Hydrogen Sulfide Preserves Endothelial Nitric Oxide Synthase Function by Inhibiting Proline-Rich Kinase 2: Implications for Cardiomyocyte Survival and Cardioprotection

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Received June 13, 2017; accepted October 11, 2017

ABSTRACT

Hydrogen sulfide (H₂S) 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 endothelial NO synthase (eNOS). We investigated the ability of H₂S to relieve PYK2-mediated eNOS inhibition and evaluated the importance of the H₂S/PYK2/eNOS axis on cardiomyocyte injury in vitro and in vivo. Exposure of H9c2 cardiomyocytes to H₂O₂ or pharmacologic inhibition of H₂S production increased PYK2 (Y402) and eNOS (Y656) phosphorylation. These effects were blocked by treatment with Na₂S or by overexpression of cystathionine γ-lyase (CSE). In addition, PYK2 overexpression reduced eNOS activity in a H₂S-reversible manner. The viability of cardiomyocytes exposed to H₂O₂ was reduced and declined further after the inhibition of H₂S production. PYK2 downregulation, l-cysteine supplementation, or CSE overexpression alleviated the effects of H₂O₂ on H9c2 cardiomyocyte survival. Moreover, H₂S promoted PYK2 sulfhydration and inhibited its activity. In vivo, H₂S administration reduced reactive oxygen species levels, as well as PYK2 (Y402) and eNOS (Y656) phosphorylation. Pharmacologic blockade of PYK2 or inhibition of PYK2 activation by Na₂S reduced myocardial infarct size in mice. Co-administration of a PYK2 inhibitor and Na₂S did not result in additive effects on infarct size. We conclude that H₂S relieves the inhibitory effect of PYK2 on eNOS, allowing the latter to produce greater amounts of NO, thereby affording cardioprotection. Our results unravel the existence of a novel H₂S-NO interaction and identify PYK2 as a crucial target for the protective effects of H₂S under conditions of oxidative stress.

Introduction

Hydrogen sulfide (H₂S) has emerged as an important gaseous signaling molecule in mammalian cells regulating a multitude of basic biologic processes, including bioenergetics, proliferation, apoptosis, and necrosis (Mustafa et al., 2009; Li et al., 2011; Szabó and Papapetropoulos, 2011; Wang, 2012; Módis et al., 2014). Endogenous H₂S is produced by three enzymes—namely, cystathionine γ-lyase (CSE), cystathionine β-synthase, and 3-mercaptopyruvate sulfur transferase (Kimura, 2011; Kabil and Banerjee, 2014; Papapetropoulos et al., 2015). Although all three enzymes are expressed in the cardiovascular system, existing data suggest that CSE plays a major role in cardiovascular physiology (Wang, 2012; Polhemus and Lefer, 2014; Katsouda et al., 2016). CSE exerts angiogenic (Papapetropoulos et al., 2009), hypotensive (Yang et al., 2008), cardioprotective (Elrod et al., 2007; Bibli et al., 2015a), as well as antioxidant and anti-inflammatory effects in the myocardium and the vessel wall (Calvert et al., 2009; Kimura, 2011; Szabó et al., 2011; Shibuya et al., 2013; Salloum, 2015; Kanagy et al., 2017). Reduced generation or increased breakdown of H₂S leads to lower levels 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; Wang et al., 2015; Greaney et al., 2017; Kanagy et al., 2017).

This work was supported by European Union FP7 REGPOT CT-2011-285900 (SEE-DRUG), by the Cooperation in Science and Technology COST Action BM1005 (EUGAS: European network on gasotransmitters), and by the Deutsche Forschungsgemeinschaft (SFB 834/A9).

https://doi.org/10.1124/mol.117.109645.

Abbreviations: ANOVA, analysis of variance; AOA, aminoacyclic acid; CSE, cystathionine-γ-lyase; DHE, dihydroethidium; DMEM, Dulbecco’s modified Eagle’s medium; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; GFP, green fluorescent protein; HEK, human embryonic kidney cell line; H₂S, hydrogen sulfide; KO, knockout; LAD, left anterior descending coronary artery; MDA, malondialdehyde; MOL, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; PBS, phosphate-buffered saline; PC, protein carbonyls; PYK2, proline-rich kinase 2; ROS, reactive oxygen species; Scrsi, scrambled small interfering RNA; Sol, solvent; TCA, trichloroacetic acid.
Studies from several laboratories have proven that endogenously produced and exogenously administered H₂S limit ischemia-reperfusion injury and reduces infarct size in isolated hearts and in vivo (Johansen et al., 2006; Pan et al., 2006; Elrod et al., 2007; Calvert et al., 2009; Szabó et al., 2011; King et al., 2014; Polhemus et al., 2014; Polhemus and Lefer, 2014; Bibi et al., 2015a; Das et al., 2015).

