfluent condition of maximum 70% before passaging to avoid differentiation. For differentiation, H9c2 were seeded and allowed to grow to confluence. The medium was then replaced to DMEM containing 1% FBS with 10 nM all-*trans*-retinoic acid for 7 days. After 7 days cells were elongated, connecting at irregular angles and cardiac

differentiation markers as cardiac troponin and MLC2v transcripts were elevated; reminiscent of cells with a cardiac phenotype, as described before (Bibli et al., 2017). HEK cells were cultured in MEM supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% streptomycin (100 µg/ml), and 1% penicillin (100 U/ml) at pH 7.4 in a 5% CO<sub>2</sub> incubator at 37°C.

### ***In vitro treatments***

For *in vitro* experiments differentiated H9c2 cells were pretreated with the appropriate drug as described below. Single treatments were as follows: AOAA was used at a concentration of 1mM for 45minutes, L-cysteine at 500µM for 45 min, PF-431396 5µM for 45 min, Na<sub>2</sub>S 100µM for 30 minutes. For double treatments AOAA and L-cysteine were added in the aforementioned concentrations simultaneously, whilst PF-431396 was added 15 minutes prior to Na<sub>2</sub>S. Following treatments cells were lysed for biochemical analysis or subsequent cell viability studies. To induce *in vitro* oxidative stress injury, H9c2 cells (1.5x10<sup>4</sup> per well) were treated with 500µM H<sub>2</sub>O<sub>2</sub> in serum-free DMEM for 12h in a 5% CO<sub>2</sub> incubator at 37°C.

### ***Small interfering RNA mediated downregulation of PYK2***

H9c2 cells were seeded until 80% confluence. Transient transfection of siRNAs (100 nM) was performed using Lipofectamine™ RNAiMAX, according to the manufacturer's instructions. The transfection complex was diluted into Opti-MEM medium and added directly to the cells. After 24 hrs, the Opti-MEM was replaced with complete DMEM medium with 10% FBS for cell viability assays or with serum-free DMEM for biochemical studies. The efficacy of siRNA PYK2 gene knockdown, 48 hrs post-transfection has been previously conformed (Bibli et al., 2017).

### ***Adenoviral infections***

H9c2 cells were seeded until confluence and differentiated for 7 days. On the 6<sup>th</sup> day of differentiation cells were infected with GFP or CSE adenoviruses (Bucci et al., 2010) at 10MOI for 36h.

### ***MTT measurements***

Following oxidative stress injury; cell survival was assessed in differentiated H9c2 cells by using the conversion of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan. Cells were incubated with MTT at a final concentration of 0.5 mg/ml, for 2 h at 37°C. The formazan formed was dissolved in solubilization solution (10% Triton-X 100 in acidic 0.1N HCl in isopropanol); subsequently, absorbance was measured at 595 nm with a background correction at 750 nm using a microplate reader.

### ***Western Blot Analysis***

H9c2 cells seeded in 6-well plates until confluence treated as described above were washed twice with PBS and further lysed with lysis solution (1% Triton X100, 20 mM Tris pH 7.4-7.6, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM glycerolphosphatase, 1% SDS, 100 mM PMSF, supplemented with protease and phosphatase inhibitor cocktail. Frozen ischemic samples were pulverized and homogenized with the lysis buffer. The lysates were centrifuged at 11,000 g for 15 min at 4 °C. The supernatants were collected and the protein concentration was determined based on the Lowry assay. The supernatant was mixed with a buffer containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenyl blue, 0.125 M Tris/HCl. The samples were then heated at 100°C for 10 min and stored at -80°C. An equal amount of protein was loaded in each well and then separated by sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% non-fat dry milk, membranes were incubated overnight at 4 °C with primerary antibody. The following primary antibodies were used: phospho PYK2 (Y402), phospho eNOS (Y656), total PYK2, total eNOS,

nitrotyrosine and  $\beta$ -tubulin. Membranes were then incubated with secondary goat anti-rabbit HRP antibody for 2 h at room temperature and developed using the Supersignal chemiluminescence ECL Western Blotting Detection Reagents. Relative densitometry was determined using a computerized software package (NIH Image) and the values for phosphorylated were normalized to the values for total proteins respectively. All presented total proteins are derived from the same gel/experiment as the phosphoprotein presented after stripping of the membrane.

### ***eNOS activity measurements***

The plasmids used were: myc-tagged human eNOS cDNA (GenBank accession no. NM\_000603) cloned in pcDNA3.1myc/His, PYK2 cDNA (NC\_000008.11) and a dominant negative PYK2 mutant (Fisslthaler et al., 2008). For the transfection experiments, HEK293 cells were plated in 12-well plates, grown overnight, and transfected with the indicated plasmids, using a total of 2  $\mu$ g DNA and 4  $\mu$ L of Lipofectamine transfection reagent per well in Opti-MEM medium. After 24 hours cells were used for LC-MS and western blot measurements. For western blot studies, cells were starved for 4h in serum free medium with addition of 0.1% of BSA. For NO measurements, HEK cells were treated either with sepiapterin 10  $\mu$ mol for 2h. Arginine depletion was performed by addition of SILAC medium for 12 hours pretreatment. Heavy labeled Arg (13C6, 15N4) was added for 2 hours before collection for activity measurements. Cells were immediately emerged in liquid nitrogen and 85% HPLC grade MeOH was added for cell disruption. The lysates were centrifuged in 12000xg for 15 minutes in 4°C. 50 $\mu$ L of supernatant was mixed with 50 $\mu$ L of MeOH containing 1mg/ml glutamine as internal standard. Conversion of heavy labeled Arg (13C6, 15N4) to heavy citrulline (13C6, 15N3) were analyzed by hydrophilic interaction chromatography coupled to mass spectrometry. Liquid chromatography separation was performed on an Agilent 1290 Infinity pump system (Agilent, Waldbronn, Germany), using a Phenomenex (Aschaffenburg, Germany) Kinetex HILIC column (100 mm  $\times$  2.1 mm, 2.6  $\mu$ m) at a column

oven temperature of 35 °C. The gradient between solvent A (10 mM ammonium formate) and solvent B (acetonitrile + 0.15% formic acid) was as follows: 0.0 to 0.5 min 10% A, then increase to 85% A from 0.5 to 6 min, then increase to 98% A to 6.1 min, which was held until 13.6 min. Subsequently, the column was reconditioned with 90% A for 4.4 min. The flow rate was set to 350 µl/min and the injection volume was 2.5 µl. Mass spectrometry was performed using a QTrap 5500 mass spectrometer (Sciex, Darmstadt, Germany) with electrospray ionization at 300°C with 3500 V in positive mode. MS parameters were set to CUR 30 psi, GS1 50 psi, and GS2 40 psi. Data acquisition and instrument control were managed through the software Analyst 1.6.2. Peak integration, data processing, and analyte quantification were performed using MultiQuant 3.0 (Sciex, Darmstadt, Germany). The area under the peak was used as the quantitative measurement. The specific MRM transition for every compound was normalized to appropriately isotope labeled internal standards.

### ***H<sub>2</sub>S measurements***

Intracellular levels of H<sub>2</sub>S were measured by monitoring the reaction of SSP4 with H<sub>2</sub>S. In brief, cells were seeded in 12 or 48 well plates and allowed to reach confluence. The culture medium was replaced with phenol red-free DMEM supplemented with 0.1 % BSA. For inhibition of endogenous H<sub>2</sub>S production, cells were pretreated with AOAA (1mM) for 45 minutes. To enhance H<sub>2</sub>S production cells were incubated with L-cysteine (500µM) for 45 min. Subsequently, medium was replaced and SSP4 (10 µmol/L) was added for 60 minutes. Thereafter, the cell supernatant was collected and floating cells were removed by centrifugation (16,000Xg, 10 minutes, 4°C). The specific products of the reaction of H<sub>2</sub>S with SPP4 were quantified by LC-MS/MS (for details see submitted attached manuscript provided as supplemental file)

### **Oxidative stress detection in cardiomyocytes**

Hydrogen peroxide levels were measured in cardiomyocytes by using the Amplex® Red Assay Kit according to the manufacturer instructions. In brief, differentiated H9c2 cells were infected with a GFP or CSE expressing adenovirus, or treated with the Na<sub>2</sub>S salt as described. In some wells, 50µM H<sub>2</sub>O<sub>2</sub> was added to the cells for 10 minutes. The reaction was stopped on ice. Cells were collected and washed 3 times with ice cold PBS to wash out the exogenous H<sub>2</sub>O<sub>2</sub>. 10<sup>6</sup> cells per condition were used for H<sub>2</sub>O<sub>2</sub> determination. A standard curve for H<sub>2</sub>O<sub>2</sub> was used to quantify the endogenously produced H<sub>2</sub>O<sub>2</sub>.

### ***PYK2 activity assay***

The effects of H<sub>2</sub>S on PYK2 activity were determined using the ADP-Glo™ Kinase Assay kit. The assay was performed in the presence of solvent or different concentrations of Na<sub>2</sub>S on purified PYK2 protein, according to the manufacturer's instructions.

