

Hypoxic pulmonary vasoconstriction in isolated rat pulmonary arteries is not inhibited by antagonists of H₂S-synthesizing pathways

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Key points

- We evaluated the hypothesis that an increase in the hydrogen sulphide concentration in pulmonary artery smooth muscle cells (PASMCs) causes hypoxic pulmonary vasoconstriction (HPV) by examining the effects of the sulphide donor cysteine and sulphide-synthesis blockers on HPV in isolated rat intrapulmonary arteries (IPAs).
- Cysteine (1 mM) enhanced HPV and also the contraction to prostaglandin F_{2α} (PGF_{2α}) and both effects were abolished by the cystathionine γ -lyase (CSE) blocker propargylglycine (PAG, 1 mM), which had little or no non-selective effect on contraction at this concentration.
- Neither PAG nor the cysteine aminotransferase (CAT) antagonist aspartate affected HPV in normal physiological saline solution (PSS), or in PSS containing physiological concentrations of cysteine, cystine and glutamate, whereas dithiothreitol (DTT), proposed to enhance HPV by converting mitochondrial thiosulphate to sulphide, instead abolished HPV.
- PAG markedly diminished whereas DTT did not affect cysteine-induced sulphide release from liver pieces.
- The results do not support the proposal that hydrogen sulphide plays a role in HPV.

Abstract An increase in the H₂S (hydrogen sulphide, hereafter sulphide) concentration in pulmonary artery smooth muscle cells (PASMCs) has been proposed to mediate hypoxic pulmonary vasoconstriction (HPV). We evaluated this hypothesis in isolated rat intrapulmonary arteries (IPAs) by examining the effects of the sulphide precursor cysteine and sulphide-synthesis blockers on HPV and also on normoxic pulmonary vasoconstriction (NPV) stimulated by prostaglandin F_{2α} (PGF_{2α}) and by the drug LY83583, which causes contraction in IPAs by increasing cellular reactive oxygen species levels. Experiments with several blockers of cystathionine γ -lyase (CSE), the enzyme responsible for sulphide synthesis in the vasculature, demonstrated that propargylglycine (PAG, 1 mM) had little or no effect on the NPV caused by PGF_{2α} or LY83583. Conversely, other CSE antagonists tested, aminooxyacetic acid (AOAA, 100 μ M), β -cyanoalanine (BCA, 500 μ M) and hydroxylamine (HA, 100 μ M), altered the NPV to PGF_{2α} (BCA increased, HA inhibited) and/or LY83583 (BCA increased, AOAA and HA inhibited). Preincubating IPAs in physiological saline solution (PSS) containing 1 mM cysteine increased the amplitude of the NPV to PGF_{2α} by ~50%, and had a similar effect on HPV elicited by hypoxic challenge with 0% O₂. The enhancement of both responses by cysteine was abolished by pretreatment with 1 mM PAG. Measurements carried out with an amperometric electrode demonstrated that incubation with 1 mM cysteine under anoxic conditions (to minimize sulphide oxidation) greatly potentiated the release of sulphide from pieces of rat liver and that this release was strongly antagonized by PAG, indicating that at this concentration PAG could enter cells intact and antagonize CSE. PAG at 1 mM had no effect on HPV recorded in control PSS, or in PSS supplemented with physiological concentrations of cysteine (10 μ M), cystine (50 μ M) and glutamate (100 μ M) in order to prevent the possible depletion of intracellular cysteine during experiments. Application of a combination of 1 mM cysteine and 1 mM

α -ketoglutarate to promote sulphide synthesis via the cysteine aminotransferase/mercaptopyruvate sulphurtransferase (CAT/MST) pathway caused an increase in HPV similar to that observed for cysteine. This was partially blocked by the CAT antagonist aspartate (1 mM) and also by PAG. However, HPV was not increased by 1 mM α -ketoglutarate alone, and HPV in the absence of α -ketoglutarate and cysteine was not attenuated by aspartate. Pretreatment of IPAs with dithiothreitol (DTT, 1 mM), proposed to promote the conversion of mitochondrial thiosulphate to sulphide, did not increase the release of sulphide from pieces of rat liver in either the presence or the absence of 1 mM cysteine, and virtually abolished HPV. The results provide evidence that the sulphide precursor cysteine can promote both NPV and HPV in rat IPA by generating sulphide via a PAG-sensitive pathway, presumably CSE. However, HPV evoked under control conditions was unaffected by the blockade of CSE. Moreover, HPV was not affected by the CAT antagonist aspartate and was blocked rather than enhanced by DTT. The data therefore indicate that sulphide generated by CSE or CAT/MST or from thiosulphate is unlikely to contribute to O₂ sensing during HPV in these arteries.

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Abbreviations AOAA, aminooxyacetic acid; BCA, β -cyanoalanine; CAT, cysteine aminotransferase; CBS, cystathionine β -synthase; CSE, cystathionine γ -lyase; DHLA, dihydrolipoic acid; DTT, dithiothreitol; HA, hydroxylamine; HPV, hypoxic pulmonary vasoconstriction; IPA, intrapulmonary artery; MST, mercaptopyruvate sulphurtransferase; NPV, normoxic pulmonary vasoconstriction; PA, pulmonary artery; PAG, propargylglycine; PASMC, pulmonary artery smooth muscle cell; PGF_{2 α} , prostaglandin F_{2 α} ; PSS, physiological saline solution; ROS, reactive oxygen species.

Introduction

Hydrogen sulphide (H₂S, hereafter sulphide) has recently emerged as a biologically active gas with multiple effects on the cardiovascular system. Although these are not as well understood as those of nitric oxide (NO), it appears that the two gases generally cause similar responses with regard to vasodilatation, cardioprotection, angiogenesis and inhibition of smooth muscle cell proliferation (Wang, 2011). There seem to be multiple mechanisms by which sulphide can potentially cause vasodilation, including hyperpolarization of endothelial and/or vascular smooth muscle cells through activation of several types of K⁺ channels; inhibition of cGMP phosphodiesterase may also make a contribution (Wang, 2011).

Sulphide, however, differs from NO in that it can cause vasoconstriction in some systemic arteries, notably those from non-mammalian species such as duck, alligator and lamprey (Dombkowski *et al.* 2005; Olson *et al.* 2006). Sulphide also constricts bovine pulmonary arteries (PAs), and causes a complex triphasic response in pre-constricted rat PAs consisting of a small transient contraction followed by relaxation and then a second more sustained constriction. In both arteries, the response to sulphide resembled the profile of hypoxic pulmonary vasoconstriction (HPV), the constricting response evoked by hypoxia (Olson *et al.* 2006; Aaronson *et al.* 2012). Likewise, hypoxia and sulphide show a remarkable similarity in their constricting or relaxing effects on various arteries

from a wide range of mammalian (e.g. cow, sea lion) and non-mammalian (e.g. hagfish, shark, toad, trout) species (Olson *et al.* 2006; Olson & Whitfield, 2010).

