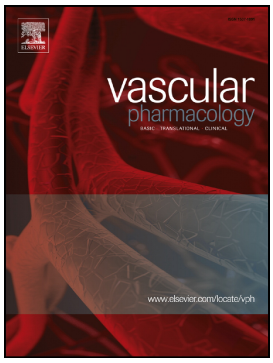


A critical role for cystathionine-β-synthase in hydrogen sulfide-mediated hypoxic relaxation of the coronary artery

J. Donovan, P.S. Wong, R. Roberts, M.J. Garle, S.P.H. Alexander, W.R. Dunn, V. Ralevic

PII: S1537-1891(17)30046-0
DOI: doi: 10.1016/j.vph.2017.05.004
Reference: VPH 6404

To appear in: *Vascular Pharmacology*

Received date: 22 February 2017
Revised date: 22 May 2017
Accepted date: 22 May 2017

Please cite this article as: J. Donovan, P.S. Wong, R. Roberts, M.J. Garle, S.P.H. Alexander, W.R. Dunn, V. Ralevic , A critical role for cystathionine-β-synthase in hydrogen sulfide-mediated hypoxic relaxation of the coronary artery, *Vascular Pharmacology* (2017), doi: 10.1016/j.vph.2017.05.004

A critical role for cystathionine-β-synthase in hydrogen sulfide-mediated hypoxic relaxation of the coronary artery

J Donovan, P S Wong, R Roberts, M J Garle, S P H Alexander, W R Dunn, V Ralevic.

School of Life Sciences, University of Nottingham, Nottingham, UK

Short title: Hydrogen sulfide and coronary artery hypoxia

Correspondence: Vera Ralevic, School of Life Sciences, University of Nottingham, Medical School, Queen's Medical Centre, Nottingham, NG7 2UH, UK

vera.ralevic@nottingham.ac.uk

Tel: 0115 8230183

Fax: 0115 9513251

Abstract

Hypoxia-induced coronary artery vasodilatation protects the heart by increasing blood flow under ischemic conditions, however its mechanism is not fully elucidated. Hydrogen sulfide (H_2S) is reported to be an oxygen sensor/transducer in the vasculature. The present study aimed to identify and characterise the role of H_2S in the hypoxic response of the coronary artery, and to define the H_2S synthetic enzymes involved. Immunoblotting and immunohistochemistry showed expression of all three H_2S-producing enzymes, cystathionine-ß-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (MPST), in porcine coronary artery. Artery segments were mounted for isometric tension recording; hypoxia caused a transient endothelium-dependent contraction followed by prolonged endothelium-independent relaxation. The CBS inhibitor amino-oxyacetate (AOAA) reduced both phases of the hypoxic response. The CSE inhibitor DL-propargylglycine (PPG) and aspartate (limits MPST) had no effect alone, but when applied together with AOAA the hypoxic relaxation response was further reduced. Exogenous H_2S (Na_2S and NaHS) produced concentration-dependent contraction followed by prolonged relaxation. Responses to both hypoxia and exogenous H_2S were dependent on the endothelium, NO, cGMP, K^+ channels and Cl^-/HCO_3^- exchange. H_2S production in coronary arteries was blocked by CBS inhibition (AOAA), but not by CSE inhibition (PPG). These data show that H_2S is an endogenous mediator of the hypoxic response in coronary arteries. Of the three H_2S-producing enzymes, CBS, expressed in the vascular smooth muscle, appears to be the most important for H_2S generated during hypoxic relaxation of the coronary artery. A contribution from other H_2S-producing enzymes only becomes apparent when CBS activity is inhibited.

Keywords

Coronary artery; heart; hydrogen sulfide; hypoxia

Abbreviations

AOAA, amino-oxyacetate; CBS, cystathionine-ß-synthase; CAT, cysteine aminotransferase; cGMP, cyclic guanosine 5'-monophosphate; CSE, cystathionine-γ-lyase; H_2S, hydrogen sulfide; MPST, 3-mercaptopyruvate sulfurtransferase; NO, nitric oxide; PPG, D,L-propargylglycine.

1. Introduction

In the heart, hypoxia due to ischemia is a dangerous insult which compromises cardiac function. Hypoxic coronary vasodilatation is an important pathophysiological response that increases blood flow to the heart and, therefore, oxygenation to the hypoxic or ischemic myocardium. Although extensively investigated, the mechanisms have not been fully elucidated. It has been reported that the gasotransmitter hydrogen sulfide (H_2S), a novel signalling molecule found throughout the cardiovascular system as multiple forms (H_2S, HS^-, S^{2-}) and polysulfides, may act as an oxygen sensor/transducer of the hypoxic response in the aorta and pulmonary arteries, with different enzymes involved in the synthesis of H_2S during hypoxia amongst these tissues/species [1-3]. Little is known, however, about the role of H_2S in the hypoxic response of the coronary artery.

H_2S is synthesised endogenously principally from L-cysteine by the enzymes cystathionine-ß-synthase (CBS) and cystathionine-γ-lyase (CSE), and by the sequential actions of cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (MPST) [1,4]. CSE and CBS are found in many tissues, with the consensus being that CSE is primarily located within the vasculature and CBS in the brain [5]. More recent evidence has shown that CBS and 3-MPST are also present in vascular endothelium and smooth muscle [6-8]. In the vasculature, H_2S may cause vasoconstriction and/or vasodilation depending on factors including concentration and vessel origin, and the responses are oxygen-dependent [3,9,10]. Mice genetically devoid of CBS show a loss of hypoxia-induced vasodilation in cerebral microvessels illustrating the importance of this enzyme in the brain [11], and have elevated plasma homocysteine levels, which has been associated with the development of cardiovascular disease [12]. The effect of genetic deletion of CSE has been shown in some studies to produce lower H_2S levels, hypertension and to reduce endothelium-dependent vasorelaxation [13] but in others the CSE knockout mice were normotensive [14].

Studies of the mechanism of coronary hypoxic relaxation have shown an involvement of ATP-sensitive potassium (K_{ATP}) channels [15] and K_v7 channels [16]. Exogenous H_2S-mediated coronary artery relaxations have also been shown to involve K_{ATP}, K_v7 and BK_{Ca} channels [16-18]. However, the role of endogenous H_2S in coronary hypoxic vasorelaxation has not been systematically investigated and which, if any, of the H_2S synthesising enzymes is involved is unclear. There is a precedent for an important protective role of endogenous H_2S in the ischemic heart, as an antioxidant and in affording protection against reperfusion injury [19-22]; if H_2S is also identified as a hypoxic coronary vasodilator, this would increase its therapeutic potential in the treatment of heart disease. If treatments leading to upregulation of endogenous H_2S are to be developed in order to offer protection to the heart from hypoxia (thereby preventing or reducing the symptoms of angina and heart failure), it is fundamental that the enzyme(s) involved in the enzymatic synthesis of H_2S are identified. Accordingly, the aims of this study were to identify and characterise the role of

endogenous H_2S in the coronary arterial response to hypoxia, and the H_2S synthetic enzymes involved.

2. Methods

2.1 Tissue preparation

All experimental procedures were approved by the Animal Care and Use Committee of University of Nottingham, United Kingdom. Pig hearts (either sex, age < 6 months, ~50 kg) were obtained on ice from a local abattoir (G Wood & Sons Ltd, Mansfield, UK). The left circumflex coronary artery was dissected and stored overnight at 4 °C in gassed (95% O_2, 5% CO_2) Krebs–Henseleit solution, as previously described [23]. The coronary artery was cut into rings of ~5 mm which were suspended in organ baths in gassed (95% O_2, 5% CO_2) Krebs–Henseleit solution. The endothelium of some arteries was removed by gentle rubbing [23]; successful removal was tested using substance P (10 nM) (control 75 ± 11%; endothelium removal 3 ± 2% (n = 8)).

2.2 Responses in the porcine isolated coronary artery

Arterial rings were mounted onto wires in 20 ml tissue baths and tensioned to 10 g. Each ring was attached to an isometric force transducer connected to a Powerlab with tension tension changes captured using LabChart software (ADInstruments, Sydney, Australia). Tissue viability was assessed using 60 mM KCl (2-3 additions). The thromboxane A_2 analogue U46619 (11α,9α-epoxymethano-PGH_2) was added to contract the vessels to ~50-60% (57 ± 1%, n = 144) of the final KCl response (6 ± 0.2 nM U46619, n = 144; arteries in the presence of glibenclamide required a 10 x higher concentration, 55 ± 6 nM U46619, n = 24)

2.3 Experimental protocols

U46619 contracted vessels were exposed to hypoxia (95% N_2/ 5% CO_2) for 30 min. In separate experiments, concentration response curves were constructed to H_2S donors (Na_2S and NaHS). The effects of the CSE inhibitor DL-propargylglycine (PPG), CBS inhibitor amino-oxyacetate (AOAA), MPST pathway inhibition (aspartate; inhibits CAT activity), nitric oxide synthase inhibitor (L-NAME), endothelium removal, soluble guanylyl cyclase inhibitor (ODQ), adenylyl cyclase inhibitor (SQ22536), Cl^-/HCO_3^- exchange inhibitor (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid, DIDS), high K^+ (50 mM), non-selective K^+ channel blocker (tetraethylammonium, TEA) and inhibitors of K_{IR} ($BaCl_2$), K_{Ca} (charybdotoxin, intermediate and large conductance Ca^{2+}-activated K channel inhibitor; TRAM-34, intermediate conductance Ca^{2+}-activated K channel inhibitor; apamin, small conductance Ca^{2+}-activated K channel inhibitor), K_V (4-AP) and K_{ATP} (glibenclamide) channels were examined by application prior to the addition of U46619, which ensured a minimal contact time of 1h prior to induction of hypoxia or H_2S application. For each of the different experimental conditions separate control experiments were carried out on vessel segments from the same coronary arteries.

2.4 Cultured porcine coronary artery smooth muscle cells

Porcine coronary arteries were dissected and stored overnight at 4 °C in gassed (95% O_2, 5% CO_2) Krebs–Henseleit solution containing 1X antibiotic antimycotic solution (100 units penicillin, 0.10 mg streptomycin and 0.25 μg amphotericin B per ml) (Antibiotic Antimycotic Solution (100×), A5955, Sigma-Aldrich, Dorset, UK). The next day, PCAs were finely dissected and cut open longitudinally. The vessels were then washed 3x with 5 ml DMEM (D6429, Sigma-Aldrich, Dorset, UK) containing 1X antibiotic antimycotic solution in a 30 ml universal tube. To isolate smooth muscle cells, each vessel was incubated at 37 °C for 40 min in a 1.5 ml Eppendorf containing 1 ml DMEM with type II collagenase (2mg/mL, C6885, Sigma-Aldrich, Dorset, UK) [24]. Each vessel was then washed twice with 1 ml DMEM, 1X antibiotic antimycotic solution. In the third wash, to isolate the smooth muscle cells, the mixture was vortex and pipetted onto a (60 mm Costar) Petri dish and 5 ml of DMEM containing 1X antibiotic antimycotic solution, L-glutamate (59202C, Sigma-Aldrich, Dorset, UK) and FBS (F6178, Sigma-Aldrich, Dorset, UK) were added. At day 6, cells were split onto cover slips for immunocytochemistry and onto 24-well plates for immunoblot studies.

2.5 Immunohistochemistry

Tissue sections were fresh frozen in OCT using liquid nitrogen cooled isopentane. Sections were cut using a cryostat (Leica CM1900) and applied to APES slides (20 μm). Staining was achieved using a VECTASTAIN Elite ABC Kit Cat no. PK6100 Series (Vector Laboratories Inc., US). Briefly sections were fixed (acetone, 4^{o}C) and quenched for endogenous peroxidase (0.3% H_2O_2 0.3% normal serum in PBS). Tissue was then incubated with 2% normal blocking serum followed by incubation with primary antibody (1 μl per 500ml MPST, 1.5 μl CBS/CSE in 500 ml PBS with 1 % BSA) and left at 4^{o}C overnight. The tissue was then incubated with the biotinylated secondary antibody at room temperature. A further peroxidase quenching step was performed followed by incubation with VECTASTAIN® *Elite*® ABC reagent. The tissue was then incubated with DAB for ~2 min, rinsed and mounted (95 % glycerol/5 % distilled water). Sections were imaged using a light microscope (Leica DM4000 B LED).