### ***S-Sulphydration detection***

Sulphydration was detected using a modified biotin switch assay. In brief, H9c2 differentiated cells were treated with Na<sub>2</sub>S (100µM) for 30 minutes. Reactions were stopped on ice and cells were washed with ice cold PBS. Subsequently, samples were precipitated with 20% trichloroacetic acid (TCA) and stored at -80°C. TCA precipitates were washed with 10% and then 5% TCA and then centrifuged (16,000g, 30 minutes, 4°C) before being suspended in HENs buffer (250 mmol/L HEPES-NaOH, 1 mmol/L EDTA, 0.1 mmol/L neocuproine, 100 µmol/L deferoxamine, 2,5% SDS) containing 20 mmol/L methanethiosulfonate to block free thiols and protease and phosphatase inhibitors. Acetone precipitation was performed and pellets were re-suspended in 300 µL qPerS-SID lysis buffer (6 mol/L urea, 100 mmol/L NaCl, 2 % SDS, 5 mmol/L EDTA, 200 mmol/L Tris pH 8.2; 50 mmol/L iodoacetyl-PEG2-biotin, 2.5 mmol/L dimedone), sonified and incubated for 2 hours at room temperature in the dark. Lysates (500 µg) were precipitated with acetone and protein pellets were re-suspended in 50 µl Tris/HCl (50 mmol/L, pH 8.5) containing guanidinium chloride (GdmCl 6 mmol/L), and

incubated at 95°C for 5 minutes. A negative control was generated for each sample by adding DTT (1 mmol/L) during biotin cross-linking. Biotin was then immunoprecipitated using a high capacity streptavidin resin (Thermo Scientific, Heidelberg, Germany) overnight at 4°C. Elution was performed by addition of 3% SDS, 1%  $\beta$ -mercaptoethanol, 8 mol/L Urea and 0.005% bromophenol blue in PBS for 15 minutes at room temperature followed by 15 minutes at 95°C. Sulfhydrated proteins were detected following SDS-PAGE by Western blotting.

### ***cGMP enzyme immunoassay***

Cyclic nucleotides were extracted by HCl and measured using a commercially available EIA kit (Enzo Life Sciences) following the manufacturer's instructions. For tissue samples, frozen ischemic tissue was pulverized. Powdered samples from myocardial ischemic tissue were lysed with 0.1N HCl (1:5 vol/w) to extract cGMP. cGMP content was measured using enzyme immunoassay according to the manufacturer's instructions. Protein concentration was determined by the Lowry method and results were expressed as pmol cGMP/mg protein.

### ***MDA and Protein Carbonylation Assessment***

Tissue samples homogenates were used to measure malondialdehyde (MDA) and protein carbonyls (PC). MDA was determined spectrophotometrically as previously described. A spectrophotometric measurement of 2,4-dinitrophenylhydrazine (DNPH) derivatives of protein carbonyls (PC) was used to quantify PC content as previously described (Andreasson et al., 2014).

### ***DHE staining of cardiac RONS formation***

Cardiac ROS production was qualitatively detected by DHE (1  $\mu$ M)-derived fluorescence in heart tissue cryo-sections of 8 $\mu$ m as described previously for vascular RONS formation (Andreadou et al., 2014).

### ***Animals***

All animal procedures were in compliance with the European Community guidelines for the use of experimental animals; experimental protocols were approved by the Ethical Committee of the Prefecture of Athens (790/2014). Animals received standard rodent laboratory diet. In the present study male mice C57BL/6J mice were used.

### ***Surgical Procedures***

#### ***Murine *in vivo* model of ischemia-reperfusion injury***

Male mice 10-12 weeks old were anesthetized by intraperitoneal injection with a combination of ketamine, xylazine and atropine (0.01 mL/g, final concentrations of ketamine, xylazine and atropine 10 mg/mL, 2 mg/mL, 0.06 mg/kg respectively). A tracheotomy was performed for artificial respiration at 120-150 breaths/min and PEEP 2.0 (0.2 mL tidal volume) (Flexivent rodent ventilator, Scireq, Montreal, Ontario, Canada). Electrocardiogram recordings were performed by a Lead I ECG recording with PowerLab 4.0 (ADInstruments, UK). Recordings were analyzed by LabChart 7.0 software. A thoracotomy was then performed between the fourth and fifth ribs and the pericardium carefully retracted to visualize the LAD, which was ligated using a 8-0 prolene monofilament polypropylene suture placed 1 mm below the tip of the left ventricle. The heart was allowed to stabilize for 15 minutes prior to ligation to induce ischemia. After the ischemic period, the ligature was released allowed reperfusion of the myocardium. Throughout experiments, body temperature was maintained at 37°C  $\pm$  0.5°C by way of a heating pad. After reperfusion hearts were rapidly excised from mice and directly cannulated and washed with 2.5 mL saline-heparin 1% for blood removal. 5 mL of 1% TTC

phosphate buffer 37<sup>0</sup> C were infused via the cannula into the coronary circulation followed by incubation of the myocardium for 5 minutes in the same buffer; 2.5 ml of 1% Evans blue, diluted in distilled water was then infused into the heart. Hearts were kept in -20<sup>0</sup>C for 24h and then sliced in 1mm sections parallel to the atrio-ventricular groove, and then fixed in 4% formaldehyde overnight. Slices were then placed between glass plates 1 mm apart and photographed with a Cannon Powershot A620 Digital Camera through Zeiss 459300 microscope and measured with the Scion Image program. Infarct and risk area volumes were expressed in cm<sup>3</sup> and the percent of infarct to risk area ratio (%I/R) was calculated.

### ***Experimental protocol***

In animals treated with the pharmacological inhibitor of PYK2, PF-431396 was administrated at 5µg/g in 2% DMSO (100µl) iv, while Na<sub>2</sub>S was given as iv bolus at 100µg/kg as described before (Bibli et al., CVR 2015). In the first experimental series animals received the indicated drugs either 10 minutes prior to sacrifice for tissue collection (sham operated groups), or 10 minutes prior to reperfusion (Ischemia-Reperfusion injury groups). The left ventricle was isolated and submerged in liquid nitrogen for preservation, prior to additional analysis.

In a second series of experiments, mice were subjected to 30 minutes regional ischemia of the myocardium, followed by 2 hours of reperfusion and were randomized into 4 groups as follows. 1) Sol group (n=8): administration of solvent (water for injection containing 2% DMSO [100µl] iv 10 minutes prior to the ischemic insult); 2) PF-431396 group (n=8): administration of 5µg/g PF-431396 (dissolved in water for injection containing 2% DMSO; (100µl) iv 10 minutes prior to reperfusion; 3) Na<sub>2</sub>S group (n=8): administration of 100µg/kg Na<sub>2</sub>S (100µl iv 10 minutes prior to reperfusion); 4) PF-431396+Na<sub>2</sub>S group (n=6): administration of PF-431396 and Na<sub>2</sub>S as in groups 2 and 3.

### ***Statistical analysis***

One- or two-way analysis of variance (ANOVA) was used to detect differences between multiple groups or unpaired two-tailed Student's t test to compare two groups. A value of  $p < 0.05$  was considered statistically significant. All statistical calculations were performed using Prism 4 analysis software (GraphPad Software, Inc., La Jolla, CA). Data are shown as mean  $\pm$  SEM values.

## Results

### Endogenously generated H<sub>2</sub>S inhibits PYK2 in cultured cardiomyocytes

To evaluate the role of endogenous H<sub>2</sub>S on PYK2 activation, we evaluated PYK2 phosphorylation in differentiated H9c2 cardiomyocytes in the presence of a pharmacological inhibitor of H<sub>2</sub>S synthesis (AOAA) (Asimakopoulou et al., 2013) (**Figures 1A&B**) or the substrate for H<sub>2</sub>S synthesis (L-cysteine) (**Figures 1A&B**). In an alternative approach, endogenous H<sub>2</sub>S production was enhanced via the adenoviral-mediated overexpression of CSE (**Figures 1C&D**). In agreement with our recently published observations (Bibli et al., 2017), H<sub>2</sub>O<sub>2</sub> treatment resulted in the increased phosphorylation of PYK2 on Y402 (**Figure 1A**). Moreover, H<sub>2</sub>O<sub>2</sub> treatment increased eNOS phosphorylation on the eNOS Y656 inhibitory site (**Figure 1B**). The effects of H<sub>2</sub>O<sub>2</sub> treatment were mimicked by inhibiting endogenous H<sub>2</sub>S production with AOAA (**Figure 1A&B**). Interestingly, PYK2 and eNOS phosphorylation were not enhanced by the combined treatment of H<sub>2</sub>O<sub>2</sub> and AOAA. In addition, supplementation with the H<sub>2</sub>S substrate L-cysteine (**Figures 1A&B**), or CSE overexpression (**Figure 1C&D**) abrogated the biochemical changes on PYK2 (**Figures 1A&C**) and eNOS (**Figures 1B & D**) triggered by H<sub>2</sub>O<sub>2</sub>.