These findings prompted the proposal (Olson *et al.* 2006, 2010) that sulphide is the universal mediator of both hypoxic constriction and hypoxic vasodilation. This idea is also supported by observations that levels of sulphide produced by rat PAs (Madden *et al.* 2012) and lung homogenate (Olson & Whitfield, 2010) increased under anoxic conditions and fell in the presence of oxygen, an effect consistent with the concept that hypoxia increases cellular [sulphide] by attenuating its metabolism, which involves oxidation to sulphite and sulphate. In addition, incubation of bovine PAs in the sulphide precursor cysteine increased the amplitude of HPV, with an even more marked enhancement observed when arteries were treated with a combination of cysteine and α -ketoglutarate, which would favour the production of sulphide via cysteine aminotransferase (CAT) and mercaptopyruvate sulphurtransferase (MST) (Olson *et al.* 2010). Crucially, HPV was also seen to be blocked by antagonists of the enzymes which metabolize cysteine and other thiol-containing amino acids to produce sulphide (Olson *et al.* 2006, 2010; Madden *et al.* 2012). These antagonists include propargylglycine (PAG) and β -cyano-L-alanine (BCA), claimed to be selective inhibitors of cystathionine γ -lyase, and hydroxylamine (HA) and aminooxyacetic acid (AOAA), which have been used as blockers of cystathionine β -synthase (CBS) although recent

work indicates that BCA, HA and AOAA block both cystathionine γ -lyase (CSE) and CBS whereas PAG is selective for CSE (Asimakopoulou *et al.* 2013). Olson *et al.* (2013) have also shown that HPV is enhanced by the reducing agents dithiothreitol (DTT) and dihydrolipoic acid (DHLA), both of which also increased sulphide production by tissues supplemented with thiosulphide. Based on this evidence, they proposed that reduction of the mitochondrial redox state, which occurs during hypoxia (Waypa *et al.* 2010), reduces mitochondrial thiosulphate to sulphide, thereby contributing another potential mechanism by which hypoxia might raise cellular [sulphide].

Sulphide is also suggested to play an analogous role in the carotid body, as evidenced by the inhibitory effect of CSE knockout and PAG on O₂ sensing *in vivo* and in isolated glomus cells (Peng *et al.* 2010), although this concept has been disputed (Buckler, 2012).

Although provocative, the proposal that sulphide acts as an oxygen sensor in HPV remains to be verified independently. The inhibitors of sulphide synthesis used to study its involvement in HPV have been used at very high concentrations (10 mM PAG, 1 mM AOAA, 10 mM BCA, 1 mM HA, 10 mM aspartate; Olson *et al.* 2006; Madden *et al.* 2012), and the possibility that these drugs cause non-specific effects on contraction at these concentrations has not been carefully assessed. It is also the case that the CSE/CBS blocker AOAA blocked HPV in bovine PAs (Olson *et al.* 2006) and the more selective CSE inhibitor PAG did not (Olson *et al.* 2006), whereas it was subsequently shown that CSE but not CBS is expressed in bovine pulmonary artery smooth muscle cells (PASMCs) (Olson *et al.* 2010). In the present study, we therefore examined the contractile effects of sulphide synthesis inhibitors on HPV and also on contractions evoked under normoxic conditions by the agonist prostaglandin F_{2 α} (PGF_{2 α}) and the drug LY83583, which generates reactive oxygen species (ROS) within cells, in rat intra-pulmonary arteries (IPAs). We find that the sulphide synthesis inhibitors BCA, AOAA and HA have marked effects on contractions. PAG and aspartate, which lack these non-selective effects on contraction, have no effect on HPV.

Methods

Ethical approval

This study conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and is in accordance with UK Home Office regulations (Animals (Scientific Procedures) Act, 1986). Male Wistar rats (200–300 g; 6–8 weeks old) were killed by lethal injection (i.p.) of sodium thiopental.

IPA mounting and measurement of tension development

The heart and lungs were excised and placed in cold physiological salt solution (PSS), which contained (mmol l⁻¹): 118 NaCl, 24 NaHCO₃, 1 MgSO₄, 0.435 NaH₂PO₄, 5.56 glucose, 1.8 CaCl₂ and 4 KCl. Rings of IPA (inner diameter 0.5–1.0 mm) were dissected free of adventitia and parenchyma under a dissection microscope, mounted on a conventional small vessel wire myograph, and stretched to give a basal tension of 5–6 mN (equivalent to an internal pressure of ~15 mmHg). They were then equilibrated with three brief exposures to PSS containing 80 mmol l⁻¹ KCl (80KPSS; isotonic replacement of NaCl by KCl). Hypoxia was induced by switching from 95% air/5% CO₂ to either 0% O₂/5% CO₂/balance N₂ or 2% O₂/5% CO₂/balance N₂. All experiments were conducted at 37°C.

RT-PCR

RNA was isolated from homogenates of freshly isolated rat IPAs and reverse transcription carried out as previously described (Knock *et al.* 2008). RT-PCR primer pairs were designed using Primer3web version 4.0 (<http://primer3.ut.ee>) and synthesized by Sigma-Aldrich (St Louis, MO, USA). MPT (accession no. NM_138843): sense CCTTCATCAAGACCCACGAG, antisense TGGACAGGTCCACCTTCTTC; C β S (accession no. NM_012522): sense ATGCTGCAGAAAGGCTTCAT, antisense CAACA-CCAAACACCATCAG; C γ L (accession no. NM_017074): sense GGTTTTGTATACAGCCGCTCTGG, antisense TGCAAATGACTTCATCTCCTGCT. PCR was carried out using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Piscataway, NJ, USA) and the PCR products (reaction equivalent on 20 ng reverse transcribed RNA) were analysed by electrophoresis on 2.8% agarose gels run in TAE buffer (National Diagnostics, Hessele, UK) with PhiX174 DNA/HinfI Marker (Thermoscientific Inc., Waltham, MA, USA).