2.6 Immunocytochemistry for the expression of CBS enzyme in cultured porcine coronary artery smooth muscle cells

Immunocytochemistry was carried out once the primary culture was confluent after ~3 days. The SMCs were washed with ice cold PBS and then fixed with 4% PFA at RT for 30 min. The SMCs were then permeabilised using 0.15% Triton (T9284, Sigma-Aldrich, Poole, UK) at RT for a further 30 min followed by blocking with 5% milk in PBS for 1 h. The SMCs were incubated in a humidifier at 4°C overnight with primary mouse monoclonal anti-actin α-smooth muscle antibody (A5228, Sigma-Aldrich, Dorset, UK) (1:200) and rabbit anti-CBS (1:200) (H00000875-D01P). The SMCs were washed 3x with PBS and incubated with TRITC goat anti-rabbit secondary

antibody (1:200) (A16101, Thermo Fisher Scientific, Waltham, MA U.S.A) and goat anti-rabbit FITC conjugated secondary antibody (1:200) (AP307F, Sigma-Aldrich, Dorset, UK) at RT for 45 min. The SMCs were then washed once with PBS then nuclear stain with DAPI (1µg/ml) for 5 min and then washed 3x with PBS followed by distilled water once. The SMCs were imaged using Leica DMRB fluorescent microscope.

2.7 Immunoblotting

Coronary arteries were dissected and frozen on dry ice. Segments were then homogenized in lysis buffer [20 mM Tris, 1 mM EGTA, 320 mM sucrose, 0.1% (v/v) Triton X-100, 1 mM NaF, 10 mM β-glycerophosphate, pH 7.6], containing protease inhibitor cocktail tablets, EDTA free. The protein concentration in each sample was assessed using the Bradford method and normalised. Samples, with 6× solubilization buffer [24% (w/v) SDS, 30% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.5% (v/v) bromophenol blue, 1.5 M Tris–HCl, pH 6.8], were heated at 95 °C for 5 min. Subsequently, electrophoresis was carried out on 4–20% Tris-glycine PAGE Gold Precast Gels (Bio-Rad, Hercules, CA, USA), with approximately 5 µg protein per lane. Samples were transferred to nitrocellulose membranes, which were incubated in blocking solution [6% fish skin gelatin in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T)] for 1h at room temperature. Blots were incubated with primary antibodies: rabbit anti-CSE (1:1000) (ab80643) rabbit anti-CBS (1:1000) (H00000875-D01P) rabbit anti-MPST (1:1000) (HPA001240) and mouse monoclonal anti-β-actin (1:40,000) (A2228) diluted in blocking solution at 4°C overnight. After washing in TBS-T, the blots were incubated with an appropriate IRDye®-conjugated secondary antibody (Li-Cor Biosciences, Biotechnology, Lincoln, NE, USA) at 37°C for 1h. Proteins were visualized and quantified using the Li-Cor/Odyssey infrared imaging system.

2.8 Endogenous production of H_2S

Segments of porcine coronary artery were homogenized in ice-cold Tris-EDTA buffer (50 mM, 1 mM, pH 7.4) and centrifuged at 4°C at 1000× g for 20 min. The supernatant was removed and centrifuged at 30 000× g at 4°C for 60 min. The supernatant layer was removed and assessed for H_2S production using the methylene blue method [25]. Briefly samples were incubated with 1 mM pyridoxal phosphate in the absence and presence of 10 µM PPG or AOAA for 15 min at 37°C in a shaking water bath. 10 mM L-cysteine was added and the samples were incubated for 90 min. As a control, blank samples were prepared using Tris-EDTA buffer containing pyridoxal phosphate and L-cysteine, but no tissue (tissue blanks); and tissue samples containing pyridoxal phosphate, but no L-cysteine (substrate blanks). Reactions were stopped by the addition of 0.5 M NaOH followed by 4% (w/v) zinc acetate in distilled water and kept on ice for 30 min. A colorimetric change was achieved by addition of 0.1% (w/v) dimethylphenylene-diamine sulphate in 5 M HCl and 50 mM ferric chloride. Samples were mixed and then centrifuged at 5000× g to clear the precipitated protein. After 20 min, samples were analysed at 670 nm.

MPST activity was examined by measuring H_2S production from 3-mercaptopyruvate [25]. Porcine coronary artery homogenates were prepared as described above. In duplicate, supernatant samples were incubated with 0.3 mM 3-mercaptopyruvate and 0.5 mM dithiothreitol in Tris buffer (0.1 M, pH 9.0) in a total volume of 150 µL in a shaking water bath at 37°C for 30 min. Reactions were stopped and H_2S production were estimated using the methylene blue method as described above.

2.9 Data analysis

Data derived from the use of H_2S salts (Na_2S, NaHS) were expressed as log concentration–response plots with contractions/relaxations expressed as a percentage of the U46619 response. Hypoxic responses were measured in grams (g) and area under the curve was also measured. Responses to hypoxia, pinacidil and substance P were expressed as a percentage of the U46619 response. Data shown are mean ± SEM and were assessed for statistical significance via two-way ANOVA with Bonferroni post-test, Student's *t*-test or one-way ANOVA with Tukey's post-test (Prism; GraphPad, San Diego, CA, USA). Differences were considered as significant when $P<0.05$, where n is the number of animals.

2.10 Materials

Krebs–Henseleit buffer (mM): NaCl 118, KCl 4.8, $CaCl_2 \cdot H_2O$ 1.3, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4 \cdot 1.2$ and glucose 11.1 (Fisher, Loughborough). U46619 (Tocris Bioscience, Bristol) was dissolved in ethanol at 10 mM, then diluted in distilled water. Stock solutions of PPG, AOAA, aspartic acid, L-NAME, 4-AP, $BaCl_2$, TEA (Sigma-Aldrich, Poole Dorset) and substance P and ChTX (Tocris Bioscience, Bristol UK) were prepared in distilled water. Stock solutions of ODQ, SQ22536, apamin, glibenclamide, TRAM-34 and DIDS were prepared in DMSO. NaHS and Na_2S were made immediately before application and kept on ice. Pinacidil (Sigma-Aldrich) was dissolved in DMSO as a 10 mM stock concentration, and then further dilutions made in distilled water. Primary antibodies for immunohistochemistry and Western blotting were from: CSE (ab80643; rabbit polyclonal) Abcam® (Cambridge, UK); CBS (H00000875-D01P; rabbit polyclonal) Abnova (Taipei, Taiwan); MPST (HPA001240; rabbit polyclonal) and β-actin (mouse monoclonal) Sigma-Aldrich (Poole, Dorset, UK).

3. Results

3.1 Hypoxia produces a biphasic vasomotor response which is mimicked by exogenous hydrogen sulfide in the coronary artery

In the porcine isolated coronary artery, hypoxia (95% N_2, 5% CO_2; measured oxygen tension of 30 µM) caused a biphasic response (Figure 1A) consisting of an initial transient contraction, followed by a prolonged and pronounced vasorelaxation. A biphasic response (Figure 1B,C), contraction at 1-100 µM and relaxation at 300-1000 µM, was also seen upon application of the H_2S salts; Na_2S and NaHS.

3.2 Three hydrogen sulfide synthesising enzymes, CSE, CBS and MPST are expressed in the coronary artery

Using antibodies we have previously characterized for cross-reactivity with porcine enzymes [25], immunoblot analysis showed that all three enzymes CBS (63 kDa), CSE (44 kDa) and MPST (33 kDa) are present within the porcine coronary artery (using actin (45 kDa) as a control to normalise for protein level variation) (n=4) (Figure 2A). Full blots are given in Online resource 1. Immunoblot analysis also showed the presence of CBS in cultured porcine and human coronary artery smooth muscle cells but the expression of CBS enzymes in cultured tissue was much reduced (only 12-15% compared to intact porcine coronary arteries) (Online resource 2).

Immunohistochemistry (Figure 2B) showed that CBS, CSE and MPST are located within the coronary artery. CBS and CSE staining appeared dispersed throughout the smooth muscle and endothelium, however MPST staining was more punctate, appearing at the base of the endothelium and within discrete regions of the smooth muscle. Immunocytochemical staining of cultured porcine coronary artery smooth muscle cells showed cytoplasmic colocalisation of CBS and α-actin (Figure 2C).

3.3 The contractile phase of the response to both hypoxia and exogenous hydrogen sulfide involves endothelial nitric oxide

The hypoxic contraction was significantly inhibited following endothelium removal, in the presence of the NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM), and in the presence of a soluble guanylyl cyclase inhibitor [1H-[1,2,4]oxadiazol[4,3,a]quinoxaline-1-one] (ODQ, 10 μM) (Figure 3A,C,E, Table 1), in agreement with previous studies [15]. Similarly, the contractile response to the H_2S salts, Na_2S and NaHS (1-100 μM) was abolished in endothelium denuded vessels and in the presence of L-NAME or ODQ (Figure 4A-F).

3.4 The relaxation phase of the response to both hypoxia and exogenous hydrogen sulfide is endothelium-independent, and involves K^+ channels and Cl^-/HCO_3^- exchange

3.4.1 Involvement of NO

The hypoxic relaxation was unaltered in endothelium-denuded vessels and in the presence of ODQ (Figure 3B,F, Table 1). However in the presence of L-NAME (100 μM), the contractile phase was absent with a shorter latency of the relaxation phase which was significantly increased, indicative of functional antagonism (Figure 3C, 3D, Table 1). Thus, when the contractile phase of the response is inhibited, the relaxation phase is augmented suggesting that they are at least partly coincident. Relaxatory effects elicited by the H_2S salt Na_2S were also increased by pharmacological interference with NO signalling (L-NAME and ODQ), and by removal of the endothelium, also indicative of functional antagonism (Figure 4A-F, Table 2). Relaxatory effects of NaHS were also increased by ODQ, but not by L-NAME or endothelium removal (Table 2).

3.4.2 Involvement of potassium channels

Pretreatment with 50 mM KCl significantly reduced both the hypoxia-induced contraction and relaxation (Table 1). However, the non-selective K^+ channel blocker tetraethylammonium (TEA) had no effect on the response to hypoxia at 1 mM TEA, but caused a significant increase in the contractile phase when used at 10 mM (Table 1). Similarly, a combination of K^+ channel blockers; glibenclamide (10 µM), charybdotoxin (ChTX 10 nM), apamin (100 nM), 4-aminopyridine (4-AP 1 mM) and $BaCl_2$ (30 µM), caused a significant increase in the hypoxia-induced contraction, but had no effect on relaxation (Table 1). Subtype selective K^+ channel inhibitors for K_{IR} ($BaCl_2$) and K_{Ca} (ChTX and apamin) had no effect on the overall hypoxic response (Table 1). An inhibitor of K_V (4-AP) caused a significant increase in the contractile response. The K_{ATP} channel inhibitor glibenclamide significantly increased the maximum relaxation induced by hypoxia (Table 1).

Relaxation responses to Na_2S and NaHS were also significantly attenuated in the presence of 50 mM KCl (Table 2). TEA (1 mM) had no effect on the concentration response curves to Na_2S and NaHS. TEA (10 mM) abolished the contractile responses to Na_2S and NaHS (data not shown) and shifted the curves to the left, producing relaxation at lower concentrations (1-100 µM) with the maximum relaxation to Na_2S, but not NaHS, being increased compared to the control (Table 2). In the presence of a combination of K^+ channel blockers, there was no effect on the concentration response curves of Na_2S and, while the effect on the response to NaHS was significant overall, with the curve shifting to the left, no individual concentrations were significantly altered (Table 2). Blockade of K_{IR} ($BaCl_2$) had no effect on relaxation responses to NaHS, however the relaxation response to Na_2S was blunted and the maximum response was significantly reduced (Table 2). K_{Ca} (TRAM-34 10 µM and apamin 100 nM), K_V (4-AP) and K_{ATP} (glibenclamide) channel inhibitors had no significant effect on the relaxation response to Na_2S or NaHS (Table 2).