### Pharmacological administration of H<sub>2</sub>S results in PYK2 inhibition and activation of eNOS

Having established that endogenously produced H<sub>2</sub>S regulates PYK2 activation, we sought to determine whether or not H<sub>2</sub>S supplementation could mitigate the effects of H<sub>2</sub>O<sub>2</sub> on the PYK2/eNOS pathway. We pretreated H9c2 cells with a sulfide salt, Na<sub>2</sub>S. In these experiments we observed that Na<sub>2</sub>S eliminated both the H<sub>2</sub>O<sub>2</sub>-induced PYK2 activation (**Figure 2A**) and eNOS phosphorylation on Y656 (**Figure 2B**). To prove that the effects observed on eNOS Y656 phosphorylation were mediated by PYK2, we used a heterologous expression system. HEK cells were co-transfected with wild-type eNOS and either an empty pcDNA3 vector, a wild-type PYK2, or a dominant negative PYK2 plasmid as in our previous

studies (Fisslthaler et al., 2008). Co-transfection with wild-type eNOS and wild-type PYK2 resulted in an increase in p-eNOS on Y656, due to the basal activity of overexpressed PYK2 (**Figure 2C**); Na<sub>2</sub>S administration inhibited eNOS Y656 phosphorylation. Exposure of HEK cells to H<sub>2</sub>O<sub>2</sub> further increased eNOS tyrosine phosphorylation; the effect of was reversed by incubation with Na<sub>2</sub>S. In contrast to what was observed with wild-type PYK2, HEK cells transfected with the dominant negative PYK2 showed no increase in eNOS phosphorylation under the conditions studied, confirming that eNOS Y656 phosphorylation depends on PYK2 activity. To evaluate the effect of the pharmacological treatments on eNOS activity we measured the conversion of L-arginine to L-citrulline (**Figure 3**). While exposure to Na<sub>2</sub>S increased eNOS activity, incubation of cells with H<sub>2</sub>O<sub>2</sub> did not alter L-citrulline formation in cells that did not express PYK2. PYK2 overexpression reduced the L-citrulline/L-arginine ratio in line with our biochemical data (enhanced eNOS phosphorylation on Y656); this effect was reversed by Na<sub>2</sub>S. Incubation of PYK2 transfected cells with H<sub>2</sub>O<sub>2</sub> potentiated the inhibitory effect on eNOS activity. Exogenous application of H<sub>2</sub>S to PYK2-transfected cells treated with H<sub>2</sub>O<sub>2</sub> restored eNOS activity, confirming that H<sub>2</sub>S inhibits PYK2 activation and alleviates its inhibitory effect on eNOS.

### Mechanisms of PYK2 inhibition by H<sub>2</sub>S

To study the mechanisms through which H<sub>2</sub>S inhibits PYK2 preventing eNOS inhibition, we determined the effect of H<sub>2</sub>S levels on the levels of ROS, a known trigger for PYK2 activation. Overexpression of CSE or L-cysteine supplementation increased H<sub>2</sub>S levels, while AOAA reduced them (Fig.4 A & B). When H<sub>2</sub>S production was enhanced, H<sub>2</sub>O<sub>2</sub> levels were reduced and vice versa (Fig. 4 C & D). Increased H<sub>2</sub>O<sub>2</sub> levels lead to PYK2 Y402 and eNOS Y657 phosphorylation (Fig.1) and H<sub>2</sub>S reversed this effect. To study if H<sub>2</sub>S also has direct effects on PYK2, we determined its ability to inhibit PYK2 activity. H<sub>2</sub>S elicited a robust inhibitory effect on recombinant PYK2 with an IC<sub>50</sub> in the sub-μM range (**Fig.5A**). The inhibition of PYK2 by H<sub>2</sub>S was associated with enhanced sulfhydrylation of the kinase (**Fig.5B**)

### **H<sub>2</sub>S salvages PYK2-induced cardiomyocyte death *in vitro***

To study the effects of PYK2 on cardiomyocyte survival, we used an *in vitro* model of H<sub>2</sub>O<sub>2</sub>-triggered oxidative stress injury and cell death. Inhibition of CSE/CBS-derived H<sub>2</sub>S by AOAA resulted in augmented cardiomyocyte death both under baseline conditions (**Supplementary Figure 1A**) as well as after H<sub>2</sub>O<sub>2</sub> (**Figure 6A**) administration. Providing additional L-cysteine in the culture media increased cardiomyocyte survival in the presence of H<sub>2</sub>O<sub>2</sub> did not but did not reverse the effect of AOAA (**Supplementary Figure 1A, Figure 6A**). To evaluate the ability of endogenous H<sub>2</sub>S production to regulate PYK2 activity in the context of cell survival, PYK2 was silenced using a siRNA approach (Bibli et al., 2017). In line with our previous findings (Bibli et al., 2017), PYK2 silencing increased cardiomyocyte survival in cells treated with H<sub>2</sub>O<sub>2</sub>. Moreover, the deleterious effect of AOAA treatment was not observed after PYK2 knockdown (**Figure 6A**). L-cysteine supplementation partially reversed the effect of H<sub>2</sub>O<sub>2</sub> in non-transfected and scrambled RNA-transfected cells, but not in PYK2 silenced cells (**Figure 6A**). We next overexpressed CSE and exposed cells to H<sub>2</sub>O<sub>2</sub> to determine the contribution of PYK2 to the protective effect of endogenously produced H<sub>2</sub>S. When PYK2 was expressed (non transfected and scrambled ScrsiRNA transfected conditions), CSE restricted the deleterious effect of H<sub>2</sub>O<sub>2</sub> on cardiomyocyte survival (**Figure 6B**). However, no additional effect of CSE overexpression was observed in cells in which PYK2 was silenced, indicating that H<sub>2</sub>S protects cardiomyocytes by inhibiting PYK2 under oxidative stress conditions. Finally, experiments were conducted in the presence of Na<sub>2</sub>S, as an exogenous source of H<sub>2</sub>S and PF-431396 as a pharmacological inhibitor of PYK2. In this series of experiments we observed that both the PYK2 inhibitor and Na<sub>2</sub>S improved cell survival after H<sub>2</sub>O<sub>2</sub> exposure; however, combining the sulfide salt with PF-431396 did not exert an additional effect (**Figure 6C**).

### **H<sub>2</sub>S alleviates eNOS inhibition during reperfusion *in vivo***

To test if the observed *in vitro* findings could be extrapolated *in vivo*, we used a LAD ligation model. In these experiments we observed that administration of Na<sub>2</sub>S in sham operated animals does not affect the basal levels of PYK2 and eNOS Y656 phosphorylation (**Supplementary Figure 2A,2B**) or cGMP levels (**Supplementary Figure 2C**). However, when Na<sub>2</sub>S was administered intravenously 10 minutes prior to reperfusion, a 50% reduction on the phosphorylation of PYK2 was observed in the early minutes of reperfusion (**Figure 7A**). At the same time, we also noted a reduction in Y656 phosphorylation of eNOS (**Figure 7B**) along with an increase in the levels of the surrogate NO marker, cGMP (**Figure 7C**). In addition, administration of Na<sub>2</sub>S during ischemia resulted in a reduction in the oxidative and nitrosative stress biomarkers malondialdehyde (MDA; **Figure 8A**) and protein carbonyls (**Figure 8B**) in the early minutes of reperfusion. Similarly, nitrotyrosine levels (**Figure 8C**) and DHE-reactive products (**Figure 8D**) were attenuated in Na<sub>2</sub>S-treated animals compared to the solvent treated animals. These findings taken together demonstrate that Na<sub>2</sub>S inhibits oxidative stress, limits PYK2 activity and de-represses eNOS activity.

### **The cardioprotective effects of H<sub>2</sub>S are dependent on the PYK2/eNOS pathway**

Next we tested if the increase in eNOS activity brought about by the H<sub>2</sub>S-mediated inhibition of PYK2 yields functionally relevant outcomes *in vivo*. To do so, we assessed the infarct size in mice subjected to ischemia/reperfusion injury in the presence of a pharmacological PYK2 inhibitor or/and a H<sub>2</sub>S source in a dose previously reported by our group not to affect haemodynamic parameters (Chatzianastasiou et al., 2016). In agreement with our previously published observations (Bibli et al., 2017) the pharmacological inhibition of PYK2 reduced myocardial infarct size (36.8±2.0% for Sol group, 18.0±0.9% for PF-431396 group). Similarly, we observed that the administration of Na<sub>2</sub>S was protective (36.8±2.0% for Sol group and 17.8±1.6% for Na<sub>2</sub>S group, \*p<0.05). However, the simultaneous administration of PF-431396 and Na<sub>2</sub>S exerted no additional beneficial effects with respect to infarct size (20.2±2.5%), implying that these two agents rely on the same downstream molecular targets

(**Figure 9A, 9C**). No statistically significant differences were observed in the area at risk to whole myocardial area among the studied groups (**Figure 9B**).

## Discussion

Myocardial ischemia induces cellular damage via maladaptive biochemical responses in the ischemic organ (Yellon and Hausenloy, 2007). Subsequent reperfusion, although beneficial, leads to further paradoxical intracellular injury and increased myocardial death. No pharmacological strategies have been introduced so far in to routine clinical practice to reduce infarct size in patients undergoing acute myocardial infarction (AMI) (Hausenloy and Yellon, 2016). Better understanding of the intracellular signaling of ischemia/reperfusion injury is expected to lead to novel therapeutic strategies for AMI patients. Herein, we investigated the impact of H<sub>2</sub>S on the PYK2/eNOS axis and its relevance to cardioprotection.