Measurement of sulphide

The sulphide concentration was measured and recorded using an amperometric sensor and an amplifier and SensorTrace Basic software from Unisense A/S (Aarhus, Denmark). Pieces of liver tissue weighing 175 mg were pre-incubated in normal Krebs solution (previously equilibrated with anoxic gas 5% CO₂/N₂) for 20 min in a sealed vial. L-cysteine (1 mM) and/or DTT (3 mM) were then added, and the sulphide concentration was recorded for 30 min. To check if the magnitude of the response to cysteine was affected by L-PAG the tissue was pre-incubated in 1 mM L-PAG for 30 min before

starting the recording. Tissues from the same animal were always paired for control recording (L-cysteine or DTT) or treatment (L-PAG on L-cysteine or DTT responses).

Drugs and chemicals

PGF_{2α} was from Enzo Life Sciences (Exeter, UK), BCA was from VWR International (Radnor, PA, USA), hydroxylamine was from MP Biomedicals (Santa Ana, CA, USA), L-propargylglycine was from Santa Cruz (Santa Cruz, CA, USA), α-ketoglutarate was from Fisher Scientific (Loughborough, UK), and D,L-propargylglycine and all other drugs were from Sigma-Aldrich (St Louis, MO, USA). All drug stock solutions were made up in distilled water, except for cystine which was dissolved in 1 M HCl and aspartate which was dissolved in 0.5 M HCl. At their final concentrations used for experiments, none of the drugs used had an effect on pH, which was maintained at 7.4.

HPV experimental protocol, data representation and statistical analysis

IPAs were exposed to hypoxia for three periods, each separated by ≥30 min. The duration of each hypoxic episode was 45 min when the arteries were challenged with 0% O₂ and 20 min when 2% O₂ was used; the shorter period was used in the latter case to minimize rundown of the response. The O₂ concentration in the myograph chambers, measured using a NeoFox oxygen sensing system with a AL300 sensor (Ocean Optics, Dunedin, FL, USA) fell within several minutes to steady-state values of 6–9 mmHg when gassing with 0% O₂ and 22–25 mmHg when gassing with 2% O₂. Tension was measured immediately before the imposition of hypoxia, every 5 min during the hypoxic periods and then 5 min after re-oxygenation, at which point HPV had largely subsided. Hypoxia under these conditions typically resulted in a rapid increase of force development within 1–3 min, which then plateaued, although in some tissues the plateau was preceded by a small transient peak in tension. Experiments were included for analysis if the amplitude of the control HPV (measured at 45 min) was ≥ 0.2 mN. To adjust for the slow progressive drift in the baseline tension which occurred in some experiments, an extrapolated line was drawn connecting the trend of the baseline tension just before imposing hypoxia with the trend of baseline tension following the exchange of the solution after re-oxygenation. Where the baseline tension after the solution exchange was clearly displaced away from the trend, the line was instead extrapolated along the trend of the baseline tension over ~5 min prior to the imposition of hypoxia. The change in tension during HPV and after 5 min of re-oxygenation was then calculated as

the difference between the tension recorded at each time point and the baseline tension values at that time as defined by the line.

When 0% O₂ is used for multiple successive hypoxic challenges, the initial HPV differs in its shape from the 2nd and 3rd HPVs, which are indistinguishable (Connolly *et al.* 2013). We therefore used the 2nd HPV as the control response, and experimental interventions (e.g. application of inhibitors or cysteine) were made 30 min prior to the 3rd hypoxic challenge. In some cases where we examined the effect on HPV of a combination of two agents (e.g. PAG and cysteine), the first was applied starting 30 min before the 2nd hypoxic challenge and remained present during the 3rd hypoxic challenge, whereas the second was then applied after the 2nd HPV, starting 30 min before the 3rd hypoxic challenge. For illustration of the results, increases in tension above baseline throughout the 2nd and 3rd hypoxic challenges were expressed as a percentage of the increase in force above baseline recorded at 45 min of hypoxia during the 2nd (control) response. When arteries were repeatedly challenged with 2% O₂, the first two HPVs were indistinguishable whereas the 3rd HPV demonstrated some rundown, so in these experiments we used the 1st and 2nd HPVs as the control and intervention responses. The amplitudes of the control and intervention HPVs were compared over 45 or 20 min using repeated measures ANOVA followed by a Bonferroni *post hoc* test to determine whether the intervention had significantly changed the response.

A protocol similar to that used to record HPV in 0% O₂ was used to record the effects of sulphide donors and synthesis inhibitors on the maximal normoxic pulmonary vasoconstriction (NPV) to 30 μM PGF_{2α}. Effects of sulphide synthesis inhibitors on the NPV to 10 μM LY83583 were also examined. In this case, because repeated responses to LY83583 were not always of consistent amplitude, the inhibitor was applied once the contraction elicited by a single application of LY83583 had reached a stable level. Tension in the presence of LY83583 just before the application of, and at the point of maximal response to the sulphide synthesis inhibitor, was measured. These results were analysed using Student's paired *t* test. The threshold for statistical significance for all comparisons was set at *P* < 0.05. GraphPad Prism was used for all analyses. Summary data values shown in the figures are the mean ± SEM.

Results

Effects of sulphide synthesis inhibitors on NPV to PGF_{2α} and LY83583

In initial experiments carried out to determine whether inhibitors of sulphide-synthesizing enzymes had non-selective effects on contraction, we examined their

effects on the presumably sulphide-independent contractions of IPAs evoked under normoxic conditions by the agonist PGF_{2 α} (30 μ M) and the drug LY83583 (10 μ M), which causes constriction of IPAs by generating ROS (Hasegawa *et al.* 2004; Knock *et al.* 2009).

As shown in Fig. 1A, neither AOAA (100 μ M) nor D,L-PAG (1 mM) had any effect on the NPV to 30 μ M PGF_{2 α} , whereas HA (100 μ M) strongly inhibited and BCA (500 μ M) markedly potentiated the PGF_{2 α} response, respectively. L-PAG (1 mM) caused a small but significant potentiation. Figure 1B shows that D,L-PAG also had no effect on the NPV evoked by LY83583, whereas L-PAG caused a small further contraction; this was observed to wane with time (data not shown). On the other hand, the NPV to LY83583 was markedly inhibited by AOAA, abolished by HA and strongly potentiated by BCA.

Cysteine enhances NPV to PGF_{2 α} and this effect is blocked by sulphide synthesis inhibitors

Application of the sulphide precursor L-cysteine (1 mM), although it seldom affected basal tone, consistently caused a further increase in tension when applied to IPAs that had been precontracted by PGF_{2 α} (Fig. 2A). This contraction was concentration-dependent, with a threshold near 3 μ M L-cysteine (Fig. 2B and C). Similarly, preincubation of arteries for 30 min in 1 mM L-cysteine increased the NPV to PGF_{2 α} by ~50% (Fig. 2D and Fig. 2E, leftmost bar).