3.4.3 Involvement of chloride channels

A Cl^-/HCO_3^- exchange inhibitor DIDS (4,4-diisothiocyanatostilbene-2,2'-disulfonic acid, 100 µM) significantly reduced the relaxation phase of the hypoxic response (Table 1). In addition, DIDS significantly reduced the relaxations induced by Na_2S and NaHS (Table 2).

3.4.4 Involvement of adenylyl cyclase

An adenylyl cyclase inhibitor, SQ 22536 (10 µM), had no significant effect on the hypoxic response (Table 1). SQ 22536 caused a significant leftward shift in the response to Na_2S; however, the contractile response at 30 µM Na_2S (data not shown) and relaxant response at 1 mM Na_2S (Table 2) were not significantly different. SQ 22536 caused no alteration in the vascular responses to NaHS (Table 2).

3.5 Hydrogen sulfide production from CBS is involved in the coronary artery hypoxic response and dominates over CSE and MPST

The CSE inhibitor, DL-propargylgylcine (PPG 10 µM, 30 µM, 100 µM, 1 mM) and the MPST pathway inhibitor, aspartate (1 mM), alone or in combination had no effect

on the vascular response to hypoxia (Figure 5F,G,H). The CBS inhibitor amino-oxyacetate (AOAA) caused a significant reduction in the contractile phase at 100 µM (control 26 ± 4%, +AOAA 16 ± 4%, P<0.03, Figure 5A, B), and significantly reduced the relaxation phase of the hypoxic response at higher concentrations (control 62 ± 5%, +300 µM AOAA 46 ± 7%, P=0.01; control 60 ± 4%, +1 mM AOAA 34 ± 5%, P=0.008). Further experiments in endothelium-denuded vessels demonstrated that 300 µM AOAA had no effect on the contractile phase (Figure 5D) but significantly reduced the relaxation phase of the hypoxic response (P=0.01) from 56.6 ± 4.0% under control conditions to 28.6 ± 4.9% in the presence of AOAA (n=8) (Figure 5E).

Combining PPG (10 µM) and AOAA (100 µM) caused a significant reduction in both the contraction (control 12 ± 2%, PPG/AOAA 6 ± 1%; P=0.0213) and the relaxation phase (control 62 ± 5%, PPG/AOAA 46 ± 6%; P=0.0210) of the hypoxic response (Figure 6A). Combinations of higher concentrations of PPG and AOAA (30 µM PPG/ 300 µM AOAA and 100 µM PPG/ 1mM AOAA, data not shown) also significantly reduced both phases of the hypoxic response. A combination of aspartate (1 mM) and AOAA (100 µM) caused a significant reduction in the hypoxia-induced relaxation (control 76 ± 6%, Aspartate/AOAA 46 ± 6%, P=0.0013) (Figure 6B). Combining PPG (10 µM), AOAA (100 µM) and aspartate (1 mM) significantly reduced both the contractile (control 14 ± 2%, combination 7 ± 2%, P=0.0105) and relaxation (control 60 ± 5%, combination 42 ± 5%, P=0.0057) phases of the hypoxic response (Figure 6C). Calculation of area under the curve (AUC) similarly showed that 100 µM AOAA had no significant effect on the AUC of the relaxation response (Figure 6D), however the relaxation phase was significantly reduced when AOAA was combined with PPG (Figure 6E), aspartate (Figure 6F), or both PPG and aspartate (Figure 6G).

We sought to investigate the selectivity of the enzyme inhibitors using pinacidil (potassium channel opener), given the evidence for an involvement of potassium channels in vasorelaxation to both hypoxia and H_2S shown in the present study and by others [15,16,18]. PPG, AOAA and aspartate, alone or in combination, had no significant effect on vasorelaxation to pinacidil in the porcine coronary artery (see Online resource 3).

3.6 Endogenous production of hydrogen sulfide through CBS and MPST, but not CSE

Endogenous production of H_2S was detected in porcine coronary arteries using the methylene blue method (Figure 7). Generation of H_2S from these coronary samples in solutions containing L-cysteine and pyridoxal phosphate was effectively abolished in the presence of AOAA (10 µM), but not PPG (10 µM) (Figure 7A). No colorimetric changes were measured in blank samples (in the absence of tissue or L-cysteine). H_2S production involving MPST activity was also measured using 3-mercaptopyruvate as a substrate (Figure 7B). L-cysteine produced concentration-dependent relaxation in coronary artery segments which was blocked by AOAA (100 µM) but not by PPG (10 µM), consistent with an involvement of CBS in the synthesis of endogenous H_2S (Online resource 4).

4. Discussion

The main findings of this study are that H_2S is an endogenous mediator of the hypoxic response in porcine coronary arteries and the most important enzyme responsible for H_2S generation during the hypoxic response in the coronary artery appears to be CBS, expressed in the vascular smooth muscle. Thus, we have provided evidence of a patho/physiological setting within the cardiovascular system where CSE is *not* the principle H_2S-synthesizing enzyme. There is some contribution to the hypoxic response from H_2S produced by the other H_2S-producing enzymes, CSE and MPST, which were also expressed in the coronary artery, but the contribution of these enzymes only becomes apparent following CBS blockade. A summary of the main mechanisms is shown in Figure 8.

CBS is the most important H_2S synthesizing enzyme involved in the coronary artery hypoxic response

A combination of inhibitors of H_2S synthesis attenuated coronary hypoxic vasorelaxation, consistent with a role of H_2S in the hypoxic response, as reported in preliminary studies by us and others [16,26]. When the enzyme inhibitors were investigated individually, AOAA (CBS inhibitor) alone attenuated the hypoxic response, but PPG (CSE inhibitor) alone and aspartate (which inhibits CAT and therefore indirectly MPST activity) alone were without effect. Thus, CBS appears to be the most important enzyme involved in hypoxic generation of H_2S in the porcine coronary artery. AOAA was also reported to reduce hypoxic constriction of bovine pulmonary arteries and hagfish aorta, but PPG was ineffective, similarly suggesting a predominant involvement of CBS [2,3]. In contrast, hypoxic relaxation of the rat thoracic aorta was essentially abolished by PPG [2] pointing to a predominant involvement of CSE in this tissue. Our data are in line with those of Kuo et al. who reported a minor role of CSE of H_2S generation in mouse coronary artery; they did not investigate a possible involvement of CBS (or hypoxia) and concluded that MPST was most important [27]. These data identify fundamental differences between tissues and/or species with regard to which enzymes are involved in the hypoxic synthesis of H_2S.

Interestingly, we found that combinations of the inhibitors that included AOAA (namely AOAA plus PPG; AOAA plus aspartate; and AOAA plus PPG and aspartate), produced a larger (versus AOAA alone) inhibitory effect. Thus, CBS may compensate for inhibition of CSE and/or MPST. Reciprocal compensatory effects of CBS and CSE in the regulation of renal functions and urinary haemodynamics have similarly been observed in kidney [28]. AOAA has been shown to have limited selectivity for CSE [29,30]; however, the response seen here is unlikely to involve CSE as the relatively selective CSE inhibitor, PPG, had no effect alone on the hypoxic response at concentrations up to 1 mM. None of the inhibitors, or combinations of inhibitors, affected pinacidil induced relaxations of the coronary artery, consistent with them having selective inhibitory actions at the H_2S synthesizing enzymes. In line with these functional data, measurement of H_2S formation showed that AOAA, but not PPG, blocked the pyridoxal phosphate-dependent generation of H_2S from the

porcine coronary artery, indicating a direct effect on the H_2S synthetic pathway, consistent with an involvement of CBS. In aortic and pulmonary vessel homogenates, production of H_2S was similarly attenuated by AOAA, but not by PPG [2]. It should be noted, however, that AOAA can inhibit pyridoxal phosphate-dependent enzymes in addition to CBS [31], and can also inhibit mitochondrial malate-aspartate shuttles leading to an increase in the cytoplasmic NADH/NAD ratio and redox state [32], which may contribute to its effects. The development and use of specific CBS inhibitors is needed to determine its precise contribution to the coronary artery hypoxic response.

CBS is expressed predominantly in the coronary artery smooth muscle

We carried out experiments in endothelium-denuded coronary artery segments to investigate which cells (endothelial or smooth muscle) are involved in the CBS-dependent hypoxic production of H_2S. Endothelium removal had no effect on the hypoxic relaxation response, and in artery segments without endothelium, the hypoxic relaxation response was attenuated by AOAA. These data point to CBS within smooth muscle as the most significant contributor of H_2S to hypoxic relaxation of the porcine coronary artery. Our antibody-based studies in cultured cells confirmed the expression of CBS in porcine coronary artery smooth muscle cells, and we also observed expression of CBS in cultured human coronary artery smooth muscle cells by the same method (Online resource 2). Our data show for the first time the expression of CBS in the coronary vasculature, and identify a role for vascular smooth muscle CBS in the coronary arterial response to hypoxia. In contrast, the hypoxic contraction was greatly attenuated by endothelium removal and the small residual contraction in the endothelium-denuded segments was not significantly affected by AOAA. Thus, with regard to the hypoxic contraction, the response is endothelium-dependent (it involves NO, see below) and involves CBS-dependent production of H_2S by the endothelium and/or smooth muscle.

H_2S as an oxygen sensor and mediator of the arterial hypoxic response

In line with a role for endogenous H_2S as a mediator of the hypoxic response, we found that exogenous H_2S (Na_2S and NaHS) produced a response which mimicked the response to hypoxia. The response to hypoxia was biphasic, consisting of a transient contraction followed by relaxation, reflecting changes in the local concentrations of endogenous metabolites and their actions due to changes in their generation/accumulation with hypoxia over time. The H_2S salts, Na_2S and NaHS, similarly produced a biphasic response; vessel contraction at low concentrations followed by relaxation at higher concentrations. A number of vasoactive mediators have been suggested to be involved in the hypoxic response, and it is likely that it is multifactorial. However, to the best of our knowledge, only H_2S produces a vasomotor response that matches so precisely the biphasic profile of the hypoxic response. Adenosine, for example, produces only vasodilatation in the coronary artery; other mediators (e.g. purine nucleotides) may elicit a biphasic response, but these typically cause vasodilatation at low concentrations and vasoconstriction at high concentrations. Moreover, the same mechanisms were involved in the biphasic responses to both the

H_2S donors and hypoxia, namely involvement of endothelial NO, K^+ channels and Cl^- /$HOCl_3^-$ exchange (discussed below).

Our finding that H_2S is an important endogenous mediator during a hypoxic insult is consistent with evidence that H_2S acts as an oxygen sensor and transducer of the hypoxic response in rat aorta, lamprey and hagfish dorsal aorta, rat and bovine pulmonary arteries [2,3] and, importantly, extend these findings to the coronary vasculature. Olson and colleagues showed in lung tissue that H_2S concentration and pO_2 are inversely related; H_2S was produced at low pO_2 but was reduced as pO_2 was increased [7]. It was proposed that under normoxic (normal pO_2) conditions, H_2S production is tightly regulated and rapidly quenched via oxidation, however in hypoxic conditions, where pO_2 is low, oxidation and therefore conversion of H_2S is reduced [7,33]. A reduction in H_2S metabolism will increase H_2S availability enabling it to produce its cardioprotective effects. In addition to the loss of metabolism of H_2S, it has been suggested that CBS and CSE activities are altered depending on the redox environment [34,35] implying that their activity may also be governed by the availability of oxygen. The time course of the current study, 30 min exposure to hypoxia, was not sufficiently long to induce changes in expression levels of the H_2S producing enzymes [36], but the activity of existing endogenous enzymes may well be altered within this time frame.