Initially we set out to determine whether endogenously produced H<sub>2</sub>S regulates PYK2 phosphorylation. When H9c2 were treated acutely with the CSE/CBS inhibitor AOAA (Asimakopoulou et al., 2013) we observed an increase in PYK2 tyrosine phosphorylation, indicative of enhanced PYK2 activation. Since PYK2 is activated by ROS (Jones and Bolli, 2006) and H<sub>2</sub>S exhibits both direct and indirect anti-oxidant effects, (Xie et al., 2016; Ju et al., 2013) AOAA-triggered PYK2 activation might be the result of increased oxidative stress upon lowering H<sub>2</sub>S levels. In line with this hypothesis, exogenously added H<sub>2</sub>O<sub>2</sub> mimicked the effects of AOAA on PYK2. Moreover, co-incubation of cells with AOAA and H<sub>2</sub>O<sub>2</sub> exerted no additional effects on PYK2 phosphorylation, indicating a common mechanism of action for AOAA and H<sub>2</sub>O<sub>2</sub>. Finally, supplementation with the H<sub>2</sub>S synthesis substrate L-cysteine, CSE overexpression or exogenously added H<sub>2</sub>S reversed the effects of H<sub>2</sub>O<sub>2</sub> on PYK2, providing further evidence that H<sub>2</sub>S limits PYK2 activation by counteracting the action of oxidant molecules. In addition to preventing PYK2 activation by reducing oxidative stress, we observed that H<sub>2</sub>S is able to directly inhibit PYK2 activity. In agreement with the fact that PYK2 phosphorylates eNOS on Y656, in all of the experiments performed, changes in eNOS tyrosine phosphorylation paralleled those in PYK2 phosphorylation. Direct evidence for the involvement of PYK2 on H<sub>2</sub>O<sub>2</sub>-induced eNOS Y656 phosphorylation was provided by a

dominant negative approach. While most researchers study S1177 phosphorylation (human eNOS sequence, corresponds to murine S1176) as a surrogate marker of eNOS activity, (Fulton et al., 1999;Dimmeler et al., 1999) we believe it is more appropriate to study Y657. We have previously shown that phosphorylation of Y657 exerts a dominant effect compared to S1177; while S1177 phosphorylation leads to eNOS activation, dual phosphorylation of Y657/S1177 abolishes eNOS activity (Bibli et al., 2017). The observed changes on eNOS phosphorylation triggered by H<sub>2</sub>S were accompanied by changes in activity. H<sub>2</sub>O<sub>2</sub> reduced eNOS activity only in cells expressing PYK2; this effect was reversible by H<sub>2</sub>S.

To determine the ability of H<sub>2</sub>S to inhibit PYK2-mediated cell toxicity, we exposed H9c2 cells to H<sub>2</sub>O<sub>2</sub>. Treatment of cardiomyocytes with H<sub>2</sub>O<sub>2</sub> significantly reduced cell survival; the effect of H<sub>2</sub>O<sub>2</sub> could be ameliorated by increasing H<sub>2</sub>S production via L-cysteine, CSE overexpression or exogenous addition of Na<sub>2</sub>S. Our findings are in agreement with previously published findings that H<sub>2</sub>S donors protect H9c2 cells from H<sub>2</sub>O<sub>2</sub> toxicity (Szabo et al., 2011;Chatzianastasiou et al., 2016;Bibli et al., 2017;Zhao et al., 2015). We recently reported that the toxicity of H<sub>2</sub>O<sub>2</sub> in cultured cardiomyocytes could be inhibited by pharmacological PYK2 inhibition or PYK2 silencing and that the effect of PYK2 was eNOS-dependen. (Bibli et al., 2017). In the present series of experiments we found that H<sub>2</sub>S was unable to improve cell survival in H9c2 when PYK2 was silenced or inhibited. Similarly, reducing endogenous H<sub>2</sub>S production did not lead to greater toxicity when PYK2 was silenced. Taken together, the above-mentioned observations suggest that H<sub>2</sub>S signals via PYK2 to protect cardiomyocytes against oxidative stress-induced toxicity. Based on our findings that i) the protective effect of PYK2 inhibition in H9c2 is linked to de-repression of eNOS (Bibli et al., 2017) and ii) the herein reported observation that H<sub>2</sub>S blocks PYK2 activation we propose that the pro-survival effect of H<sub>2</sub>S is mediated by an increase in NO bioavailability that results from PYK2 inhibition.

Nitric oxide is considered a key player in myocardial ischemia/reperfusion injury *in vivo* (Andreadou et al., 2015; Jones and Bolli, 2006). We and others have shown that eNOS is required for the cardioprotective actions of H<sub>2</sub>S *in vivo* (Kondo et al., 2013; King et al., 2014; Karwi et al., 2017; Chatzianastasiou et al., 2016; Bibli et al., 2015a). Our group has recently demonstrated that the capability of eNOS to produce NO during ischemia/reperfusion injury is regulated by the redox-sensitive kinase PYK2 (Bibli et al., 2017). PYK2 phosphorylation peaks roughly 3 minutes after reperfusion, returning to baseline within 10 minutes. The time course of eNOS phosphorylation on Y656 parallels that of PYK2 activation, resulting in reduced NO production contributing to myocardial death. Herein, we found that administration of H<sub>2</sub>S was able to inhibit PYK2 phosphorylation in the early minutes of reperfusion in the infarcted left ventricle. This resulted in alleviation of the inhibitory eNOS tyrosine phosphorylation and a subsequent increase of the cardiac levels of the NO surrogate marker cGMP. The observed biochemical changes translated to functional outcomes, as inhibition of PYK2 kinase either via Na<sub>2</sub>S or via its pharmacological inhibitor PF-431396 resulted in a reduction of myocardial infarct size in the murine hearts. In compliance with our *in vitro* cell survival studies simultaneous administration of Na<sub>2</sub>S and PF-431396 did not exert additional beneficial effects in myocardial survival, further suggesting that Na<sub>2</sub>S exerts its effects through PYK2 inhibition.

As ROS are a likely trigger for PYK2 activation after reperfusion, we assessed their levels 3 minutes after ischemia/reperfusion injury when PYK2 activity is maximal (Bibli et al., 2017), by determining 4 different indexes of oxidative stress namely MDA, protein carbonyls, nitrotyrosine levels and DHE-reactive species. All of the indexes measured were lower in mice receiving Na<sub>2</sub>S compared to vehicle-treated mice at a time. Moreover, the reduced oxidative stress at early time points of reperfusion correlated with lower levels of PYK2 and eNOS tyrosine phosphorylation. Several reports have demonstrated that the cardioprotective effects of H<sub>2</sub>S depend on its anti-oxidant properties. Upregulation of antioxidant protein expression via Nrf-2 activation has been suggested as a major protective pathway utilized by

H<sub>2</sub>S (Shimizu et al., 2016;Calvert et al., 2009;Peake et al., 2013); however, the acute protective effect observed in our studies was obviously independent of transcriptional activation of antioxidant defense genes. In addition, although direct scavenging of several ROS has been shown to occur *in vitro* (Li and Lancaster, Jr., 2013;Kabil et al., 2014), the biological relevance of these reactions has not been demonstrated *in vivo* and is likely of minor, if any, importance due to their slow rates and the limited amount of free H<sub>2</sub>S compared to other reducing compounds in living cells. A more plausible mechanism for the action for H<sub>2</sub>S is persulfidation and modification of the activity of proteins involved in regulating ROS levels. One such example was provided by Sun et al., (Sun et al., 2012) who demonstrated that H<sub>2</sub>S decreases the levels of ROS by inhibiting mitochondrial complex IV and increasing Mn- and CuZn-SOD activities in cardiomyocytes; NaHS also activated CuZn-SOD in a cell-free system. The mechanism through which Na<sub>2</sub>S limits ROS production during early reperfusion and prevents PYK2 activation is a matter of future investigations.

So far, a number of synergistic interactions between NO and H<sub>2</sub>S have been reported (Szabo, 2017). For example, eNOS expression increases after treatment with H<sub>2</sub>S, (Meng et al., 2013) and exposure to H<sub>2</sub>S promotes eNOS coupling, dimerization and phosphorylation on the S1177 residue (Coletta et al., 2012;King et al., 2014). H<sub>2</sub>S can also release NO from intracellular storage pools (Bir et al., 2012;Olson, 2013), inhibit phosphodiesterase activity to boost cGMP signaling (Bucci et al., 2010;Bucci et al., 2012) and preserve the NO receptor, soluble guanylate cyclase, in its reduced, NO-responsive state (Zhou et al., 2016). In the present study, we report an additional level of H<sub>2</sub>S-NO cross talk and further underscore the importance of NO in the biological activity of H<sub>2</sub>S. In conclusion, our study confirms the importance of PYK2 inhibition for cardioprotection and provides a novel molecular pathway through which H<sub>2</sub>S donors exert their beneficial cardiovascular effects.

### **Authors contributions**

Participated in research design: SIB, CS, AP, IF

Conducted experiments: SIB, AC, SZ, BL

Performed data analysis: SIB, AP

Wrote or contributed to the writing of the manuscript: SIB, CS, IF, AP

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## Footnote

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## Legends for figures

**Figure 1. Endogenous production of H<sub>2</sub>S regulates PYK2/eNOS phosphorylation in cardiomyocytes.** Differentiated H9c2 were treated with 50μM H<sub>2</sub>O<sub>2</sub> for 10 minutes, lysed and proteins subjected to SDS-PAGE. Representative western blots along with densitometric for (A) pPYK2(Y402); and (B) peNOS(Y656) of cells treated with AOAA 1mM for 45 min or L-cysteine 500μM for 45 minutes. Cells infected with GFP Ad or CSE Ad (10MOI, 36h prior to H<sub>2</sub>O<sub>2</sub>) were evaluated for (C) pPYK2(Y402) and (D) peNOS(Y656). Phosphorylated protein levels were normalized to total protein levels. n=5 independent experiments; \*p<0.05; \*\*p<0.001; \*\*\*p<0.0001 (two-way ANOVA, Bonferroni).