In further experiments, we examined the effect of the sulphide synthesis inhibitors on the enhancement by L-cysteine of the NPV induced by 30 μ M PGF_{2 α} . IPAs were incubated with the inhibitor for 30 min, and PGF_{2 α} was applied. Immediately following washout of the agonist, the inhibitor was re-applied together with 1 mM cysteine, and after 30 min the artery was re-challenged with PGF_{2 α} . As shown in Fig. 2E, each of the inhibitors markedly inhibited or abolished the potentiation of the NPV caused by cysteine, regardless of whether the inhibitor itself had suppressed (HA), potentiated (BCA) or had little effect (AOAA and PAG) on the PGF_{2 α} response.

As it appeared that, of the inhibitors tested, only PAG was able to block the (presumably sulphide-dependent) enhancement of the NPV caused by PGF_{2 α} with little effect of its own on contraction, we also examined whether PAG was able to block sulphide synthesis in intact tissue, and found that 1 mM L-PAG strongly suppressed cysteine-induced sulphide production by rat liver (Fig. 3). Therefore, only D,L-PAG and L-PAG were used to block CSE in subsequent experiments.

Cysteine also causes an enhancement of HPV that is blocked by PAG

As shown in Fig. 4A, the amplitude of HPV evoked by 0% O₂ was significantly increased by L-cysteine (1 mM), and

this was abolished by both D,L-PAG (Fig. 4B) and L-PAG (Fig. 4C).

In contrast to the potentiation of HPV by cysteine, the putative sulphide precursors GSH and homocysteine had no effect on HPV (Fig. 5A and B), whereas GSSG significantly attenuated HPV (Fig. 5C).

HPV is not blocked by PAG

Having shown that both L- and D,L-PAG were able to block cysteine-induced enhancement of both HPV and the NPV evoked by PGF_{2 α} without appreciable non-selective effects, we used PAG to determine whether sulphide production by CSE was required for HPV under more physiological conditions. As shown in Fig. 6, preincubation with 1 mM of either D,L-PAG or L-PAG had no effect on HPV evoked by 0% O₂ in normal PSS.

To address the possibility that the lack of effect of PAG on HPV arose because PAs were unable to synthesize sulphide due to depletion of cysteine from the smooth muscle and/or endothelial cells which might have occurred during several hours of incubation in PSS, additional experiments were carried out using PAs incubated in PSS supplemented with 10 μ M cysteine, 50 μ M cystine and 100 μ M glutamate to maintain cellular sulphide synthesis (see Discussion) throughout the protocol. However, as illustrated in Fig. 7, neither D,L-PAG nor L-PAG had any effect on HPVs evoked by either 0% O₂ (Fig. 7A and B, respectively) or 2% O₂ (Fig. 7D and E) in the 'supplemented' PSS. HPV elicited by 2% O₂ was stable with repeated hypoxic challenge in the presence of the amino acids (Fig. 7C). Representative traces of these experiments are shown in Fig. 7F.

Aspartate-sensitive enhancement of HPV by cysteine and α -ketoglutarate

Incubation of IPA with the combination of L-cysteine and α -ketoglutarate (both 1 mM) also increased the amplitude of HPV. This effect appeared to be somewhat larger than that caused by L-cysteine alone (Fig. 8A), although this difference was not significant. We examined whether the CAT/MST pathway of sulphide synthesis might be operative in PAs under these conditions by using the CAT antagonist aspartate, which competes with cysteine for binding to the enzyme. As shown in Fig. 8B, 1 mM aspartate markedly attenuated the increase in HPV caused by the combination of L-cysteine and α -ketoglutarate. In contrast, the blocking effect of aspartate was not observed if only L-cysteine was used to enhance HPV (Fig. 8C; the increase in HPV is not different from that shown in Fig. 4A, $P > 0.05$). The enhancement of HPV by L-cysteine and α -ketoglutarate was also diminished in the presence of D,L-PAG (Fig. 8D), implying that under these conditions sulphide synthesis by both CSE and CAT/MST

was contributing to the enhancement of HPV. Crucially, however, application of α -ketoglutarate (1 mM) on its own had no effect on HPV elicited in normal PSS (Fig. 9A), or in PSS supplemented with 10 μ M cysteine, 50 μ M cysteine and 100 μ M glutamate so as to maintain intracellular cysteine levels (Fig. 9B). Aspartate had no significant effect on HPV in the absence of pretone (Fig. 9C), or on the NPVs elicited by either 30 μ M PGF_{2 α} or 10 μ M LY83583 (Fig. 9D).

Effects of DTT on HPV and sulphide production by liver

Olson *et al.* (2013) have recently shown that DTT enhances HPV in rat PAs, interpreting this observation as supporting the idea that hypoxia increases cellular [sulphide] by reducing mitochondrial thiosulphate. In contrast, we consistently observed that DTT (1 mM) virtually abolished HPV, whether evoked by 0% O₂ in control PSS (Fig. 10A) or by 2% O₂ in PSS supplemented with cysteine, cysteine and glutamate (Fig. 10B). Figure 10C shows that 3 mM DTT had no effect on the production of sulphide by liver samples, either under basal conditions (left) or in the presence of 1 mM cysteine to boost sulphide production.

Expression of sulphide-synthesizing enzymes in rat PAs

As shown in Fig. 11, mRNA for CSE and MST, but not CBS, is expressed in rat IPAs.

Discussion

Olson *et al.* (2006) proposed that a reduction in the ambient O₂ concentration leads to an increase in cellular [sulphide] in vascular smooth muscle cells, and that this effect constitutes an O₂ sensor that causes arterial contraction or dilatation depending on vessel type. In PAs this increase in cellular [sulphide] would trigger HPV. They initially hypothesized that hypoxia would lead to a rise in cellular [sulphide] by attenuating its oxidation, but more recently have suggested that reduction of the mitochondrial redox state, which has been shown to occur during hypoxia (Waypa *et al.* 2010), could also raise [sulphide] by promoting the conversion of mitochondrial thiosulphate to sulphide (Olson *et al.* 2013). Enzymatic pathways of sulphide synthesis and metabolism relevant to these proposals, as well as potential mechanisms by which hypoxia might increase cellular sulphide and thereby trigger HPV, are summarized in Fig. 12, which is largely based on information presented in Go & Jones (2011), Wang (2012) and Olson *et al.* (2013).