To exert its biologic responses, H₂S uses a variety of signaling pathways by regulating the activity of kinases, phosphatases, transcription factors, and ion channels (Szabó, 2007; Paul and Snyder, 2012; Wang, 2012; Polhemus and Lefer, 2014; Kanagy et al., 2017). Many of these actions are attributed to a post-translational modification of cysteine residues in a modification referred to as sulphydrylation or persulfidation (Paul and Snyder, 2012). In addition, some biologic effects exerted by H₂S require nitric oxide (NO) production. Angiogenesis, vasodilation, and cardioprotection are reduced or blunted when endothelial NO synthase (eNOS) is inhibited (Coletta et al., 2012; King et al., 2014; Bibi et al., 2015a). At the molecular level, the H₂S-NO interaction involves increased eNOS phosphorylation at the activator site S1177 (Minamishima et al., 2009; Papapetropoulos et al., 2009; Coletta et al., 2012; Altaany et al., 2013; Kondo et al., 2013; King et al., 2014; Bibi et al., 2015a; Chatzianastasiou et al., 2016; Karwi et al., 2017) and reduced phosphorylation at the T495 inhibitory site (Coletta et al., 2012; Polhemus et al., 2013; Bibi et al., 2015b). Moreover, H₂S promotes eNOS dimerization and coupling through the sulfydrylation of C443 (Altaany et al., 2014). The aforementioned post-translational modifications of eNOS enhance NO production and/or bioavailability after exposure to H₂S, as evidenced by increases in cGMP accumulation or NO metabolite levels (Coletta et al., 2012; Predmore et al., 2012; Kondo et al., 2013; King et al., 2014; Szabo, 2017). With respect to cardioprotection, the importance of S1176 phosphorylation in vivo was demonstrated using S1176A knock-in mice in which H₂S 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; Tai et al., 2002; Chappell et al., 2008; Loot et al., 2009) that has been linked to cardiac remodeling (Takeishi, 2014), hypertrophic responses (Hirotani 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 (Fisslthaler et al., 2008; Loot et al., 2009). PYK2 is activated in the early minutes of myocardial reperfusion after ischemia, resulting in increased phosphorylation of eNOS on the Y656 inhibitory residue and reduced NO output; this mechanism defines myocardial infarct size, and PYK2 is proposed to serve as a novel therapeutic target for cardioprotection (Bibi et al., 2017). Since H₂S is known to possess antioxidant 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 H₂S restrains the activation of PYK2, providing a novel mechanism of positive interaction between H₂S and NO in cardiomyocytes.

### Materials and Methods

#### Chemicals and Reagents

All chemicals and reagents— including aminoxyacetic acid (AOAA), l-cysteine, Na₂S, PF-431396, Triton x > 100, NaCl, NaF, EDTA, EGTA, phenylmethylsulfonyl fluoride, protease and phosphatase inhibitors, glycerol, phosphatase, and MITT were purchased from Sigma-Aldrich (Taufkirchen, Germany); DMSO, H₂O₂, Tris, and SDS were purchased from AppliChem (Bioline Scientific, Athens, Greece). LipofectAMINE RNAiMAX was obtained from Invitrogen (Antisel, Athens, Greece); Dulbecco’s modified Eagle’s medium (DME), sodium pyruvate, antibiotics, Opti-MEM, and fetal bovine serum (FBS) were obtained from Gibco (Antisel); the eNOS was generated by Eurogentec (Köln, Germany). SSP4 was purchased from Doinjo EU (Munich, Germany). The Amyplex 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 American Type Culture Collection (CRL-1446) (ATCC, LGC Standards, Middlesex, UK). H9c2 cells were cultured in DME containing 25 mM l-glucose and 1 mM sodium pyruvate, supplemented with 10% FBS, 2 mM l-glutamine, 1% streptomycin (100 μg/ml), and 1% penicillin (100 U/ml) at pH 7.4 in a 5% CO₂ incubator at 37°C. Cells were maintained in a subconfluent condition of a maximum of 70% before passaging to avoid differentiation. For differentiation, H9c2 cells were seeded and allowed to grow to confluence. The medium was then replaced to DME 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 such as cardiac troponin and MLC2v transcripts were elevated, reminiscent of cells with a cardiac phenotype, as described before (Bibi et al., 2017). Human embryonic kidney cells (HEK) cells were cultured in DME supplemented with 1 mM sodium pyruvate and 10% FBS, 2 mM l-glutamine, 1% streptomycin (100 μg/ml), and 1% penicillin (100 U/ml) at pH 7.4 in a 5% CO₂ incubator at 37°C.