Mechanisms involved in the coronary artery hypoxic response to H_2S

Analysis of the mechanisms involved in the vascular responses to hypoxia and exogenous H_2S produced information that was broadly consistent with a role of endogenous H_2S in the hypoxic response. Under standard oxygen conditions, constitutive production of NO by the endothelium contributes vasodilator tone to the arteries via activation of soluble guanylyl cyclase and production of cGMP. The contractile responses to hypoxia and the H_2S salts, Na_2S and NaHS, were endothelium-dependent and were blocked by L-NAME and ODQ, a soluble guanylyl cyclase inhibitor, indicating that they are mediated by hypoxia-induced suppression of NO synthesis [15] and NO and/or H_2S signalling via cGMP, but not cAMP (SQ22536 was without effect). Since the hypoxic contraction was also attenuated by inhibitors of H_2S synthesis, this suggests that H_2S is produced during hypoxia in the porcine coronary artery, and may combine with, and so inactivate, NO (through H_2S-nitrosothiol formation [37] leading to contraction, as suggested for H_2S-induced contractions in rat aorta [38]. However, this hypothesis is complicated by evidence from a number of studies showing opposing interactions between NO and H_2S, as well as additional interactions involving carbon monoxide (CO) [5,39-41], and it is likely that there is tight regulation, synergisms and antagonisms, between these so-called "gasotransmitters" [5].

H_2S-induced vasodilation involves a number of proposed mechanisms including the opening of K_{ATP} channels [9,42,43]. In the porcine coronary artery, in the presence of high potassium the vascular responses to hypoxia and the responses to Na_2S and NaHS were abolished suggesting the involvement of potassium channels and a hyperpolarising mechanism. Glibenclamide did not attenuate the hypoxic response;

the hypoxic contraction was unaltered and the relaxation was greater in the presence of glibenclamide. Glibenclamide also did not attenuate the relaxation responses to Na_2S and NaHS. Glibenclamide at 5 and 30 μM was shown to be an effective inhibitor of vasorelaxation mediated by the K_{ATP} channel opener levcromakalim in the porcine coronary artery [44,45], although depending on the concentration and conditions it can also inhibit other ion channels including Kv and L-type Ca^{2+} channels [46-48]. Our data suggest a lack of involvement of K_{ATP} channels in coronary artery hypoxia/H_2S-induced vasorelaxant responses. Our study also shows that K_{IR} and K_{Ca} are not involved in the hypoxic response, since $BaCl_2$, TRAM-34/ChTX and apamin were without effect. A combination of potassium channel blockers, TEA and 4-AP (K_V channel blocker), had no effect on the relaxation component of the hypoxic response, but significantly increased the hypoxic contractile component, suggesting that K_V channels may be involved early in the hypoxic response. A recent study reported the specific involvement of K_V7 channels in hypoxic coronary artery relaxation [16], and it is likely that these and other potassium channels are involved. The relaxations induced by hypoxia and by Na_2S and NaHS, were significantly reduced in the presence of DIDS, suggesting that Cl^-/HCO_3^- exchange channels are also involved in both the hypoxic response and the response to H_2S donors, again consistent with a role of H_2S in hypoxic coronary vasodilatation and a hyperpolarising effect. NaHS relaxations in rat and mouse aorta were also blocked by DIDS [49-51]. The mechanism may involve H_2S-mediated intracellular acidification involving activation of the Cl^-/HCO_3^- exchanger leading to relaxation [51]. Differences observed in the susceptibilities of Na_2S and NaHS to blockade by some of the inhibitors may be attributable to their different purities [52]; it is appropriate that the pharmacological profile of the more pure of the two compounds, Na_2S [52], matched most closely with that of the hypoxic response.

Limitations

Although the collective evidence (immunohistochemistry, immunocytochemistry, immunoblotting, biochemistry, pharmacology) points to an involvement of H_2S generated from CBS in the mechanism underlying hypoxic relaxation of the coronary artery, further studies are warranted. We were unable to use siRNA successfully in small arteries, possibly because of the difficulty in delivering the siRNA into multi-layered vessels as well as the lower turnover rate in arteries compared to cells in culture. Although we were able to show the presence of CBS immunoreactivity in both porcine and human cultured coronary artery smooth muscle cells, the absence of a relevant acute hypoxic response in these cells meant that experiments using siRNA could not be carried out in the cultured cells. Regardless, this is the first (to the best of our knowledge) demonstration of the expression of CBS in coronary artery smooth muscle of pigs and humans, and evidence of a role of H_2S generated from CBS in hypoxia in pig coronary arteries is presented. Future studies involving inhibitors more selective for CBS are required to further clarify the precise role of CBS in the coronary artery hypoxic response.

Conclusions

These findings are the first to show that CBS, expressed in the vascular smooth muscle, is the most important H_2S-synthesisizing enzyme involved in hypoxic relaxation of the coronary artery, and that this enzyme can compensate when there is inhibition of CSE and MPST. This is important as it challenges the widely held belief that CSE is the principal enzyme involved in production of H_2S in the cardiovascular system, a conclusion which may derive from the focus on rats as experimental models. While pig and human hearts are anatomically very similar [53], it will be important to determine whether this is also the case in human. These results point to CBS as the principal target for future treatments designed to upregulate endogenous H_2S levels, thus protecting the heart from hypoxia and preventing or reducing the symptoms of angina and heart failure.

Acknowledgements

This project was funded by the British Heart Foundation (PG/11/45/28975).

Acknowledgements

Jagdish Heer and Kathryn Ellis are thanked for their tremendous technical support.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

Experiments were performed at the School of Life Sciences, Queen's Medical Centre, University of Nottingham NG7 2UH. VR, SPHA, WRD and MJG designed the research study. JD and PSW performed the research and analysed the data together with MJG, SPHA, VR and WRD. RR assisted with the immunoblotting, immunohistochemistry and biochemistry experiments. JD and VR wrote the initial draft. All the authors read, modified and approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

References

[1] Olson KR (2015) Hydrogen sulfide as an oxygen sensor. Antioxid Redox Signal 22:377-397. doi:org/10.1089/ars.2014.5930

[2] Olson KR, Dombkowski RA, Russell MJ, Doellman MM, Head SK, Whitfield NL, Madden JA (2006) Hydrogen sulfide as an oxygen sensor/transducer in vertebrate hypoxic vasoconstriction and hypoxic vasodilation. J Exp Biol 209:4011-4023. doi:org/10.1242/jeb.02480

[3] Olson KR, Forgan LG, Dombkowski RA, Forster ME (2008) Oxygen dependency of hydrogen sulfide-mediated vasoconstriction in cyclostome aortas. J Exp Biol 211:2205-2213. doi:org/10.1242/jeb.016766

[4] Shibuya N, Koike S, Tanaka M, Ishigami-Yuasa M, Kimura Y, Ogasawara Y, Fukui K, Nagahara N, Kimura H (2013) A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells. Nat Commun 4. doi:org/10.1038/ncomms2371

[5] Li L, Moore PK (2007) An overview of the biological significance of endogenous gases: new roles for old molecules. Biochem Soc Trans 35:1138-1141. doi:org/10.1042/BST0351138

[6] Baragatti B, Ciofini E, Sodini D, Luin S, Scebba F, Coceani F (2013) Hydrogen sulphide in the mouse ductus arteriosus: a naturally occurring relaxant with potential EDHF function. Am J Physiol 304:H927-H934. doi:org/10.1152/ajpheart.00718.2012

[7] Olson KR, Whitfield NL, Bearden SE, St Leger J, Nilson E, Gao Y, Madden JA (2010) Hypoxic pulmonary vasodilation: a paradigm shift with a hydrogen sulfide mechanism. Am J Physiol Regul Integr Comp Physiol 298:R51-60. doi:org/10.1152/ajpregu.00576.2009

[8] Shibuya N, Mikami Y, Kimura Y, Nagahara N, Kimura H (2009) Vascular endothelium expresses 3-mercaptopyruvate sulfurtransferase and produces hydrogen sulfide. J Biochem 146**:**623-626. doi:org/10.1093/jb/mvp111

[9] Dunn WR, Alexander SPH, Ralevic V, Roberts R (2016) Effects of hydrogen sulfide in smooth muscle. Pharmacol Ther 158:101-113. doi:10.1016/j.pharmthera.2015.12.007

[10] Koenitzer JR, Isbell TS, Patel HD, Benavides GA, Dickinson DA, Patel RP, Darley-Usmar VN, Lancaster JR, Doeller JE, Kraus DW (2007) Hydrogen sulfide mediates vasoactivity in an O2-dependent manner. Am J Physiol 292:H1953-H1960. doi:org/10.1152/ajpheart.01193.2006

[11] Morikawa T, Kajimura M, Nakamura T, Hishiki T, Nakanishi T, Yukutake Y, Nagahata Y, Ishikawa M, Hattori K, Takenouchi T, Takahashi T, Ishii I, Matsubara K, Kabe Y, Uchiyama S, Nagata E, Gadalla MM, Snyder SH, Suematsu M (2012) Hypoxic regulation of the cerebral microcirculation is mediated by a carbon monoxide-sensitive hydrogen sulfide pathway. Proc Natl Acad Sci USA 109:1293-1298. doi:org/10.1073/pnas.1119658109

[12] Watanabe M, Osada J, Aratani Y, Kluckman K, Reddick R, Malinow MR, Maeda N (1995) Mice deficient in cystathionine beta-synthase: animal models for mild and severe homocyst(e)inemia. Proc Natl Acad Sci USA 92**:**1585-1589. doi:10.1073/pnas.92.5.1585

[13] Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R (2008) H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science 322:587-590. doi:org/10.1126/science.1162667

[14] Ishii I, Akahoshi N, Yamada H, Nakano S, Izumi T, Suematsu M (2010) Cystathionine gamma-Lyase-deficient mice require dietary cysteine to protect against acute lethal myopathy and oxidative injury. J Biol Chem 285:26358-26368. doi:org/10.1074/jbc.M110.147439

[15] Mellemkjaer S, Nielsen-Kudsk JE (1994) Glibenclamide inhibits hypoxic relaxation of isolated porcine coronary arteries under conditions of impaired glycolysis. Eur J Pharmacol 270:307-312. doi:10.1016/0926-6917(94)90006-X

[16] Hedegaard ER, Nielsen BD, Kun A, Hughes AD, Krøigaard C, Mogensen S, Matchkov VV, Fröbert O, Simonsen U (2014). KV7 channels are involved in hypoxia-induced vasodilatation of porcine coronary arteries. Br J Pharmacol 171:69-82. doi:10.1111/bph.12424

[17] Casalini ED, Goodwill AG, Owen MK, Moberly SP, Berwick ZC, Tune JD (2014). Contribution of hydrogen sulfide to the control of coronary blood flow. Microcirculation 21:104-111. doi.org/10.1111/micc.12083

[18] Chai Q, Lu T, Wang X-L, Lee H-C (2015) Hydrogen sulfide impairs shear stress-induced vasodilation in mouse coronary arteries. Pflüg Arch Eur J Physiol 467:329-340. doi.org/10.1007/s00424-014-1526-y

[19] Elsey DJ, Fowkes RC, Baxter GF (2010) L-Cysteine stimulates hydrogen sulfide synthesis in myocardium associated with attenuation of ischemia-reperfusion injury. J Cardiovasc Pharmacol Ther 15:53-59. doi:10.1177/1074248409357743

[20] Geng B, Chang L, Pan C, Qi Y, Zhao J, Pang Y, Du J, Tang C (2004) Endogenous hydrogen sulfide regulation of myocardial injury induced by isoproterenol. Biochem Biophys Res Commun 318:756-763. doi:org/10.1016/j.bbrc.2004.04.094

[21] Pan T-T, Feng Z-N, Lee SW, Moore PK, Bian J-S (2006) Endogenous hydrogen sulfide contributes to the cardioprotection by metabolic inhibition preconditioning in the rat ventricular myocytes. J Mol Cell Cardiol 40:119-130. doi:org/10.1016/j.yjmcc.2005.10.003