The proposal that sulphide is involved in HPV rests on a number of observations. Exogenous NaHS and hypoxia are seen to cause similar constricting or dilatating responses in a wide range of arteries from many species, implying the existence of a ubiquitous causal relationship between sulphide and hypoxia-induced changes in vascular tone which arose early in evolution (Olson, 2012). In rat PAs, for example, application of NaHS causes a complex tri-phasic response – a small transient

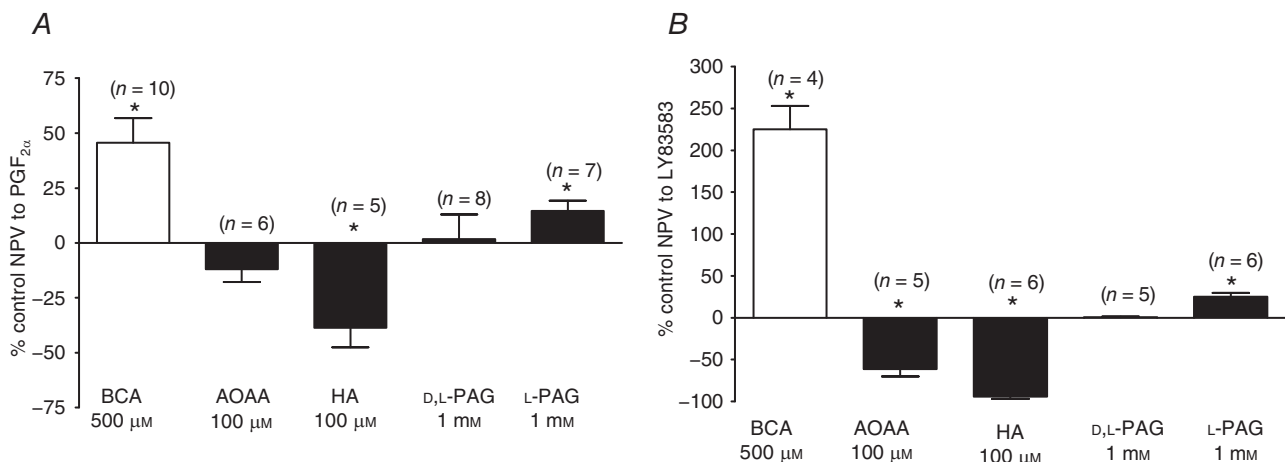


Figure 1. Effect of sulphide synthesis blockers on the responses to PGF_{2 α} and LY83583

A, effect of several CSE antagonists (BCA, 500 μ M; AOAA, 100 μ M; HA, 100 μ M; D,L-PAG, 1 mM; L-PAG, 1 mM) on contractions evoked by 30 μ M PGF_{2 α} . A control NPV to 30 μ M PGF_{2 α} was recorded, the agonist was washed out and the blocker was applied. PGF_{2 α} at 30 μ M was then re-applied 30 min later. The percentage change in the maximum amplitude of the second compared to the first PGF_{2 α} NPV is shown for each blocker. B, effect of several PAG blockers on the contraction evoked by 10 μ M LY83583. Blockers were added when the amplitude of the LY83583-induced NPV reached a constant level, and the maximum change in tension from that level was recorded and is plotted as the percentage of the LY83583 NPV immediately before addition of the blocker. **P* < 0.05 vs. control. The mean \pm SEM NPVs to 30 μ M PGF_{2 α} and 10 μ M LY83583 were 3.05 \pm 0.48 mN (*n* = 47) and 2.25 \pm 0.26 mN (*n* = 30) in the absence of inhibitors, respectively.

contraction followed by a relaxation and then a second maintained contraction – the profile of which bears some resemblance (albeit not a close one) to HPV (Olson *et al.* 2006). In addition, under anoxic conditions homogenates of rat lung supplied with L-cysteine and α -ketoglutarate

produced sulphide, which was then consumed when O₂ was introduced (Olson & Whitfield, 2010), and a similar effect occurred in pieces of isolated rat small PA (Madden *et al.* 2012). Also, HPV was greatly increased in isolated bovine PAs in the presence of a range of sulphide