#### In Vitro Treatments

For in vitro experiments, differentiated H9c2 cells were pretreated with the appropriate drug as follows. Single treatments were as follows: AOAA was used at a concentration of 1 mM for 45 minutes, l-cysteine at 500 mM for 45 minutes, PF-431396 5 μM for 45 minutes, and Na₂S 100 μM for 30 minutes. For double treatments, AOAA and l-cysteine in the previously mentioned concentrations were added simultaneously; PF-431396 was added 15 minutes before Na₂S. After treatment, cells were lysed for biochemical analysis or subsequent cell-viability studies. To induce in vitro oxidative stress injury, H9c2 cells (1.5 × 10⁶/well) were treated with 500 μM H₂O₂ in serum-free DME for 12 hours in a 5% CO₂ incubator at 37°C.

#### Small Interfering RNA-Mediated Downregulation of PYK2

H9c2 cells were seeded until 80% confluence. Transient transfection of small interfering RNA (siRNA) (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 hours, the Opti-MEM was replaced with complete DME medium with 10% FBS for cell viability assays or with serum-free DME for biochemical studies.
The efficacy of siRNA PYK2 gene knockdown, 48 hours post-transfection, has been previously confirmed (Bibli et al., 2017).

**Adenoviral Infections**

H9c2 cells were seeded until confluence and differentiated for 7 days. On day 6 of differentiation, cells were infected with green fluorescent protein (GFP) or CSE adenoviruses (Bucci et al., 2010) at 10 MOI for 36 hours.

**MTT Measurements**

After 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 hours at 37°C. The formazan formed was dissolved in solubilization solution (10% Triton-X 100 in acidic 0.1 N 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 six-well plates until confluence, treated as described already, were washed twice with phosphate-buffered saline (PBS) and further lysed with lysis solution (1% Triton × 100, 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, and 100 mM phenylmethyl sulfonyl fluoride, 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 minutes 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% bromophenol blue, 0.125 M Tris/HCl. The samples were then heated at 100°C for 10 minutes and stored at −80°C. An equal amount of protein was loaded in each well and then separated on SDS-PAGE electrophoresis and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% nonfat dry milk, membranes were incubated overnight at 4°C with primary antibody. The following primary antibodies were used: phospho PYK2 (Y402), phosphor-eNOS (Y656), total PYK2, total eNOS, nitrotyrosine, and β-tubulin. Membranes were then incubated with secondary goat anti-rabbit HRP antibody for 2 hours at room temperature and developed using the Supersignal electrogenerated chemiluminescence Western blotting detection reagents. Relative densitometry was determined using a computerized software package (National Institutes of Health Image), and the phosphorylated values were normalized to the values for total proteins respectively. All the presented total proteins were derived from the same cell line. All the presented total proteins were derived from the same totalized values were normalized to the values for total proteins repackage (National Institutes of Health Image), and the phosphorylation levels were normalized to total proteins.

**H2S Measurements**

Intracellular levels of H2S were measured by monitoring the reaction of SSP4 with H2S. 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% bovine serum albumin. For inhibition of endogenous H2S production, cells were pretreated with AOA (1 mM) for 45 minutes. To enhance H2S production, cells were incubated with L-cysteine (500 μM) for 45 minutes. Subsequently, medium was replaced and SSP4 (10 μmol/liter) was added for 60 minutes. Thereafter, the cell supernatant was collected, and floating cells were removed by centrifugation (16,000g, 10 minutes, 4°C). The specific products of the reaction of H2S with SSP4 were quantified by LC-MS/MS.