[22] Yong QC, Lee SW, Foo CS, Neo KL, Chen X, Bian J-S (2008) Endogenous hydrogen sulphide mediates the cardioprotection induced by ischemic postconditioning. Am J Physiol Heart Circ Physiol 295:H1330-H1340. doi:org/10.1152/ajpheart.00244.2008

[23] Rayment SJ, Ralevic V, Barrett DA, Cordell R, Alexander SPH (2007) A novel mechanism of vasoregulation: ADP-induced relaxation of the porcine isolated coronary artery is mediated via adenosine release. FASEB J 21:577-585. doi:org/10.1096/fj.06-7050com

[24] Almukhtar H, Garle MJ, Smith PA, Roberts RE (2016). Effect of simvastatin on vascular tone in porcine coronary artery: Potential role of the mitochondria. Toxicol Appl Pharmacol 305:176-185. doi.org/10.1016/j.taap.2016.06.024

[25] Rashid S, Heer JK, Garle MJ, Alexander SPH, Roberts RE (2013) Hydrogen sulphide-induced relaxation of porcine peripheral bronchioles. Br J Pharmacol 168:1902-1910. doi:org/10.1111/bph.12084

[26] Donovan J, Garle MJ, Alexander SPH, Dunn WR, Ralevic V (2013) Hydrogen sulfide and its effect on the hypoxic response in porcine coronary arteries. Nitric Oxide 31:S38-39. doi:org/10.1016/j.niox.2013.06.073

[27] Kuo MM, Kim DH, Jandu S, Bergman Y, Tan S, Wang H, Pandey DR, Abraham TP, Shoukas AA, Berkowitz DE, Santhanam L (2016) MPST but not CSE is the primary regulator of hydrogen sulfide production and function in the coronary artery. Am J Physiol Heart Circ Physiol 310:H71-H79. doi:10.1152/ajpheart.00574.2014

[28] Xia M, Chen L, Muh RW, Li PL, Li N (2009) Production and actions of hydrogen sulfide, a novel gaseous bioactive substance, in the kidneys. J Pharmacol Exp Ther 329:1056-1062. doi:org/10.1124/jpet.108.149963

[29] Asimakopoulou A, Panopoulos P, Chasapis CT, Coletta C, Zhou Z, Cirino G, Giannis A, Szabo C, Spyroulias GA, Papapetropoulos A (2013) Selectivity of commonly used pharmacological inhibitors for cystathionine β synthase (CBS) and cystathionine γ lyase (CSE). Br J Pharmacol 169:922-932. doi:org/10.1111/bph.12171

[30] Papapetropoulos A, Whiteman M, Cirino G (2015) Pharmacological tools for hydrogen sulphide research: a brief, introductory guide for beginners. Br J Pharmacol 172:1633-1637. doi:org/10.1111/bph.12806

[31] John RA, Charteris A, Fowler LJ (1978) The reaction of amino-oxyacetate with pyridoxal phosphate-dependent enzymes. Biochem J 171:771-779. PMCID: PMC1184026

[32] Barron JT, Gu L, Parrillo JE (1998) Malate-aspartate shuttle, cytoplasmic NADH redox potential, and energetics in vascular smooth muscle. J Mol Cell Cardiol 30:1571-1579. doi: 10.1006/jmcc.1998.0722

[33] Olson KR, Deleon ER, Gao Y, Hurley K, Sadauskas V, Batz C, Stoy GF (2013) Thiosulfate: a readily accessible source of hydrogen sulfide in oxygen sensing. Am J Physiol Regul Integr Comp Physiol 305:R592-603. doi:org/10.1152/ajpregu.00421.2012

[34] Banerjee R, Zou CG (2005) Redox regulation and reaction mechanism of human cystathionine-beta-synthase: a PLP-dependent hemesensor protein. Arch Biochem Biophys 433:144-156. doi:org/10.1016/j.abb.2004.08.037

[35] Stipanuk MH (2004) Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. Ann Rev Nutrition 24:539-577. doi:org/10.1146/annurev.nutr.24.012003.132418

[36] Wang M, Guo Z, Wang S (2014) Regulation of cystathionine γ-Lyase in mammalian cells by hypoxia. Biochem Genet 52:29-37. doi:org/10.1007/s10528-013-9624-7

[37] Whiteman M, Li L, Kostetski I, Chu SH, Siau JL, Bhatia M, Moore PK (2006) Evidence for the formation of a novel nitrosothiol from the gaseous mediators nitric oxide and hydrogen sulphide. Biochem Biophys Res Commun 343:303-310. doi:org/10.1016/j.bbrc.2006.02.154

[38] Ali MY, Ping CY, Mok YY, Ling L, Whiteman M, Bhatia M, Moore PK (2006) Regulation of vascular nitric oxide in vitro and in vivo; a new role for endogenous hydrogen sulphide? Br J Pharmacol 149:625-634. doi:org/10.1038/sj.bjp.0706906

[39] Altaany Z, Ju Y, Yang G, Wang R (2014) The coordination of S-sulfhydration, S-nitrosylation, and phosphorylation of endothelial nitric oxide synthase by hydrogen sulfide. Sci Signal 7:ra87. doi:org/10.1126/scisignal.2005478

[40] Chen PH, Fu YS, Wang YM, Yang KH, Wang DL, Huang B (2014) Hydrogen sulfide increases nitric oxide production and subsequent S-nitrosylation in endothelial cells. Sci World J 480387. doi:org/10.1155/2014/480387

[41] Taoka S, Banerjee R (2001) Characterization of NO binding to human cystathionine β-synthase:: Possible implications of the effects of CO and NO binding to the human enzyme. J Inorg Biochem 87:245-251. doi:org/10.1016/S0162-0134(01)00335-X

[42] White BJO, Smith PA, Dunn WR (2013) Hydrogen sulphide–mediated vasodilatation involves the release of neurotransmitters from sensory nerves in

pressurized mesenteric small arteries isolated from rats. Br J Pharmacol 168:785-793. doi:org/10.1111/j.1476-5381.2012.02187.x

[43] Zhao W, Zhang J, Lu Y, Wang R (2001) The vasorelaxant effect of H2S as a novel endogenous gaseous KATP channel opener. EMBO J 20:6008-6016. doi:org/10.1093/emboj/20.21.6008

[44] McPherson GA, Choi RT, Kong DCM, Iskander MN (1997) The thromboxane A2 and KATP channel antagonist actions of a series of sulphonylurea derivatives in the pig coronary artery. Eur J Pharmacol 324:193-200. doi.org/10.1016/S0014-2999(97)00086-1

[45] Kimoto Y, Kinoshita H, Nakahata K, Dojo M (2005). Inhibitory effects of lidocaine and mexiletine on vasorelaxation mediated by adenosine triphosphate-sensitive K+ channels and the role of kinases in the porcine coronary artery. Anesthesiol 102(3):581-587. PMID: 15731597

[46] Mishra SK, Aaronson PI (1999) A role for a glibenclamide-sensitive, relatively ATP-insensitive K+ current in regulating membrane potential and current in rat aorta. Cardiovasc Res 44:429-435. doi:org/10.1016/S0008-6363(99)00223-0

[47] Schaffer P, Pelzmann B, Bernhart E, Lang P, Mächler H, Rigler B, Koidl B (1999) The sulphonylurea glibenclamide inhibits voltage dependent potassium currents in human atrial and ventricular myocytes. Br J Pharmacol 128:1175-1180. doi:10.1038/sj.bjp.0702904

[48] Lee S-Y, Lee CO (2005) Inhibition of Na+-K+ pump and L-type Ca2+ channel by glibenclamide in guinea pig ventricular myocytes. J Pharmacol Exp Ther 312(1):61-68. doi:10.1124/jpet.104.074369

[49] Al-Magableh M, Hart J (2011). Mechanism of vasorelaxation and role of endogenous hydrogen sulfide production in mouse aorta. Naunyn-Schmied Arch Pharmacol 383:403-413. doi:org/10.1007/s00210-011-0608-z

[50] Kiss L, Deitch EA, Szabo C (2008) Hydrogen sulfide decreases adenosine triphosphate levels in aortic rings and leads to vasorelaxation via metabolic inhibition. Life Sci 83:589-594. doi:org/10.1016/j.lfs.2008.08.006

[51] Lee SW, Cheng Y, Moore PK, Bian JS (2007) Hydrogen sulphide regulates intracellular pH in vascular smooth muscle cells. Biochem Biophys Res Commun 358:1142–1147. doi:org/10.1016/j.bbrc.2007.05.063

[52] Hughes MN, Centelles MN, Moore KP (2009) Making and working with hydrogen sulfide, The chemistry and generation of hydrogen sulfide in vitro and its measurement in vivo: A review. Free Rad Biol Med 47:1346-1353. doi:org/10.1016/j.freeradbiomed.2009.09.018

[53] Swindle MM, Makin A, Herron AJ, Clubb FJ, Frazier KS (2012) Swine as Models in Biomedical Research and Toxicology Testing. Vet Pathol Online 49**:**344-356. doi:org/10.1177/0300985811402846

Figure legends

Fig. 1 Hypoxia produces a biphasic response in the porcine coronary artery that is mimicked by exogenous hydrogen sulfide (Na_2S and NaHS). (A) Representative trace to illustrate the effect of hypoxia, induced using 95% N_2/5% CO_2, on the porcine coronary artery. Hypoxia caused a biphasic vascular response; transient contraction followed by prolonged relaxation which was reversible. (B) Representative trace to illustrate the effect of H_2S salts, Na_2S and NaHS, on the porcine coronary artery. (C) Concentration response profile for Na_2S and NaHS (mean ± SEM n=40/38, respectively)

Fig. 2 All three hydrogen sulfide synthesizing enzymes, CBS, CSE and MPST, are expressed in the porcine coronary artery. A) Immunoblot analysis of porcine coronary artery (n=4) showed that all 3 enzymes CBS (63 kDa), CSE (44 kDa) and MPST (33 kDa) are present within the porcine coronary artery using actin (45 kDa) to indicate the level of protein. (B) Immunohistochemistry using an ABC-DAB staining showing the location of CBS, CSE and MPST within the coronary artery compared to control (no primary antibody). All panels were of equal magnification. (C) Immunocytochemistry of α-actin (1:200 antibody dilution) (green) for confirmation of smooth muscle, and CBS immunoreactivity (1:200 antibody dilution) (red) in cultured porcine coronary artery vascular smooth muscle cells. SM- smooth muscle, EC- endothelium

Fig. 3 The biphasic hypoxic response of the coronary artery involves the endothelium, nitric oxide and guanylyl cyclase. Hypoxia-induced contraction was significantly reduced in the absence of the endothelium (A) and in the presence of L-NAME (C) and ODQ (E). Hypoxia-induced relaxation was unaltered in the absence of the endothelium (B), was significantly increased in the presence of L-NAME (D) and unaltered in the presence of ODQ (F). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (mean ± SEM n=8)

Fig. 4 The biphasic response to exogenous hydrogen sulfide (Na_2S and NaHS) in the coronary artery is endothelium, nitric oxide and guanylyl cyclase dependent. Concentration response curves in response to Na_2S and NaHS in the absence of the endothelium (A, B) and in the presence of L-NAME (C, D) and ODQ (E, F). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (mean ± SEM, n=15-24)