**Figure 2. Exogenous Na<sub>2</sub>S reduces PYK2 activation and eNOS phosphorylation on Y657 during oxidative stress injury.** Differentiated H9c2 were treated with Na<sub>2</sub>S 100μM for 20 minutes prior to H<sub>2</sub>O<sub>2</sub>. Subsequently H<sub>2</sub>O<sub>2</sub> (50μM for 10 minutes) was applied. Representative western blots along with densitometric analysis of (A) pPYK2(Y402) and (B) peNOS(Y656). Phosphorylated protein levels were normalized to total proteins. n=5 independent experiments; \*\*p<0.001; \*\*\*p<0.0001. (C) HEK cells were transfected with WT eNOS with or without WT PYK2 or a kinase-dead PYK2 mutant. Representative western blots for peNOS(Y657), eNOS and PYK2. Phosphorylated eNOS levels were normalized to total eNOS levels. n=4 independent experiments; \*\*p<0.001; \*\*\*p<0.0001. (two-way ANOVA, Bonferroni).

**Figure 3. Na<sub>2</sub>S increases eNOS activity.** Heavy citrulline to heavy arginine ratio of supernatants from cells treated as described in (Fig.2C). eNOS activity was assessed by its ability to produce heavy citrulline upon depletion of arginine for 12h and addition of heavy arginine for 2h. Results were analyzed by LC-MS measurements. n=4 independent experiments; \*\*p<0.001; \*\*\*p<0.0001 (two-way ANOVA, Bonferroni).

**Figure 4. Correlation of H<sub>2</sub>S levels with oxidative stress in cardiomyocytes.** H<sub>2</sub>S produced by H9c2 cells overexpressing CSE (**A**) or following pharmacological manipulation of endogenous CSE activity (**B**) was assessed by using SSP4. (**C-D**) Hydrogen peroxide levels in cells with increased or decreased H<sub>2</sub>S production. n=5 independent experiments; \*\*p<0.001; \*\*\*p<0.0001. t test (A); one way Anova; Newman Kleus (B); two-way ANOVA, Bonferroni (C,D)

**Figure 5. H<sub>2</sub>S inhibits PYK2 activity.** (**A**). Effects of increasing concentrations of Na<sub>2</sub>S on purified PYK2 enzymatic activity. n=3 independent experiments (**B**). Effects of Na<sub>2</sub>S (100μM, 30min) on PYK2 sulfhydration in H9c2 cells. n=5 independent experiments; \*\*p<0.001; \*\*\*p<0.0001 (two-way ANOVA, Bonferroni).

**Figure 6. H<sub>2</sub>S reduces oxidative stress induced cardiomyocyte death in a PYK2 dependent manner.** Differentiated H9c2 were subjected to 12h H<sub>2</sub>O<sub>2</sub> 500 μM exposure. To inhibit endogenous H<sub>2</sub>S production cells were pretreated with AOAA 1mM (**A**). To stimulate endogenous H<sub>2</sub>S production cells were pretreated with L-cysteine 500 μM (**A**). To assess the effect of inhibition of H<sub>2</sub>S production during H<sub>2</sub>O<sub>2</sub> exposure, cells were pretreated with a combination of AOAA and L-cysteine as described above (**A**). The effects of endogenous H<sub>2</sub>S production were evaluated on both naïve cells and in cells in which PYK2 was inhibited with PYK2 siRNA for 48 hours. In (**B**) H9c2 survival was determined after infection with a GFP or CSE Ad (10 MOI, 36h) prior to H<sub>2</sub>O<sub>2</sub> addition; control cells or cells in which PYK2 was silenced were used. To increase H<sub>2</sub>S availability cells were treated with Na<sub>2</sub>S 100μM for 30 minutes and then exposed to H<sub>2</sub>O<sub>2</sub>. In the same experimental series PYK2 was pharmacologically inhibited with 5μM PF-431396 for 45 min prior to H<sub>2</sub>O<sub>2</sub> (**C**). At the end of the incubation cell viability was assessed by the MTT assay. n=6 independent experiments; \*p<0.05 vs No Injury (white circles), #p<0.05 vs H<sub>2</sub>O<sub>2</sub>, § p<0.05 vs AOAA (**A**); (two-way ANOVA, Bonferroni A-C).

**Figure 7. Exogenous Na<sub>2</sub>S regulates PYK2/eNOS activation in early reperfusion *in vivo*.** Mice were subjected to ischemia (30min) and reperfusion for 3min. One group of mice received Na<sub>2</sub>S as iv bolus at 100µg/kg 10 minutes before myocardial reperfusion. Ischemic tissues from the left ventricle were then collected and PYK2 (**A**), eNOS phosphorylation (**B**) and cGMP levels (**C**) were determined. Representative western blots and densitometric analysis of (**A**) pPYK2(Y402) and (**B**) peNOS (Y656). Phosphorylated protein levels were normalized to total protein levels. (**C**) cGMP levels in the ischemic myocardium in mice receiving solvent or Na<sub>2</sub>S treatment, n=6 animals per group; \*\*p<0.001 (t-test).

**Figure 8. Na<sub>2</sub>S reduces ROS production in early reperfusion.** Mice were subjected to ischemia (30min) and reperfusion for 3min. One group of mice received Na<sub>2</sub>S as IV bolus at 100µg/kg 10 minutes before myocardial reperfusion. Tissues were then collected and (**A**) MDA or (**B**) protein carbonyls were determined by colorimetric assays. (**C**) Nitrotyrosine semi-quantitative levels were determined with a nitrotyrosine Ab and (**D**) myocardial RONS were determined by DHE staining in 8µM cryosections of the ischemic myocardial area. n=6 animals per group. \*p<0.05, \*\* p<0.001 (t-test).

**Figure 9. Na<sub>2</sub>S limits myocardial infarct size in a PYK2-dependent manner.** Mice were subjected to LAD ligation and infarcted area, area at risk and total area were determined. (A). Infarcted to area at risk ratio as % (% I/R). n=8 for Sol group, n=8 for PF-431396 group, n=8 for Na<sub>2</sub>S; n=6 for PF-43139+ Na<sub>2</sub>S group; \*\*p<0.001. (B). Ratio of area at risk to whole myocardial area; p=NS among groups. (C). representative pictures from different treatment groups (two-way ANOVA, Bonferroni).