**Oxidative Stress Detection in Cardiomyocytes.** Hydrogen peroxide levels were measured in cardiomyocytes by using the Amplex Red Assay Kit according to the manufacturer’s instructions. In brief, differentiated H9c2 cells were infected with a GFP- or CSE-expressing adenovirus or treated with the Na2S salt as described. In some wells, 50 μM H2O2 was added to the cells for 10 minutes. The reaction was stopped on ice. Cells were collected and washed three times with ice-cold PBS to wash out the exogenous H2O2; 106 cells per condition were used for H2O2 determination. A standard curve was obtained. The specific products of the reaction of H2S with H2O2 were used to quantify the endogenously produced H2O2.

**PYK2 Activity Assay**

The effects of H2S 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 Na2S on purified PYK2 protein according to the manufacturer’s instructions.

**S-Sulfhydration Detection**

Sulphydrylation was detected using a modified biotin switch assay. In brief, H9c2- differentiated cells were treated with Na2S (100 μM) for 30 minutes. Reactions were stopped on ice, and cells were washed with ice-cold PBS. Subsequently, samples were precipitated with trichloroacetic acid.

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**Fig. 2.** Exogenous Na2S reduces PYK2 activation and eNOS phosphorylation on Y656/7 during oxidative stress injury. Differentiated H9c2 cells were treated with Na2S 100 μM for 20 minutes before H2O2. Subsequently, H2O2 (50 μM for 10 minutes) was applied. Representative Western blots along with densitometric analysis of (A) pPYK2(Y402) and (B) p-eNOS(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 human eNOS, with or without WT PYK2 or a kinase-dead PYK2 mutant. Representative Western blots for p-eNOS (Y656), 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).
20% trichloroacetic acid (TCA) and stored at −80°C. TCA precipitates were washed with 10% and 5% TCA and then centrifuged (16,000g, 30 minutes, 4°C) before being suspended in HENs buffer (250 mmol/liter HEPES-NaOH, 1 mmol/liter EDTA, 0.1 mmol/liter neocuprine, 100 μmol/liter deferoxamine, 2.5% SDS) containing 20 mmol/liter methanethiosulfonate to block free thiols and protease and phosphatase inhibitors. Acetone precipitation was performed, and pellets were resuspended in 300 μl qPerS-SID tisV buffer (6 mol/liter urea, 100 mmol/liter NaCl, 2% SDS, 5 mmol/liter EDTA, 200 mmol/liter Tris pH 8.2; 50 mmol/liter iodoacetyl-PEG2-biotin, 2.5 mmol/liter dimedone), sonified, and incubated for 2 hours at room temperature in the dark. Lysates (500 μg) were precipitated with acetone, and protein pellets were resuspended in 50 μl Tris/HCl (50 mmol/liter, pH 8.5) containing guanidinium chloride (GdmCl 6 mmol/liter), and incubated at 95°C for 5 minutes. A negative control was generated for each sample by adding dithiothritol (1 mmol/liter) during biotin crosslinking. Biotin was then immunoprecipitated using a high-capacity streptavidin resin (Thermo Scientific, Heidelberg, Germany) overnight at 4°C. Elution was performed by the addition of 3% SDS, 1% β-mercaptoethanol, 8 mol/liter urea, and 0.005% bromeophenol blue in PBS for 15 minutes at room temperature, followed by 15 minutes at 95°C. Sulphydrated proteins were detected after SDS-PAGE by Western blotting.

**cGMP Enzyme Immunoassay**

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

**Malondialdehyde and Protein Carbonylation Assessment**

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

**Dihydroethidium Staining of Cardiac Reactive Oxygen Species Formation**

Cardiac reactive oxygen species (ROS) production was qualitatively detected by dihydroethidium (DHE) (1 μM)-derived fluorescence in heart tissue cryosections of 8 μm as described previously (Andreadou et al., 2014).

**Animals**

All animal procedures complied 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, we used male mice C57BL/6J mice.

**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, QC).

**Experimental Protocol**

In animals treated with the pharmacologic inhibitor of PYK2, PF-431396 was administrated at 5 μg/g in 2% DMSO (100 μl) i.v., and Na2S was given as an i.v. bolus at 100 μg/kg as described previously (Bibli et al., 2015a). In the first experimental series, animals received the indicated drugs either 10 minutes before sacrifice for tissue collection (sham-operated groups) or 10 minutes before reperfusion (ischemia-reperfusion injury groups). The left ventricle was isolated and submerged in liquid nitrogen for preservation before analysis.