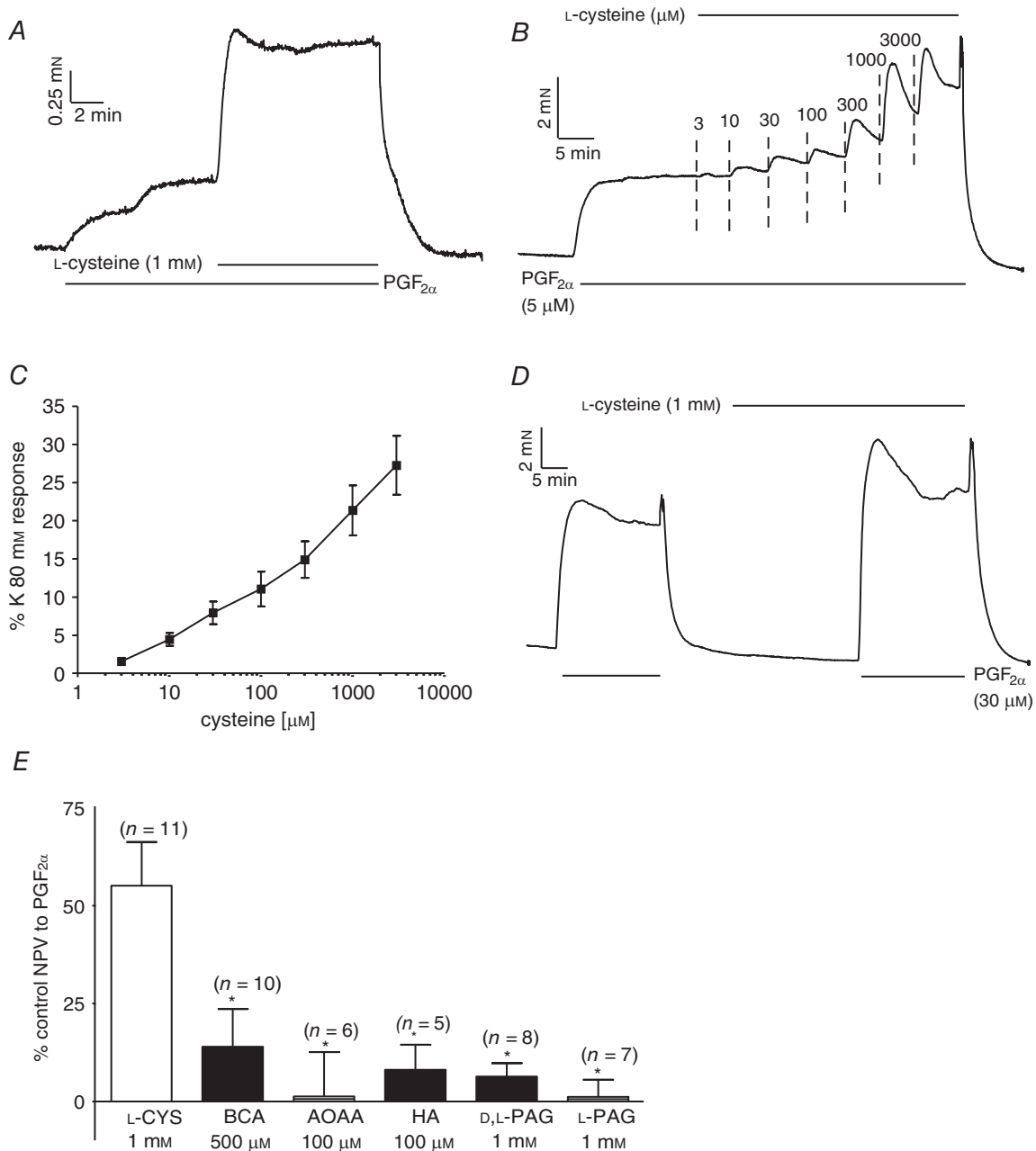


Figure 2. Effects of L-cysteine on basal and PGF_{2 α} -induced tension in IPAs
 A, example trace showing the increase in tension elicited by 1 mM L-cysteine during NPV induced by 5 μ M PGF_{2 α} .
 B, example of the concentration-dependent increase in tension evoked by cysteine (3 μ M–3 mM) during an NPV induced by 5 μ M PGF_{2 α} . C, average increases in tension recorded using the protocol described for B shown as percentage of the response to 80 mM K⁺ PSS, which represents the maximum contraction in that arterial ring (n = 15). D, example of the effect of pre-treatment with 1 mM cysteine on the contraction evoked by 30 μ M PGF_{2 α} . E, mean \pm SEM effect of 1 mM L-cysteine on the contraction evoked by 30 μ M PGF_{2 α} in the absence (leftmost bar) and presence of CSE inhibitors. *Significant effect (P < 0.05) of blocker on the response to cysteine alone. The mean \pm SEM NPV to 5 μ M PGF_{2 α} was 1.58 \pm 0.39 mN (n = 15) prior to the addition of cysteine.

donors, including cysteine, cysteine + α -ketoglutarate and glutathione (Olson *et al.* 2010), and in isolated perfused rat lungs in the presence of α -ketoglutarate (Madden *et al.* 2012).

Nonetheless, the only direct evidence for a causal relationship between sulphide and HPV is provided by observations that HPV is blocked by several antagonists of CSE and CBS, including PAG, AOAA, HA and BCA (Olson *et al.* 2006, 2010), and also by aspartate (Madden *et al.* 2012), which blocks CAT (Singh & Banerjee, 2011; Whiteman *et al.* 2011). However, the concentrations of the drugs which have been used to block CSE in studies of HPV (PAG, 10 mM; HA, 1 mM; AOAA, 1 mM; BCA, 10 mM) are much higher than those which are required to block purified CSE and CBS (Asimakopoulou *et al.* 2013). Results have also been variable, in that PAG did not block HPV in isolated bovine PAs (Olson *et al.* 2006) but abolished it in perfused rat lung (Madden *et al.* 2012). More generally, the concentration-dependency of CSE and CBS inhibition by these drugs in intact cells is not well understood, and all of them are known to have multiple non-selective effects, although when used with the proper controls they can provide meaningful information about the role of sulphide in cellular function (Whiteman *et al.* 2011; Whiteman & Winyard, 2011).

Effects of cysteine and CSE blockers on vasoconstriction under normoxic conditions

In light of these factors, we examined a number of widely used inhibitors of CSE, thought to be the most important

sulphide-synthesizing enzyme in the vasculature, to determine if any of them would be suitable for studying HPV. We used concentrations of the blockers lower than those which have previously been used to block HPV, but which have been shown to cause full inhibition of purified CSE (Asimakopoulou *et al.* 2013). Any sulphide synthesis blocker used to examine the role of sulphide in HPV should ideally have been first shown to fulfil three criteria. First, it should be selective, in the sense that it does not block contractions caused by stimuli which are not sulphide-dependent. Secondly, it should block sulphide synthesis, preferably in intact cells or tissues. Thirdly, it should demonstrate the ability to inhibit some change in tissue function that can reasonably be assumed to depend on sulphide synthesis.

Cysteine has frequently been used to stimulate sulphide synthesis in tissue homogenates, cells and intact tissues (e.g. Asimakopoulou *et al.* 2013), and has also been shown to enhance HPV, an effect ascribed to its ability to act as a sulphide precursor (Olson *et al.* 2010). It seemed reasonable that because sulphide causes contraction of PAs under normoxic conditions (Olson *et al.* 2006), cysteine might have the same effect. In accordance with this concept, cysteine caused a concentration-dependent increase in tension when applied to IPAs during an ongoing PGF_{2 α} -induced NPV, and moreover, if applied before PGF_{2 α} , increased the NPV when this drug was subsequently applied.

Each of the CSE inhibitors we tested prevented this potentiating effect of cysteine on the PGF_{2 α} -induced contraction, supporting the possibility that they were inhibiting cysteine-supported sulphide synthesis in intact