Fig. 5 A CBS inhibitor (AOAA, amino-oxyacetate) attenuates the hypoxic response of the coronary artery, but a CSE inhibitor and MPST inhibition have no effect. (A) Representative trace showing effect of a CBS inhibitor, AOAA (100 μM), on the hypoxic response. (B) AOAA, at 100 μM, inhibited the contractile phase of the hypoxic response, *P=0.02. (C) AOAA, at 100 μM, did not affect the relaxation phase of the hypoxic response (mean ± SEM n=8). In endothelium denuded coronary arteries (D,E) the contractile phase was greatly attenuated, and AOAA, at 300 μM, had no further effect (D); AOAA, at 300 μM, attenuated the relaxant phase of endothelium-denuded segments of porcine coronary artery (mean ± SEM n=8), **P=0.01. Representative traces show that: (F) the CSE inhibitor D,L-propargylglycine (PPG, 10 μM), (G) the CAT inhibitor aspartate (1 mM) (an indirect inhibitor of MPST), or (H) PPG and aspartate in combination, had no effect on the vascular response to hypoxia (mean ± SEM n=8). The representative traces are aggregates of multiple data points produced by responses of coronary arteries from 8 pigs

Fig. 6 Combination of a CBS inhibitor (AOAA) together with inhibitors of CSE (PPG) and MPST (aspartate) has pronounced inhibitory effects on the coronary artery hypoxic response. The residual hypoxic response in the presence of AOAA was further reduced in the presence of PPG (A), aspartate (B) or both PPG and aspartate (C) (mean ± SEM n=8). The representative traces are aggregates of multiple data points produced by responses of coronary arteries from 8 pigs. Area under the relaxation response curve (AUC) in response to 100 μM AOAA alone (D) was not significantly different, however in the presence of PPG (10 μM) (E), aspartate (1 mM)

(F) or both PPG and aspartate (G) the relaxation phase is significantly reduced compared to control. *P<0.05, **P<0.01, ***P<0.001 (mean ± SEM n=8)

Fig. 7 Endogenous production of hydrogen sulfide through CBS and MPST, but not CSE in the porcine coronary artery. H_2S was produced in coronary artery samples in the presence of (A) 10 mM L-cysteine and 1mM pyridoxal phosphate. H_2S generation was essentially abolished in the presence of AOAA (10 μM) but not PPG (10 μM). (B) Production of H_2S from 3-mercaptopyruvate in porcine coronary artery. *P<0.05, ***P<0.001 (mean ± SEM n=4-12)

Fig. 8 Summary of mechanisms involved in hypoxic response of the coronary artery involving H_2S. Under normoxic conditions, constitutive production of nitric oxide (NO) causes vasodilatation via generation of cGMP; levels of hydrogen sulfide (H_2S) during normoxia are relatively small. Hypoxia produces a biphasic response in the coronary artery: transient endothelium-dependent contraction followed by endothelium-independent prolonged relaxation. The contraction may involve inhibition of endothelial NO by H_2S generated by cystathionine-ß-synthase (CBS). The relaxation involves smooth muscle CBS and activation of potassium channels and chloride/bicarbonate exchange.