**Fig. 3.** Na2S 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 on depletion of arginine for 12 hours and the addition of heavy arginine for 2 hours. Results were analyzed by LC-MS measurements; n = 4 independent experiments; *P < 0.001 (two-way ANOVA, Bonferroni).

Electrocardiogram recordings were performed by a lead I ECG recording with PowerLab 4.0 (ADInstruments, Sydney, Australia). Recordings were analyzed by LabChart 7.0 software. A thoracotomy was then performed between the fourth and fifth ribs, and the pericardium was carefully retracted to visualize the left anterior descending coronary artery (LAD), which was ligated using a 8-0 Prolene (Ethicon, Somerville, NJ) monofilament polypropylene suture placed 1 mm below the tip of the left ventricle. The heart was stabilized for 15 minutes before ligation to induce ischemia. After the ischemic period, the ligature was released and allowed reperfusion of the myocardium. Throughout the experiments, body temperature was maintained at 37 ± 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 of saline-heparin 1% for blood removal. Five milliliter of 1% TTC phosphate buffer 37°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°C for 24 hours, sliced in 1-mm sections parallel to the atrioventricular 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 cubic centimeters, and the percentage of infarct-to-risk area ratio (percentage of ischemia/reperfusion) was calculated.
In a second series of experiments, mice were subjected to 30 minutes of regional ischemia of the myocardium, followed by 2 hours of reperfusion, and randomized into four groups as follows: 1) Sol group (n = 8): administration of solvent [water for injection containing 2% DMSO (100 μl) i.v. 10 minutes before the ischemic insult]; 2) PF-431396 group (n = 8): administration of 5 mg/g PF-431396 [dissolved in water for injection containing 2% DMSO; (100 μl) iv 10 minutes before reperfusion; 3) Na₂S group (n = 8): administration of 100 mg/kg Na₂S (100 μl i.v. 10 minutes before reperfusion), and 4) PF-431396 + Na₂S group (n = 6): administration of PF-431396 and Na₂S as in groups 2 and 3.

Statistical Analysis

One- or two-way analysis of variance (ANOVA) was used to detect the 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 ± S.E.M. values.

Results

Endogenously Generated H₂S Inhibits PYK2 in Cultured Cardiomyocytes. To evaluate the role of endogenous H₂S on PYK2 activation, we evaluated PYK2 phosphorylation in differentiated H9c2 cardiomyocytes in the presence of a pharmacologic inhibitor of H₂S synthesis (AOAA) (Asimakopoulou et al., 2013) (Fig. 1, A and B) or the substrate for H₂S synthesis (L-cysteine) (Fig. 1, A and B). In an alternative approach, endogenous H₂S production was enhanced via the adenoviral-mediated overexpression of CSE (Fig. 1, C and D). In agreement with our recently published observations (Bibli et al., 2017), H₂O₂ treatment resulted in the increased phosphorylation of PYK2 on Y402 (Fig. 1A). Moreover, H₂O₂ treatment increased eNOS phosphorylation on the eNOS Y656 inhibitory site (Fig. 1B). The effects of H₂O₂ treatment were mimicked by inhibiting endogenous H₂S production with AOAA (Fig. 1, A and B). PYK2 and eNOS phosphorylation were not enhanced by the combined treatment of H₂O₂ and AOAA. In addition, supplementation with the H₂S substrate L-cysteine (Fig. 1, A and B), or CSE overexpression (Fig. 1, C and D) abrogated the biochemical changes on PYK2 (Fig. 1, A and C) and eNOS (Fig. 1, B and D) triggered by H₂O₂.