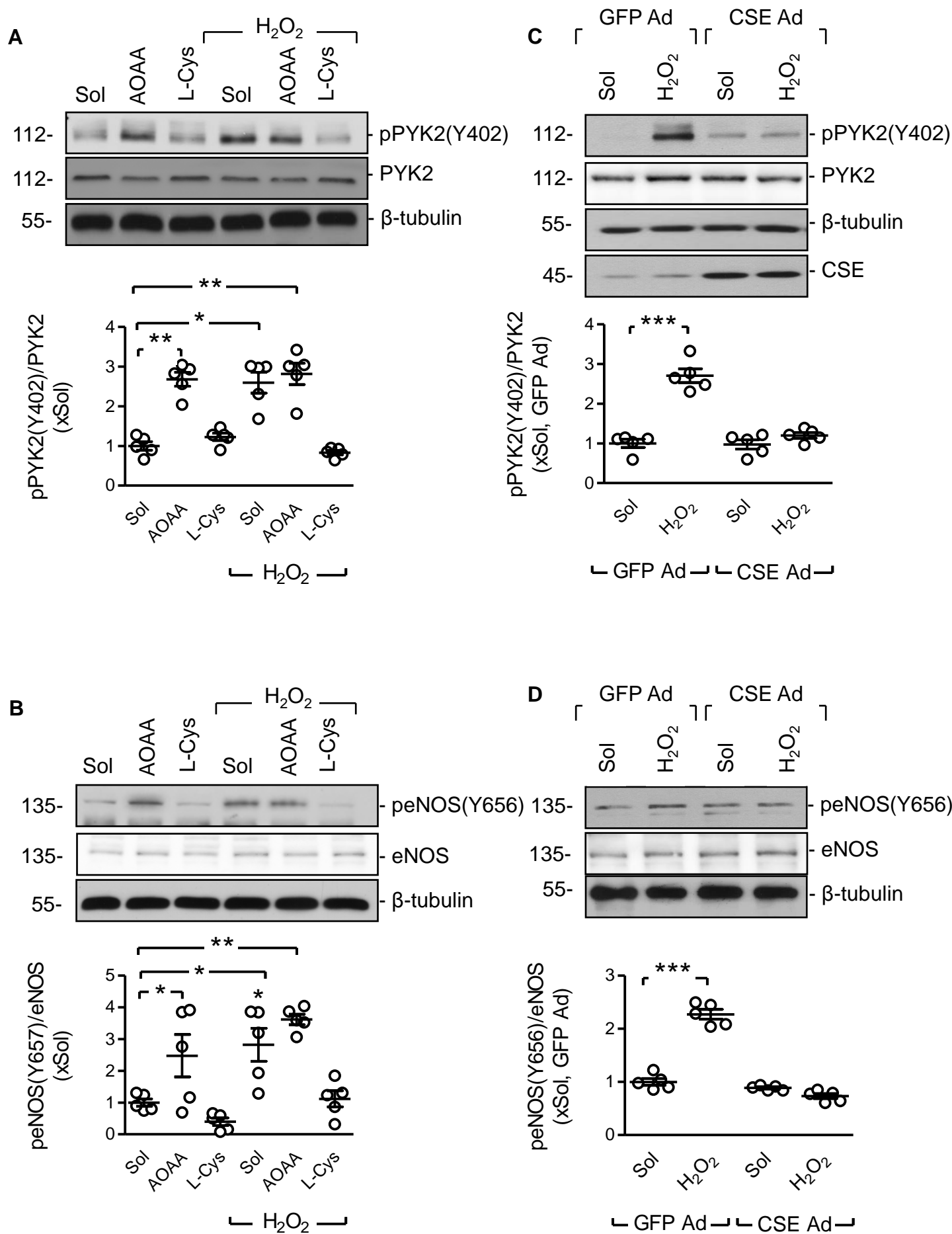
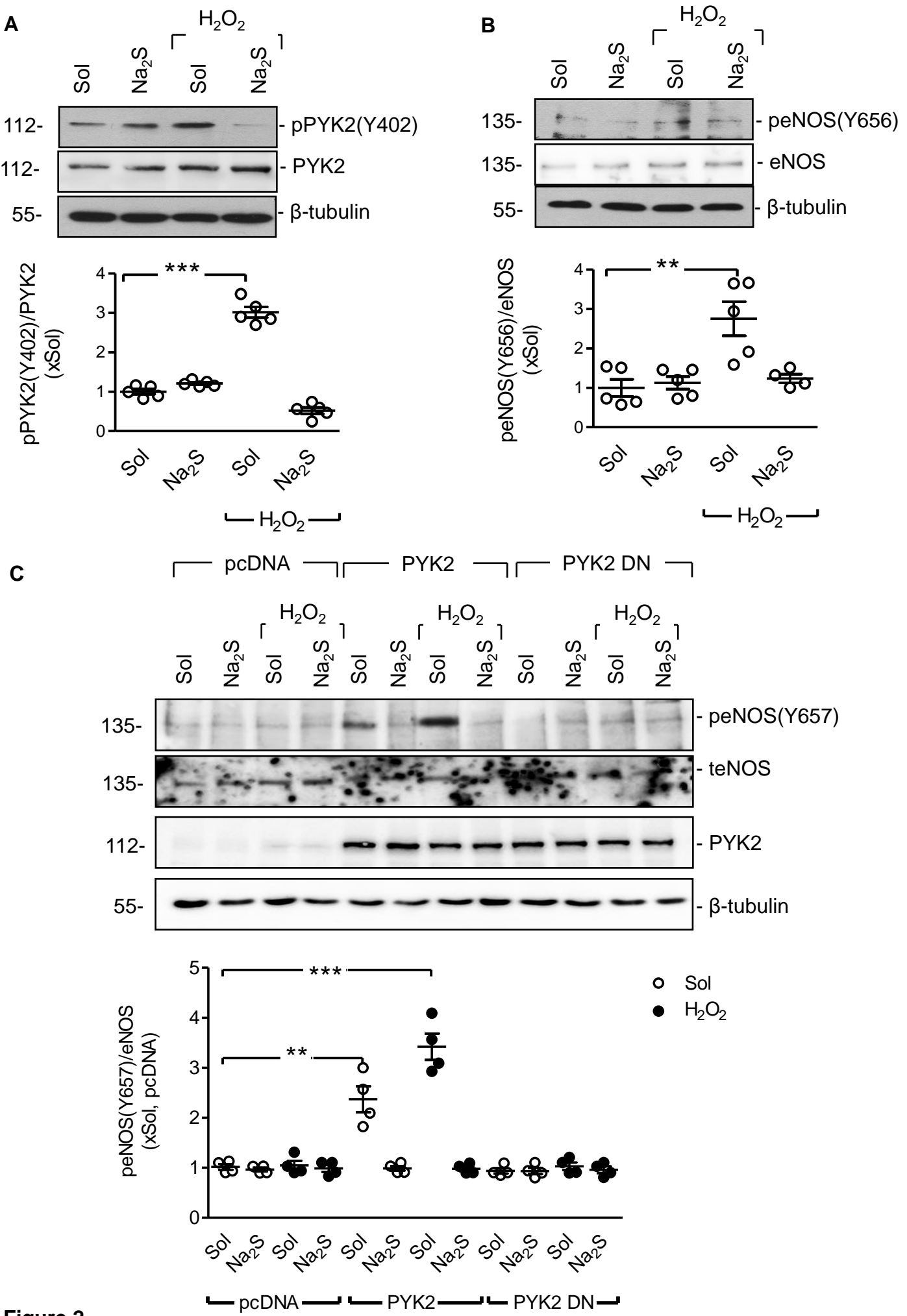