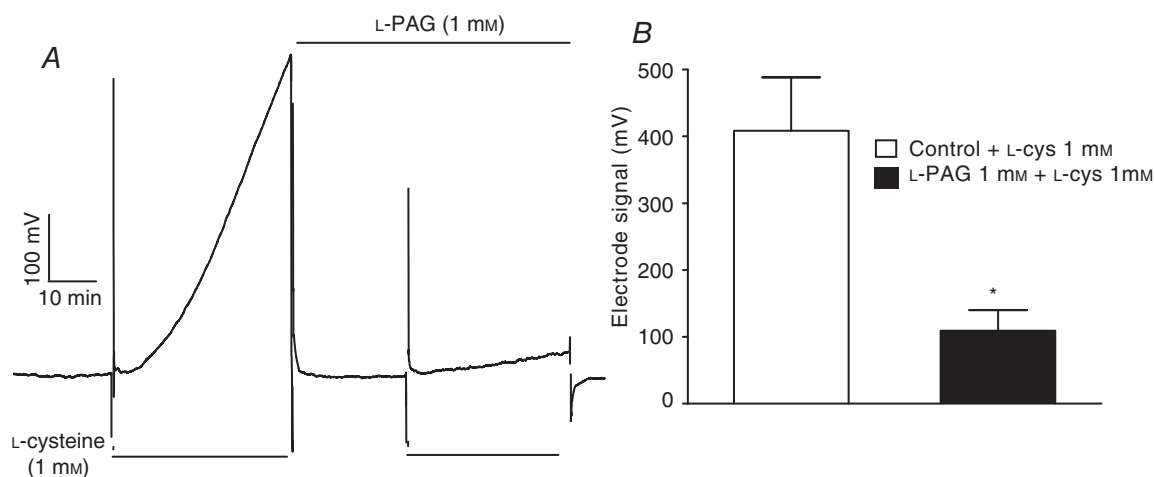


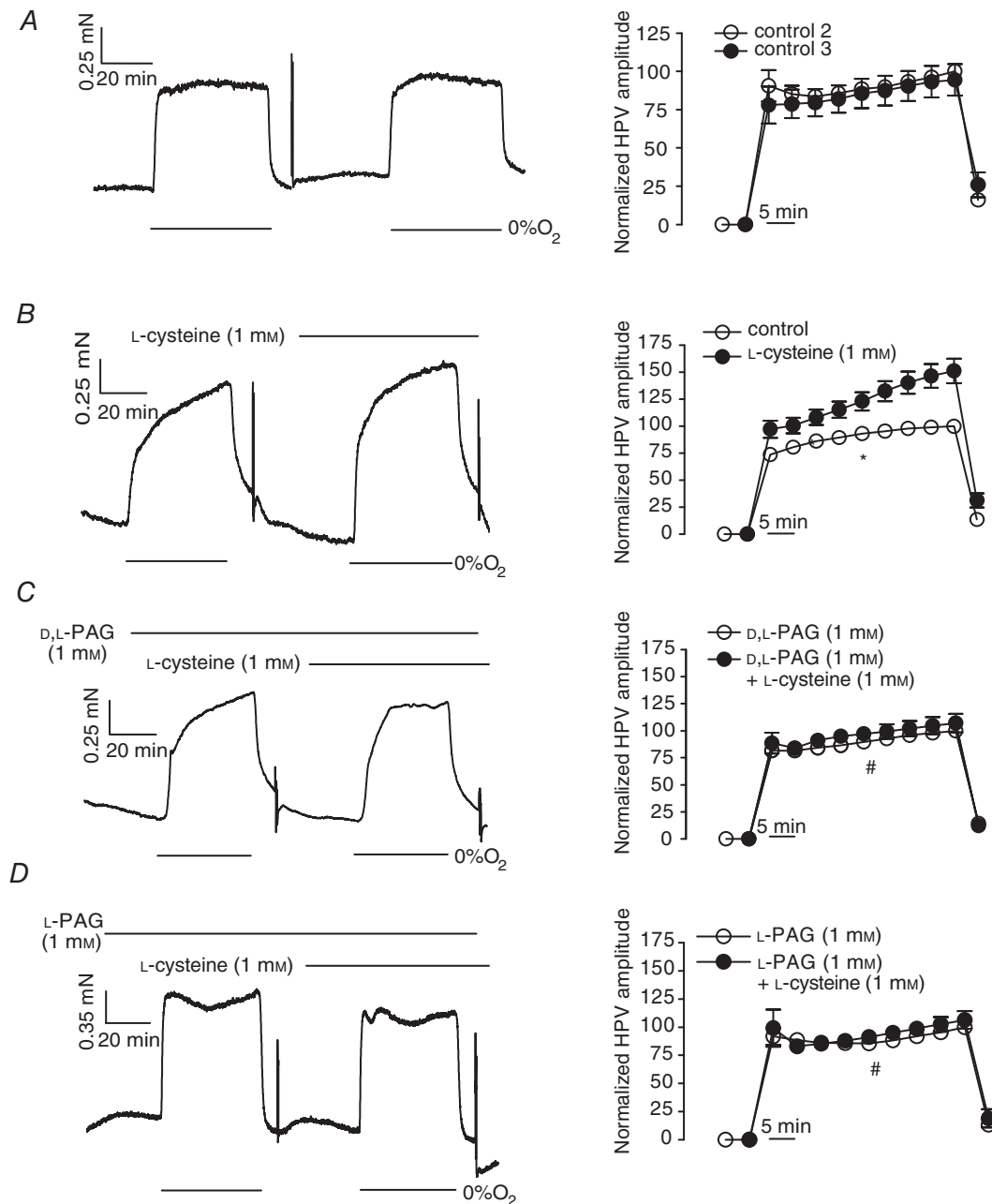
Figure 3. Block of cysteine-dependent sulphide synthesis by rat liver by L-PAG

Sulphide production and release into PSS by pieces of rat liver (175 mg) immersed in 1 ml of PSS containing 1 mM cysteine was recorded using an amperometric sulphide sensor. Vials were sealed to prevent the loss of sulphide, and the solution was pre-gassed with N₂/CO₂ to remove O₂ and thereby minimize sulphide oxidation. A, example of sulphide release in the presence of 1 mM cysteine in the absence (left) and presence (right) of 1 mM L-PAG. B, average cysteine-induced sulphide release in the absence and presence of L-PAG. Experiments were carried out in paired tissues, and results are normalized to the sulphide release observed in the control tissue ($n = 10$, mean \pm SEM). * $P < 0.05$ vs. control.

PAs. These results echo those of Elsey *et al.* (2010), who found that 1 mM cysteine mimicked exogenously applied sulphide in reducing ischaemia-reperfusion injury in isolated rat hearts, and that this effect was blocked by 1 mM PAG. L-PAG also strongly blocked sulphide release induced

by 1 mM L-cysteine in pieces of liver, a tissue in which CSE is thought to be the most important sulphide-synthesizing enzyme (Wang, 2012).

AOAA, HA and BCA demonstrated very marked non-selective effects on the NPV caused by PGF_{2α} and/or



the drug LY83583. Although LY83583 was originally introduced as a blocker of soluble guanylate cyclase, there is evidence that its contractile effect in PA is due to its ability to raise cellular ROS (Knock *et al.* 2009), consistent with it acting as a substrate for cellular oxoreductases (Hasegawa *et al.* 2004). The profound inhibition of the LY83583 contraction by HA and AOAA suggested that these drugs might be acting as anti-oxidants, in which case

they might affect HPV, which is likely to be triggered by changes in the cellular redox state (Waypa *et al.* 2010), independently of any effect on sulphide synthesis. In contrast, the racemic mixture of PAG had no effects of its own on contraction and L-PAG caused only a small potentiation of contraction, which tended to wane with time, suggesting that they might be useful agents for probing the role of sulphide in HPV.