Pharmacologic Administration of H₂S Results in PYK2 Inhibition and Activation of eNOS. Having established that endogenously produced H₂S regulates PYK2 activation, we sought to determine whether H₂S supplementation could mitigate the effects of H₂O₂ on the PYK2/eNOS pathway. We pretreated H9c2 cells with a sulfide salt, Na₂S. In these experiments, we observed that Na₂S eliminated both the H₂O₂-induced PYK2 activation (Fig. 2A) and eNOS phosphorylation on Y656 (Fig. 2B). To prove that the effects observed on eNOS Y656 phosphorylation were mediated by PYK2, we used a heterologous expression system. HEK cells were cotransfected with wild-type eNOS and an empty pcDNA3 vector, a wild-type PYK2, or a dominant negative PYK2 plasmid as in our previous studies (Fisslthaler et al., 2008). Cotransfection with wild-type eNOS and wild-type PYK2 resulted in an increase in peNOS on Y657 from the...
basal activity of overexpressed PYK2 (Fig. 2C); Na2S administration inhibited eNOS Y656 phosphorylation. Exposure of HEK cells to H2O2 further increased eNOS tyrosine phosphorylation; the effect of was reversed by incubation with Na2S. 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 Y657 phosphorylation depends on PYK2 activity. To evaluate the effect of the pharmacologic treatments on eNOS activity, we measured the conversion of L-arginine to L-citrulline (Fig. 3). Although exposure to Na2S increased eNOS activity, incubation of cells with H2O2 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 Y657); this effect was reversed by Na2S. Incubation of PYK2-transfected cells with H2O2 potentiated the inhibitory effect on eNOS activity. Exogenous application of H2S to PYK2-transfected cells treated with H2O2 restored eNOS activity, confirming that H2S inhibits PYK2 activation and alleviates its inhibitory effect on eNOS.

**Mechanisms of PYK2 Inhibition by H2S.** To study the mechanisms through which H2S inhibits PYK2 preventing eNOS inhibition, we determined the effect of H2S levels on the levels of ROS, a known trigger for PYK2 activation. Overexpression of CSE or l-cysteine supplementation increased H2S levels, whereas AOAA reduced these levels (Fig. 4, A and B). When H2S production was enhanced, H2O2 levels were reduced and vice versa (Fig. 4, C and D). Increased H2O2 levels led to PYK2 Y402 and eNOS Y657 phosphorylation (Fig. 1), and H2S reversed this effect. To determine whether H2S also has direct effects on PYK2, we determined its ability to inhibit PYK2 activity. H2S elicited a robust inhibitory effect on recombinant PYK2 with an IC50 in the sub-micromolar range (Fig. 5A). The inhibition of PYK2 by H2S was associated with enhanced sulphydrylation of the kinase (Fig. 5B).

**H2S Salvages PYK2-Induced Cardiomyocyte Death In Vitro.** To study the effects of PYK2 on cardiomyocyte survival, we used an in vitro model of H2O2-triggered oxidative stress injury and cell death. Inhibition of CSE/CBS-derived H2S by AOAA resulted in augmented cardiomyocyte death under baseline conditions (Supplemental Fig. 1A), as well as after H2O2 (Fig. 6A) administration. Providing additional l-cysteine in the culture media increased cardiomyocyte survival in the presence of H2O2 did not but did not reverse the effect of AOAA (Fig. 6A; Supplemental Fig. 1A). To evaluate the ability of endogenous H2S production to regulate PYK2 activity in the context of cell survival, PYK2 was silenced using an 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 H2O2. Moreover, the deleterious effect of AOAA treatment was not observed after PYK2 knockdown (Fig. 6A). l-cysteine supplementation partially reversed the effect of H2O2 in nontransfected and scrambled RNA-transfected cells, but not in PYK2 silenced cells (Fig. 6A). We next overexpressed CSE and exposed cells to H2O2 to determine the contribution of PYK2 to the protective effect of endogenously produced H2S. When PYK2 was expressed (nontransfected and scrambled ScrsiRNA transfected conditions), CSE restricted the deleterious effect of H2O2 on cardiomyocyte survival (Fig. 6B); however, no additional effect of CSE overexpression was observed in cells in which PYK2 was silenced, indicating that H2S protects cardiomyocytes by inhibiting PYK2 under oxidative stress conditions. Finally, experiments were conducted in the
presence of Na$_2$S as an exogenous source of H$_2$S and PF-431396 as a pharmacologic inhibitor of PYK2. In this series of experiments, we observed that both the PYK2 inhibitor and Na$_2$S improved cell survival after H$_2$O$_2$ exposure; however, combining the sulfide salt with PF-431396 did not exert an additional effect (Fig. 6C).