Figure 1



**Figure 2**

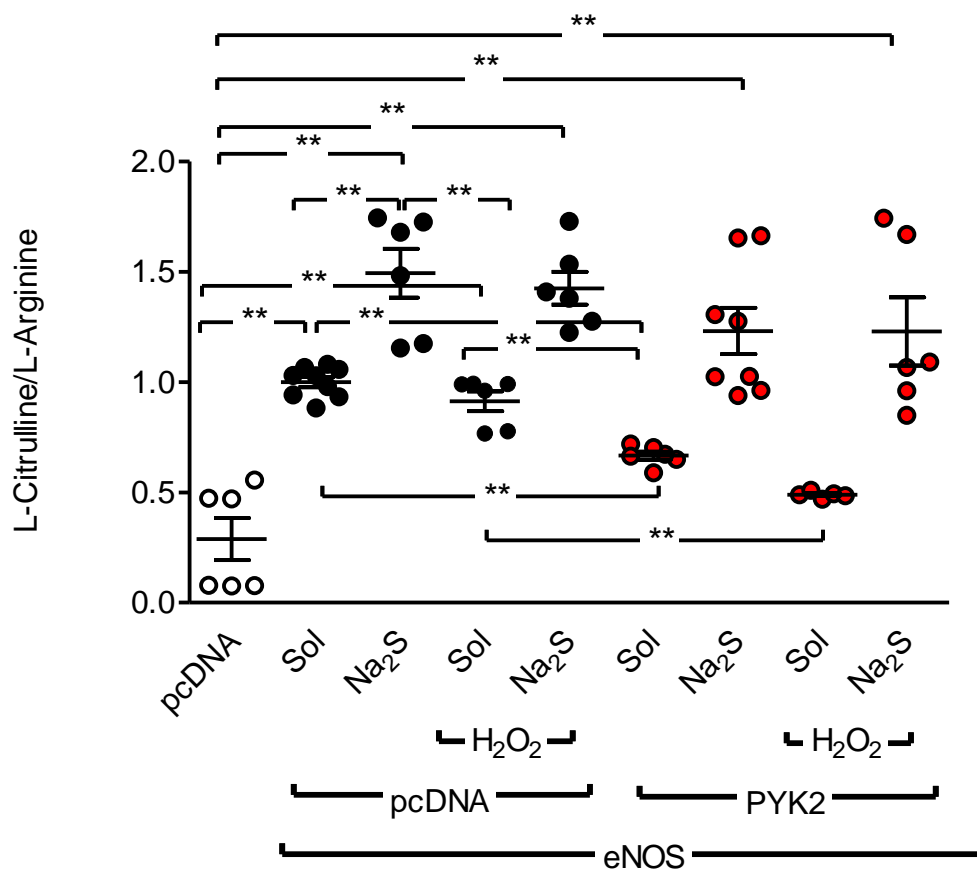


Figure 3

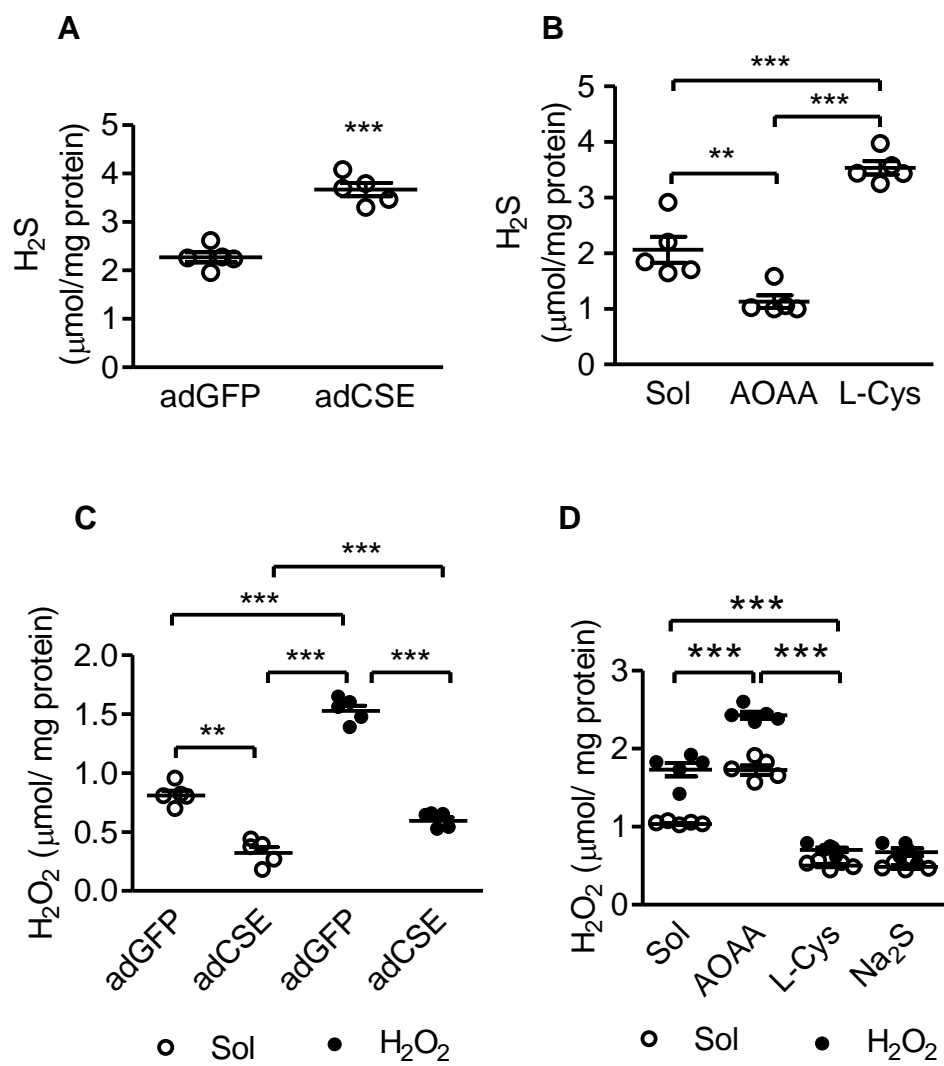


Figure 4

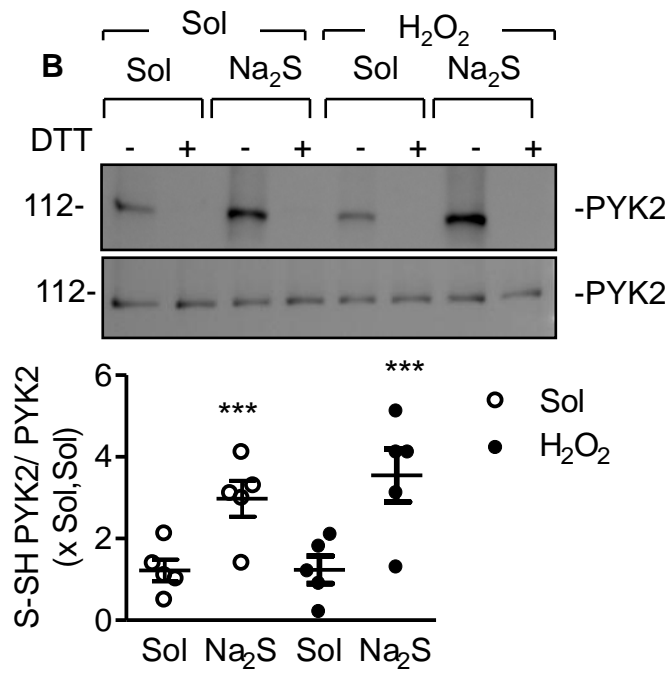
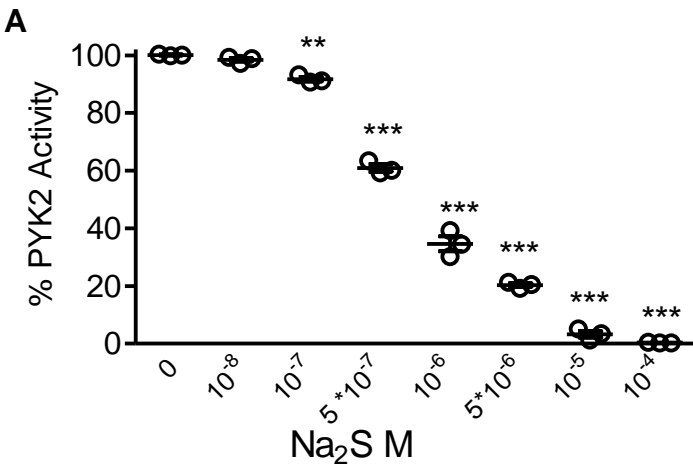


Figure 5

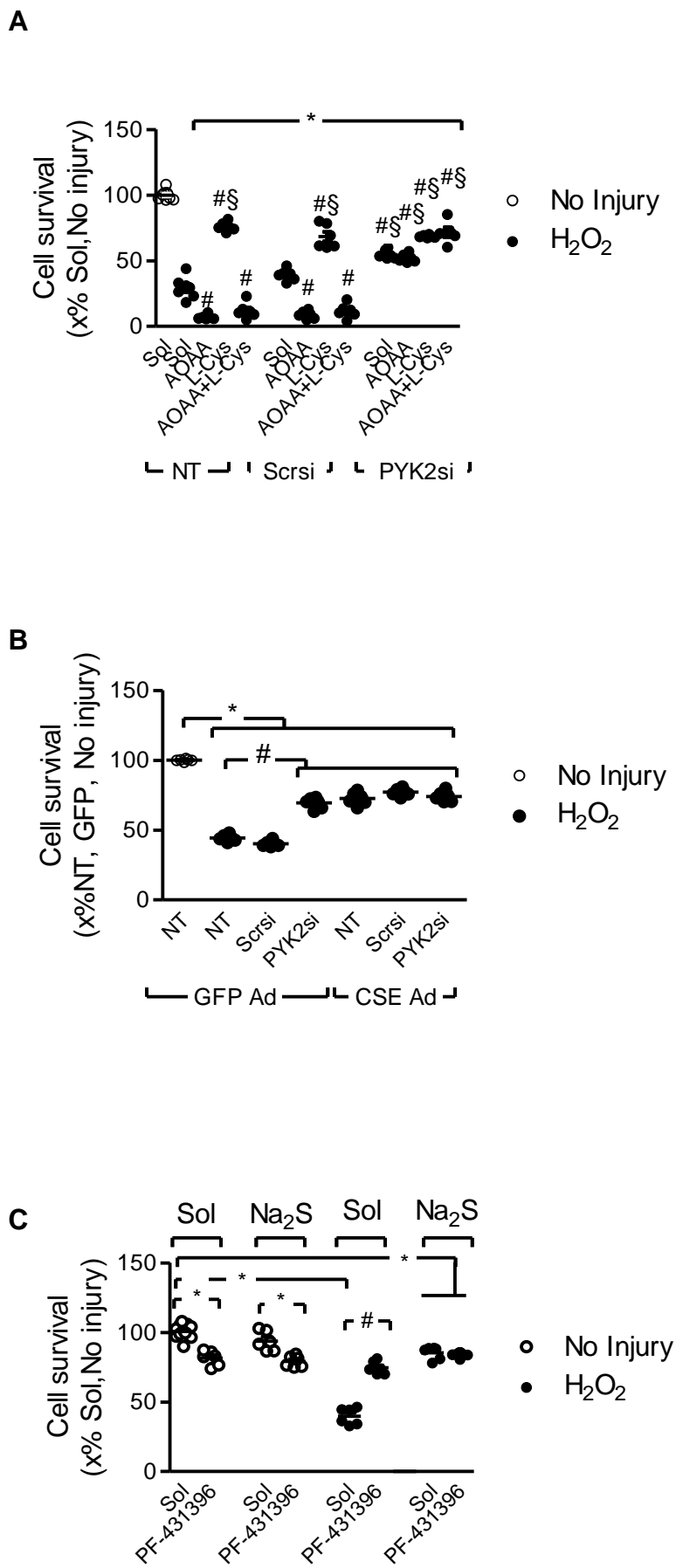
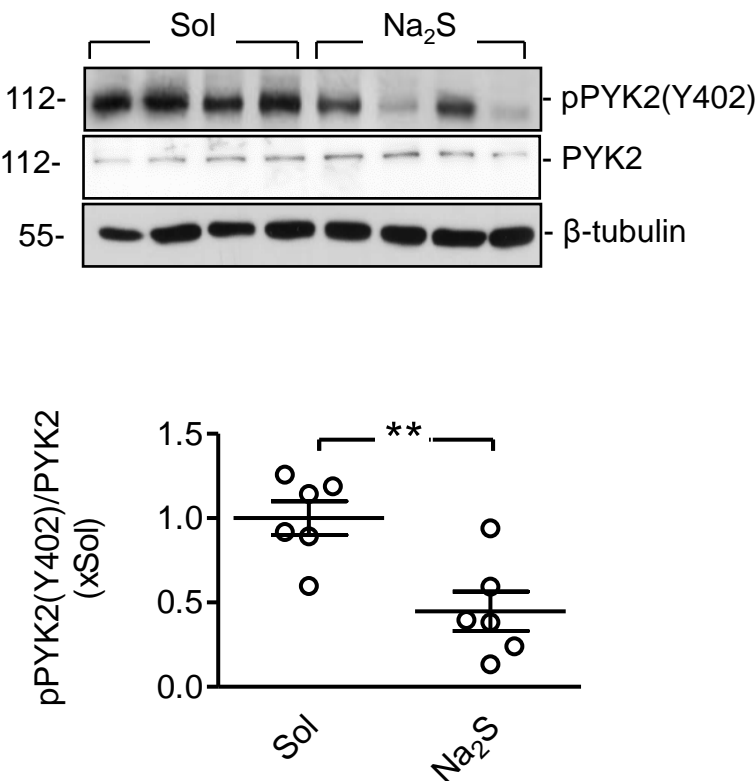
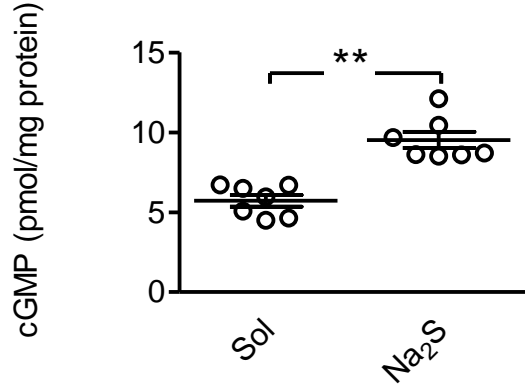