Figure 1

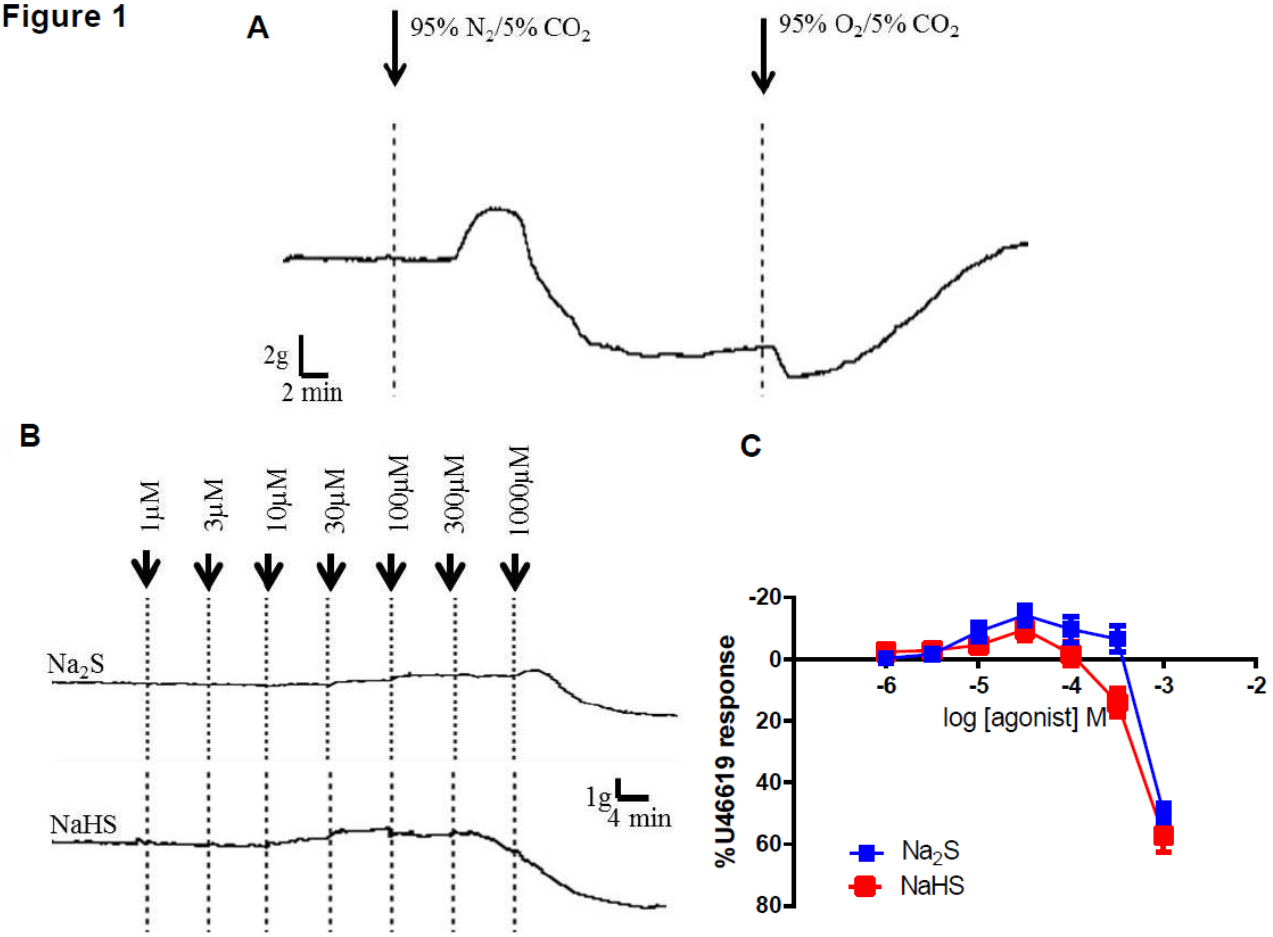


Figure 2

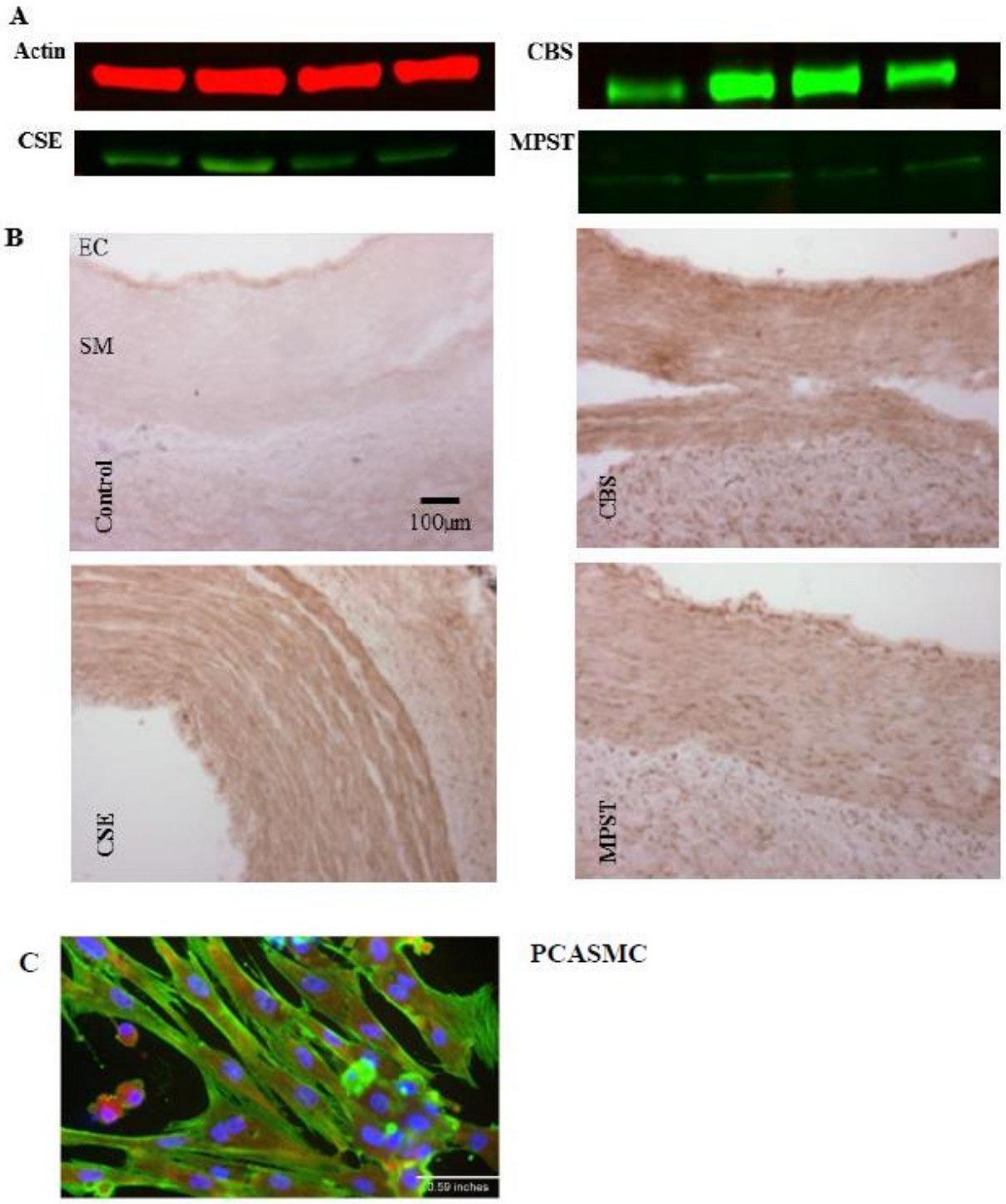


Figure 3

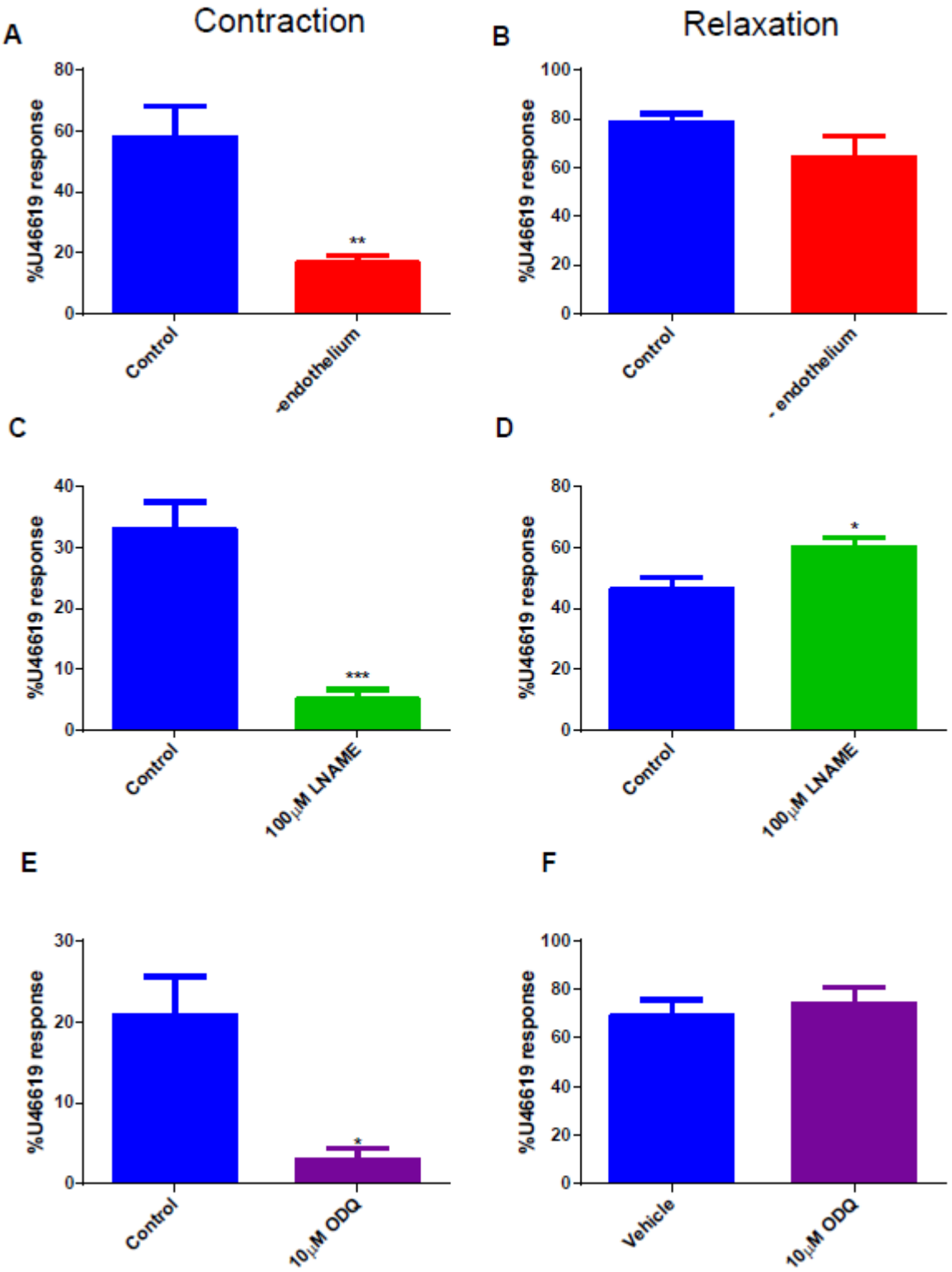
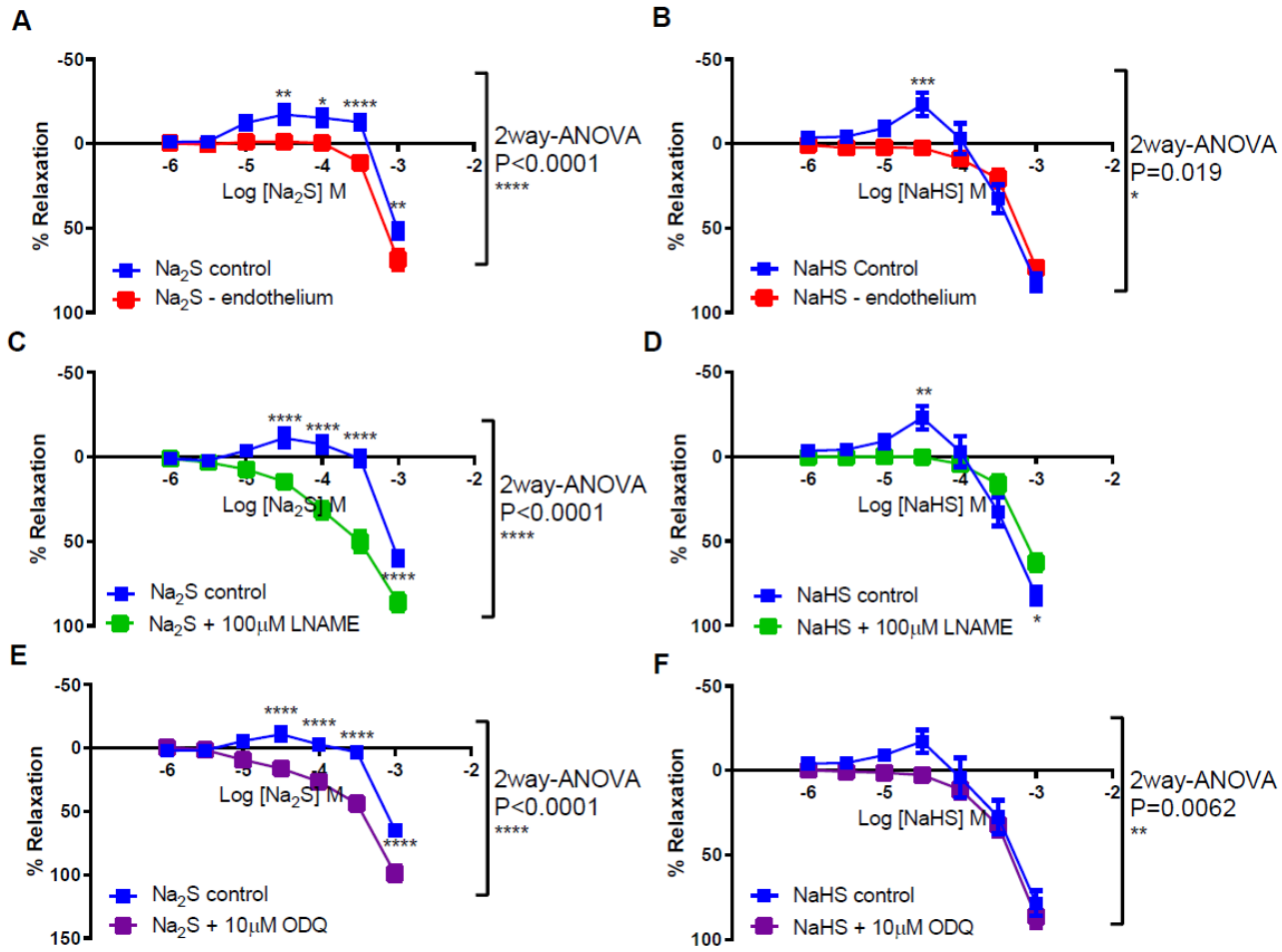