**H$_2$S Alleviates eNOS Inhibition During Reperfusion In Vivo.** To test whether the observed in vitro findings could be extrapolated in vivo, we used a LAD ligation model. In these experiments, administration of Na$_2$S in sham-operated animals did not affect the basal levels of PYK2 and eNOS Y656 phosphorylation (Supplemental Fig. 2, A and B) or cGMP levels (Supplemental Fig. 2C). When Na$_2$S was administrated intravenously 10 minutes before reperfusion, however, a 50% reduction in the phosphorylation of PYK2 was observed in the early minutes of reperfusion (Fig. 7A). At the same time, we also noted a reduction in Y656 phosphorylation of eNOS (Fig. 7B) along with an increase in the levels of the surrogate NO marker, cGMP (Fig. 7C). In addition, administration of Na$_2$S during ischemia resulted in a reduction in the oxidative and nitrosative stress biomarkers malondialdehyde (MDA; Fig. 8A) and PCs (Fig. 8B) in the early minutes of reperfusion. Similarly, nitrotyrosine levels (Fig. 8C) and DHE-reactive products (Fig. 8D) were attenuated in Na$_2$S-treated animals compared with the solvent-treated animals. These findings taken together demonstrate that Na$_2$S inhibits oxidative stress, limits PYK2 activity, and de-represses eNOS activity.

**Cardioprotective Effects of H$_2$S Are Dependent on the PYK2/eNOS Pathway.** Next, we tested whether the increase in eNOS activity brought about by the H$_2$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 pharmacologic PYK2 inhibitor or/and an H$_2$S source in a dose previously reported by our group not to affect hemodynamic parameters (Chatzianastasiou et al., 2016). In agreement with our previously published observations (Bibli et al., 2017), the pharmacologic inhibition of PYK2 reduced myocardial infarct size (36.8% ± 2.0% for the Sol group, 18.0% ± 0.9% for the PF-431396 group). Similarly, we observed that the administration of Na$_2$S was protective (36.8% ± 2.0% for the Sol group and 17.8% ± 1.6% for the Na$_2$S group, *P < 0.05). However, the simultaneous administration of PF-431396 and Na$_2$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 (Fig. 9, A and C). No statistically significant differences were observed in the area at risk to whole myocardial area among the studied groups (Fig. 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 pharmacologic strategies have been introduced so far in to routine clinical practice to reduce infarct size in patients undergoing acute myocardial infarction (Hausenloy and Yellon, 2016). Better understanding of the intracellular signaling of ischemia/reperfusion injury is expected to lead to novel therapeutic strategies for acute myocardial infarction patients. Herein, we investigated the impact of H2S on the PYK2/eNOS axis and its relevance to cardioprotection.

Initially, we set out to determine whether endogenously produced H2S regulates PYK2 phosphorylation. When H9c2 cells 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 H2S exhibits both direct and indirect antioxidant effects, (Ju et al., 2013; Xie et al., 2016), AOAA-triggered PYK2 activation might be the result of increased oxidative stress on lowering H2S levels. In line with this hypothesis, exogenously added H2O2 mimicked the effects of AOAA on PYK2. Moreover, coincubation of cells with AOAA and H2O2 exerted no additional effects on PYK2 phosphorylation, indicating a common mechanism of action for AOAA and H2O2. Finally, supplementation with the H2S synthesis substrate L-cysteine, CSE overexpression or exogenously added H2S reversed the effects of H2O2 on PYK2, providing further evidence that H2S limits PYK2 activation by counteracting the action of oxidant molecules. In addition to preventing PYK2 activation by reducing oxidative stress, we observed that H2S can directly inhibit PYK2 activity, suggesting a dual mechanism of action for H2S.

In agreement with the fact that PYK2 phosphorylates eNOS on Y656, in all the experiments performed, changes in eNOS tyrosine phosphorylation paralleled those in PYK2 phosphorylation. Direct evidence for the involvement of PYK2 on H2O2-induced eNOS Y656 phosphorylation was provided by a dominant negative approach. Whereas most researchers study S1177 phosphorylation (human eNOS sequence, corresponds to murine S1176) as a surrogate marker of eNOS activity (Dimmeler et al., 1999; Fulton 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 with S1177; whereas 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 H2S were accompanied by changes in activity. H2O2 reduced eNOS activity only in cells expressing PYK2; this effect was reversible by H2S.