Figure 6

A



C



B

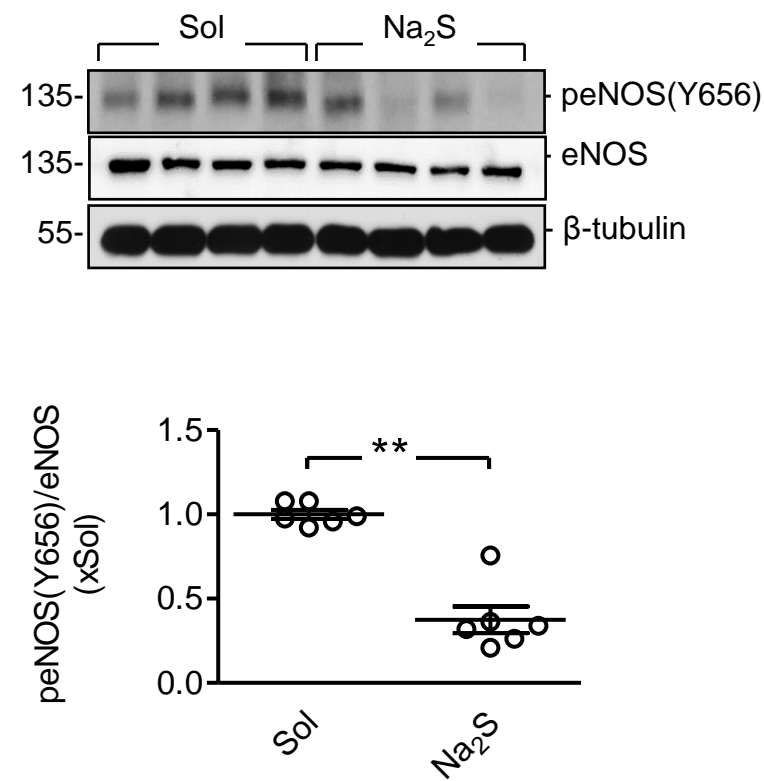
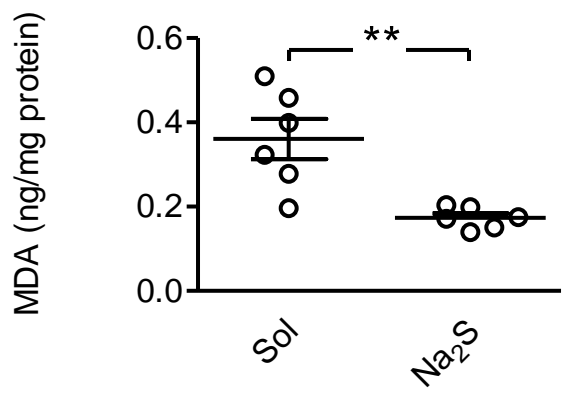
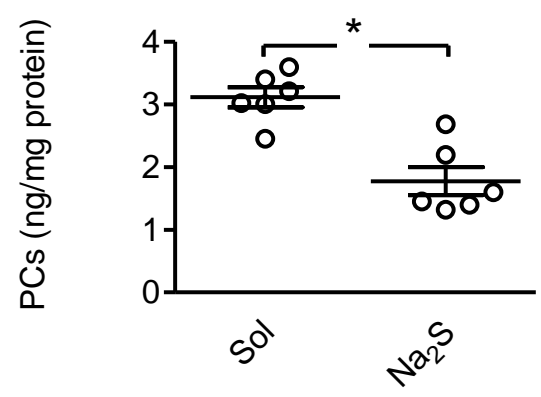


Figure 7

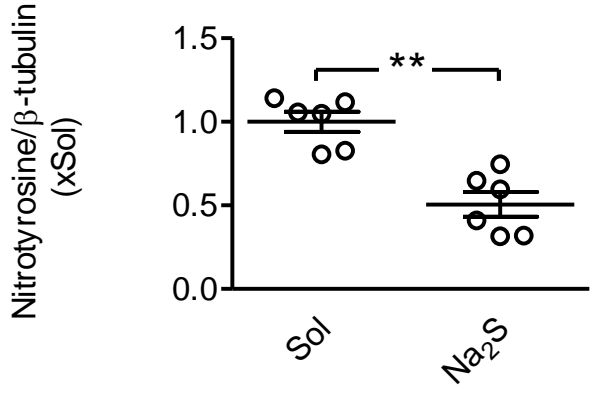
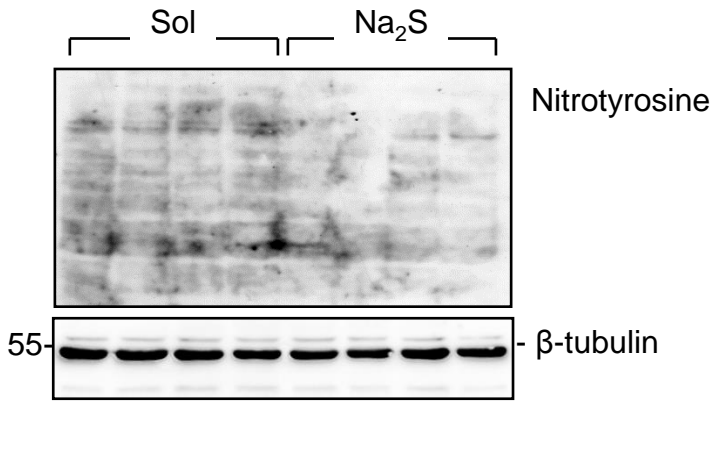
A



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C



D

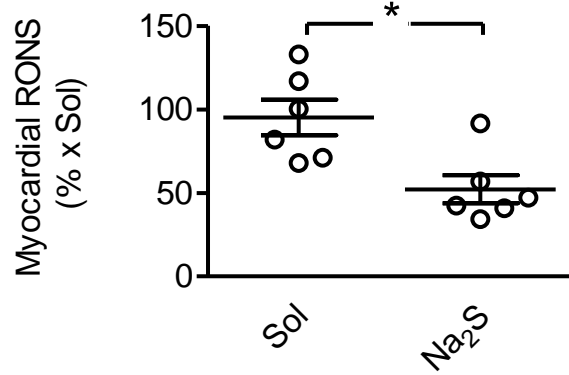
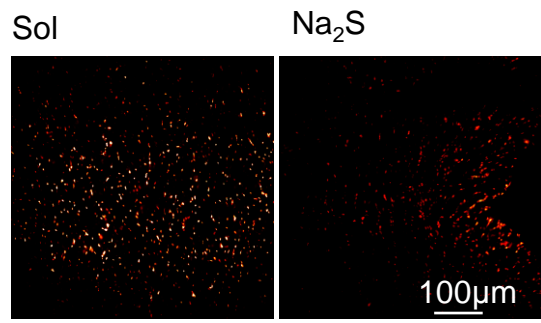
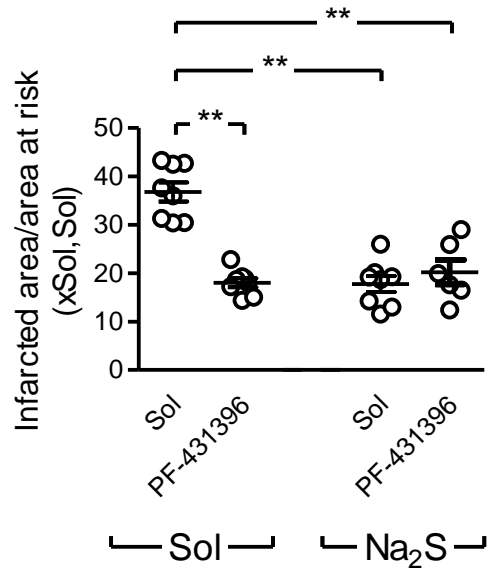
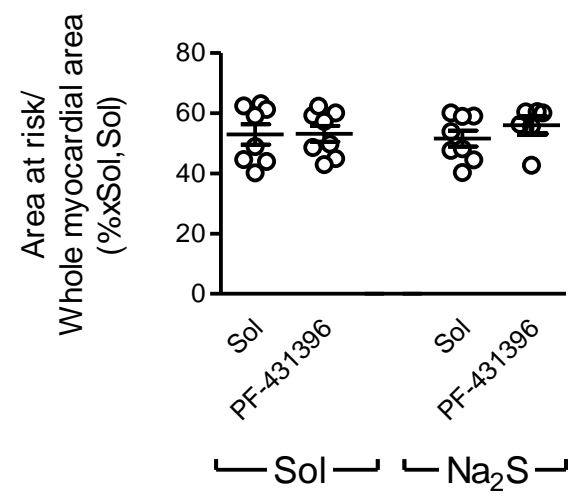


Figure 8

A



B



C

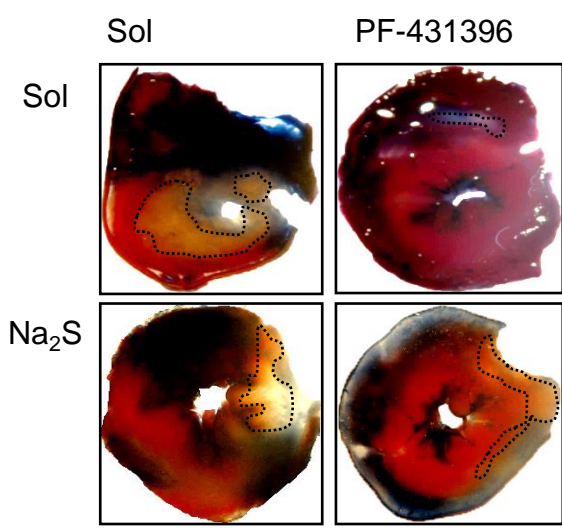


Figure 9