Figure 4



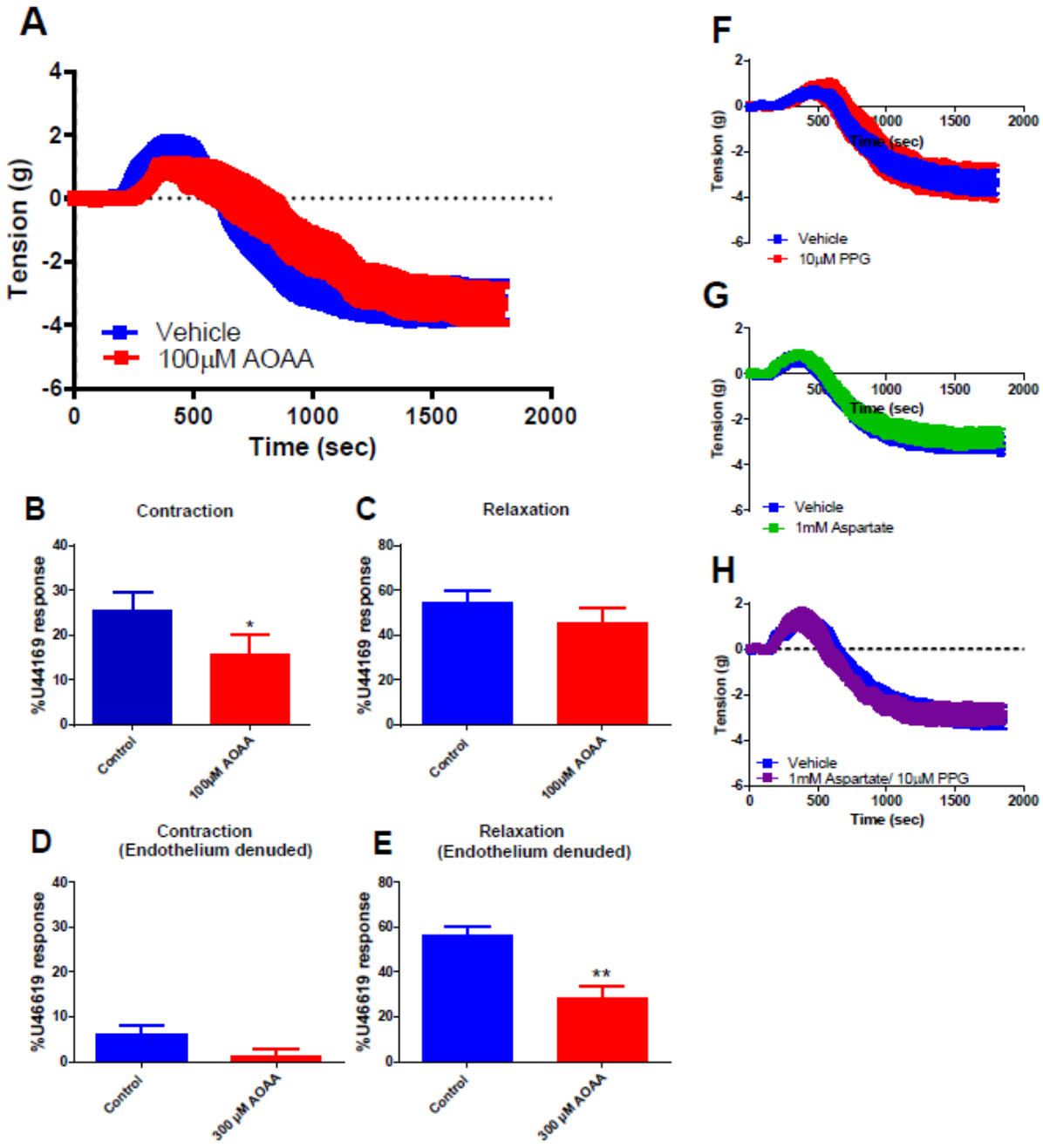


Figure 5

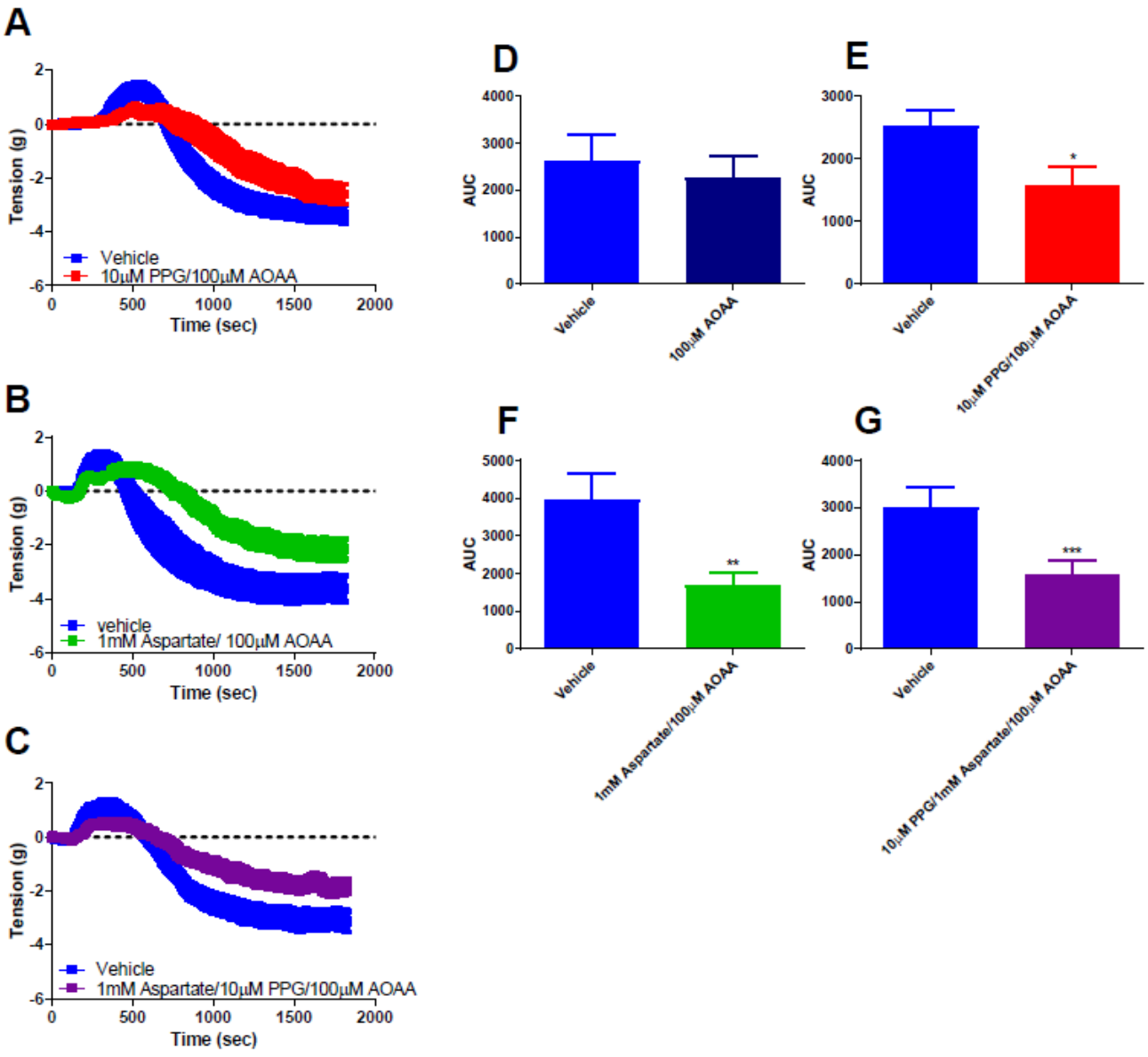


Figure 6

Figure 7

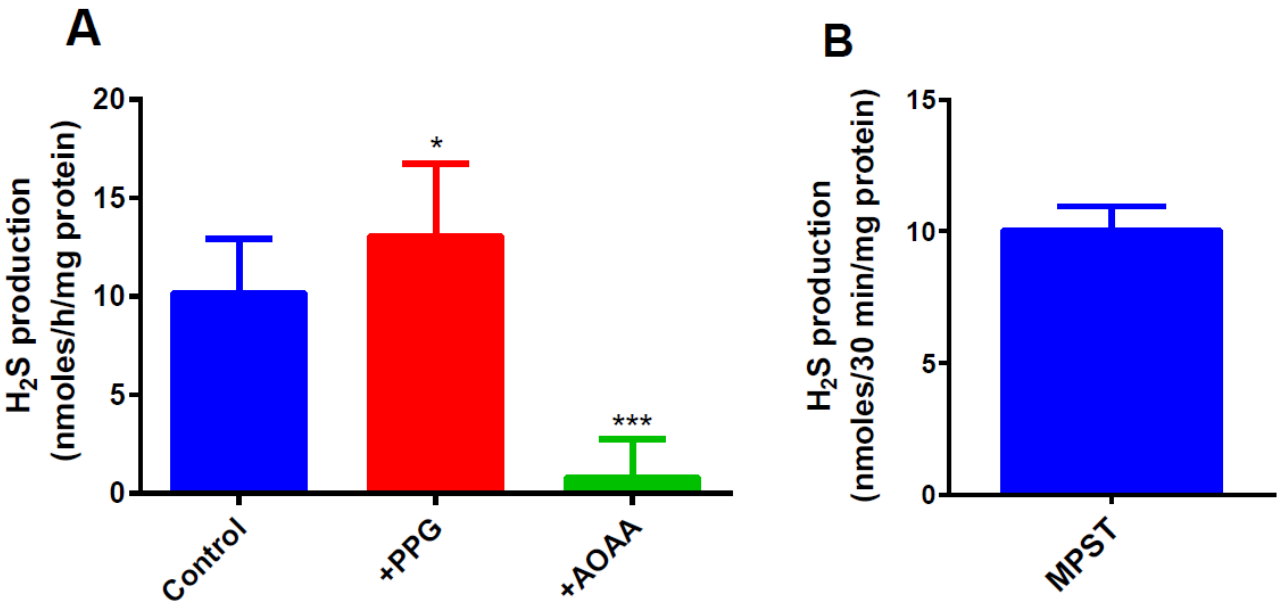


Figure 8

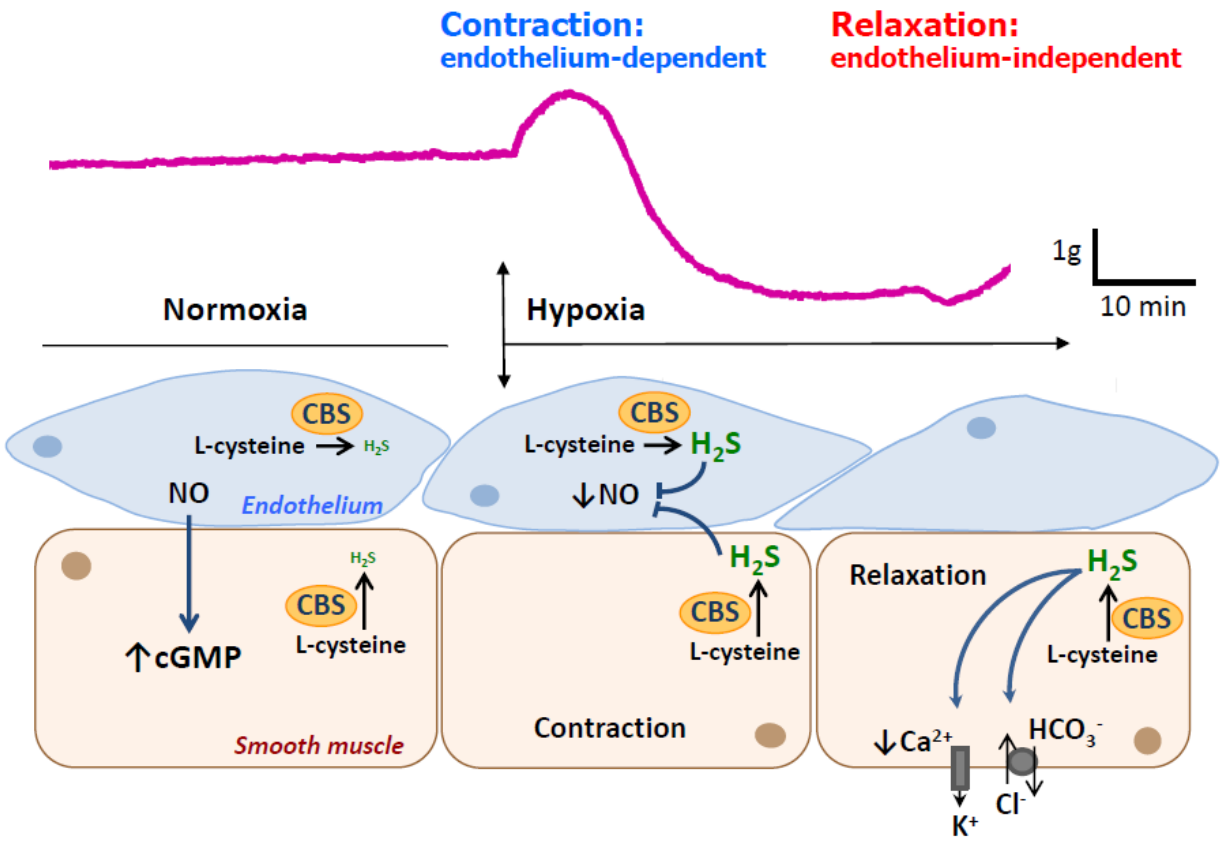


Table 1. The effect of pharmacological inhibitors on the mean relaxation responses to hypoxia in the porcine coronary artery. *<0.05, **<0.01, ***<0.001

Hypoxic contraction (% KCl)				**Hypoxic relaxation (% U46619)**			
	Control	Treatment	n		Control	Treatement	n
endothelium denuded	26 ± 4	10 ± 2 **	8	endothelium denuded	78 ± 4	64 ± 9	8
L-NAME (100 μM)	18 ± 2	3 ± 1 ***	8	L-NAME (100 μM)	46 ± 4	60 ± 3 *	8
SQ22536 (10 μM)	19 ± 2	17 ± 4	8	SQ22536 (10 μM)	73 ± 7	78 ± 5	8
ODQ (10 μM)	12 ± 2	2 ± 1 **	8	ODQ (10 μM)	69 ± 7	74 ± 7	8
K^+ (50 mM)	20 ± 4	4 ± 1 **	8	K^+ (50 mM)	84 ± 7	19 ± 2 ***	8
TEA (1 mM)	15 ± 4	21 ± 4	8	TEA (1 mM)	49 ± 4	51 ± 5	8
TEA (10 mM)	12 ± 3	22 ± 4 *	8	TEA (10 mM)	65 ± 6	74 ± 5	8
Combination of K^+ blockers	11 ± 3	32 ± 6 *	5	Combination of K^+ blockers	96 ± 3	90 ± 9	5
Glibenclamide (10 μM)	16 ± 2	18 ± 3	8	Glibenclamide (10 μM)	62 ± 8	93 ± 6*	8
ChtX (10 nM) and apamin (100 nM)	9 ± 4	18 ± 4	5	ChtX (10 nM) and apamin (100 nM)	99 ± 14	97 ± 7	8
4-AP (1 mM)	13 ± 2	20 ± 2 *	8	4-AP (1 mM)	57 ± 4	57 ± 5	5
$BaCl_2$ (30 μM)	16 ± 3	15 ± 3	8	$BaCl_2$ (30 μM)	56 ± 11	58 ± 6	8
DIDS (100 μM)	4 ± 2	4 ± 2	8	DIDS (100 μM)	109 ± 5	99 ± 5 *	8

Table 2. The effect of pharmacological inhibitors on the mean relaxation responses to Na_2S and NaHS (at 1 mM) in the porcine coronary artery. *<0.05, **<0.01, ***<0.001. Treatment is compared to Control. 2-way ANOVA indicates a shift in the concentration-response curve.

	Na_2S relaxation (% U46619)				NaHS relaxation (% U46619)			
	Control	Treatment	n	2-way ANOVA	Control	Treatment	n	2-way ANOVA
endothelium denuded	52 ± 5	69 ± 6 **	23-24	****	82 ± 5	73 ± 3	15-16	*
L-NAME (100 μM)	60 ± 4	86 ± 5 ***	23-24	****	82 ± 5	63 ± 5 *	15-16	
SQ22536 (10 μM)	68 ± 6	59 ± 5	15-16	**	61 ± 8	56 ± 5	16	
ODQ (10 μM)	65 ± 4	99 ± 6 ***	23	****	79 ± 7	87 ± 7	15-16	**
K^+ (50 mM)	62 ± 4	14 ± 3 ***	23-24	**	82 ± 5	19 ± 4 ***	15-16	****
TEA (1 mM)	61 ± 7	58 ± 10	15-16		65 ± 6	62 ± 4	16	
TEA (10 mM)	52 ± 3	84 ± 5 ***	31-32	****	82 ± 5	87 ± 6	15-16	****
Combination of K^+ blockers	83 ± 6	100 ± 15	15-16		69 ± 6	86 ± 5	15-16	**
Glibenclamide (10 μM)	83 ± 6	73 ± 5	15-16		69 ± 6	87 ± 5	15-16	
TRAM34 (10 μM)/ apamin (100 nM)	83 ± 6	69 ± 5	15-16		69 ± 6	64 ± 4	15-16	
4-AP (1 mM)	75 ± 8	60 ± 7	15-16		73 ± 4	57 ± 6	15-16	
$BaCl_2$ (30 μM)	75 ± 8	52 ± 4 **	16	*	72 ± 4	69 ± 4	16	
DIDS (100 μM)	65 ± 4	8 ± 15 ***	8	*	89 ± 19	28 ± 11 ***	8	***