To determine the ability of H2S to inhibit PYK2-mediated cell toxicity, we exposed H9c2 cells to H2O2. Treatment of cardiomyocytes with H2O2 significantly reduced cell survival; the effect of H2O2 could be ameliorated by increasing H2S.
production via l-cysteine, CSE overexpression, or the exogenous addition of Na₂S. Our findings agree with previously published findings that H₂S donors protect H9c2 cells from H₂O₂ toxicity (Szabó et al., 2011; Zhao et al., 2015; Chatzianastasiou et al., 2016; Bibli et al., 2017). We recently reported that the toxicity of H₂O₂ in cultured cardiomyocytes could be inhibited by pharmacological PYK2 inhibition or PYK2 silencing and that the effect of PYK2 was eNOS-dependent (Bibli et al., 2017). In the present series of experiments, we found that H₂S was unable to improve cell survival in H9c2 when PYK2 was silenced or inhibited. Similarly, reducing endogenous H₂S production did not lead to greater toxicity when PYK2 was silenced. Taken together, the aforementioned observations suggest that H₂S signals via PYK2 to protect cardiomyocytes against oxidative stress-induced toxicity. Based on our findings that: 1) the protective effect of PYK2 inhibition in H9c2 is linked to de-repression of eNOS (Bibli et al., 2017), and 2) the herein reported observation that H₂S blocks PYK2 activation, we propose that the prosurvival effect of H₂S 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 (Jones and Bolli, 2006; Andreadou et al., 2015). We and others have shown that eNOS is required for the cardioprotective actions of H₂S in vivo (Kondo et al., 2013; King et al., 2014; Bibli et al., 2015a; Chatzianastasiou et al., 2016; Karwi et al., 2017). 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. We found that administration of H₂S can inhibit PYK2 phosphorylation in the early minutes of reperfusion in the infarcted left ventricle, which 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 Na₂S or via its pharmacologic 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 Na₂S and PF-431396 did not exert additional beneficial effects in myocardial survival, further suggesting that Na₂S exerts its effects through PYK2 inhibition.

As ROSs 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 four different indexes of oxidative stress, namely, MDA, PC, nitrotyrosine levels, and DHE-reactive species. All the indexes measured were lower in

![Fig. 9. Na₂S limits myocardial infarct size in a PYK2-dependent manner. Mice were subjected to LAD ligation; infarcted area, area at risk, and total area were determined. (A). Infarcted to area at risk ratio as percentage; n = 8 for Sol group, n = 8 for PF-431396 group, n = 6 for Na₂S, n = 6 for PF-431396+ Na₂S 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).](molpharm.aspetjournals.org)
mice receiving Na$_2$S compared with vehicle-treated mice at a specific 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$_2$S depend on its antioxidant properties. Upregulation of antioxidant protein expression via Nrf-2 activation has been suggested as a major protective pathway used by H$_2$S (Calvert et al., 2009; Peake et al., 2013; Shimizu et al., 2016); however, the acute protective effect observed in our studies was likely independent of the transcriptional activation of antioxidant defense genes. In addition, although direct scavenging of several ROS has been shown to occur in vitro (Li and Lancaster, 2013; Kabil et al., 2014), the biologic relevance of these reactions has not been demonstrated in vivo and is likely of minor, if any, importance owing to their slow rates and the limited amount of free H$_2$S compared with other reducing compounds in living cells. A more plausible mechanism for the action for H$_2$S is persulfidation compared with other reducing compounds in living cells. A more beneficial cardiovascular effects.

NO-responsive state (Zhou et al., 2016). In the present study, NO receptor, soluble guanylate cyclase, in its reduced, (Bucci et al., 2010; Bucci et al., 2012), and preserve the inhibit phosphodiesterase activity to boost cGMP signaling intracellular storage pools (Bir et al., 2012; Olson, 2013), and modification of the activity of proteins involved in regulat-


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Wrote or contributed to the writing of the manuscript: Bibli, Szabo, Fleming, Papapetropoulos.

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References


mong used pharmacological inhibitors for cystathionine synthase (CYSH) and cystathionine $\gamma$ lyase (CSE). Br J Pharmacol 169:922–932.


diomyopathy interfering with signaling molecules and cardiomyocyte metabolism. J Mol Cell Cardiol 69:4–16.