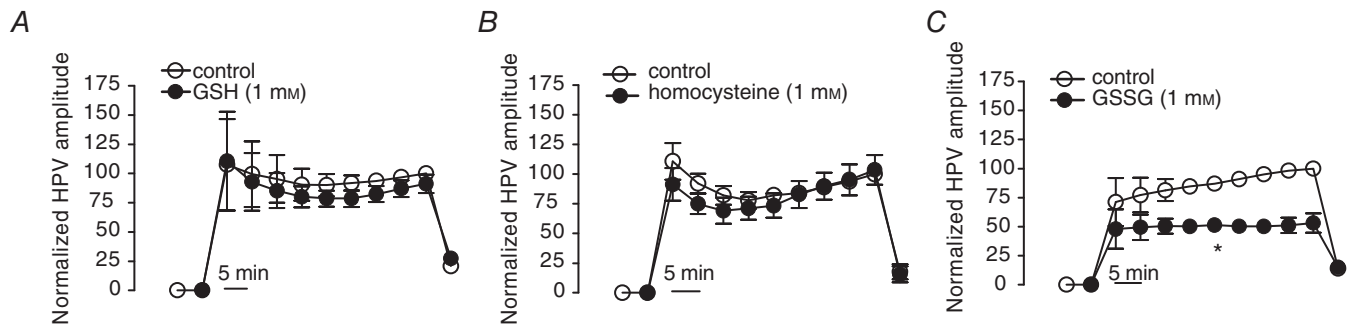


Figure 5. Effects of sulphide precursors GSH, GSSG and homocysteine on HPV evoked by gassing with 0% O₂ in the absence and presence of PAG
HPV to 0% O₂ before (open circles) and after (filled circles) incubation of IPAs with GSH (A, 1 mM, *n* = 11), GSSG (B, 1 mM, *n* = 8) or homocysteine (C, 1 mM, *n* = 5). **P* < 0.05 vs. control trace.

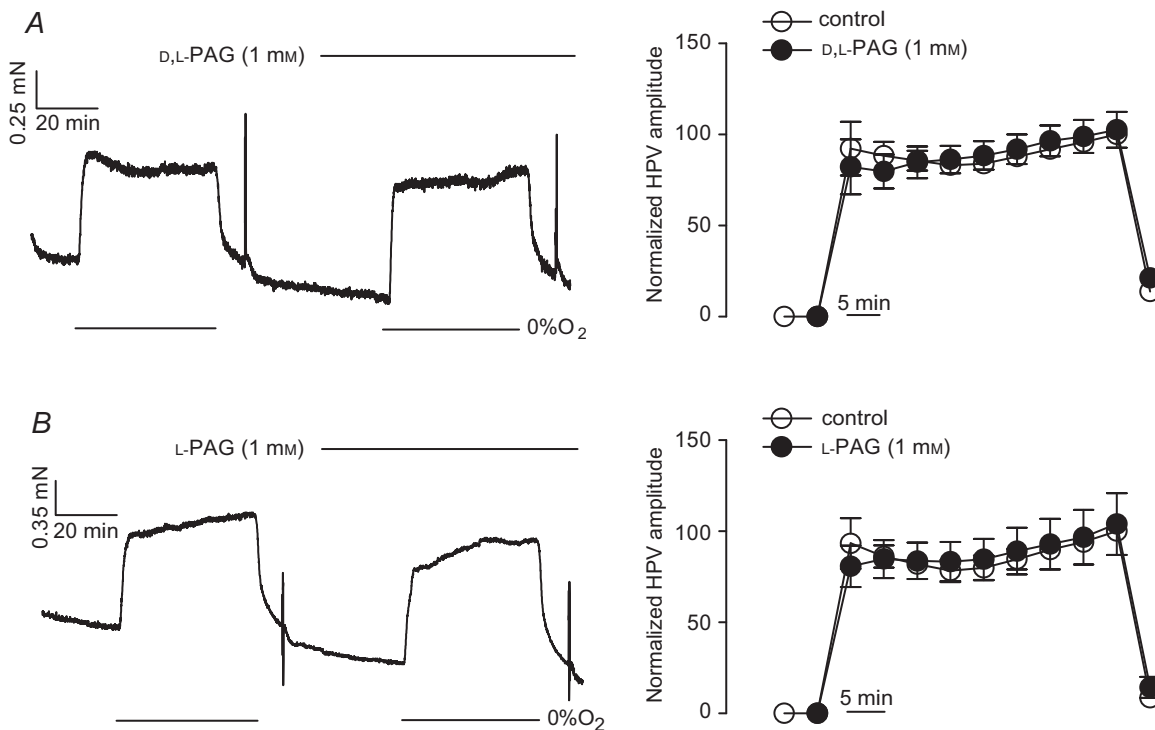


Figure 6. Effects of D,L-PAG and L-PAG on HPV in control PSS during hypoxia evoked by gassing with 0% O₂
HPV to 0% O₂ before (open circles) and after (filled circles) 30 min incubation of IPAs with D,L-PAG (A, 1 mM, *n* = 11), or L-PAG (B, 1 mM, *n* = 8). For both panels, averaged data are shown on the right and a tension trace from an experiment using the same protocol is shown on the left. No significant effect of PAG was observed at any time point.

Effects of cysteine and PAG on HPV

Similarly to its effect of the PGF_{2α}-induced NPV, 1 mM cysteine increased the amplitude of HPV by ~50%. The enhancement of HPV by cysteine was not mimicked by either reduced or oxidized glutathione (and in fact the latter inhibited HPV), suggesting that the effect of cysteine was not due to a rise in the intracellular glutathione concentration. The lack of effect of GSH on HPV also provides evidence that the effect of cysteine was not due

to its potential effect as a scavenger of HNO (Pino *et al.* 1994), as GSH shares this property (Fukuto *et al.* 2013).

The potentiation of HPV by cysteine was abolished by both D,L- and L-PAG, consistent with the idea that it was due to sulphide synthesis by CSE, and with previous observations (Olson *et al.* 2010). Both L-PAG and the racemic mixture had equivalent effects, implying that 0.5 mM L-PAG may have been sufficient to block CSE if D-PAG is not active against CSE

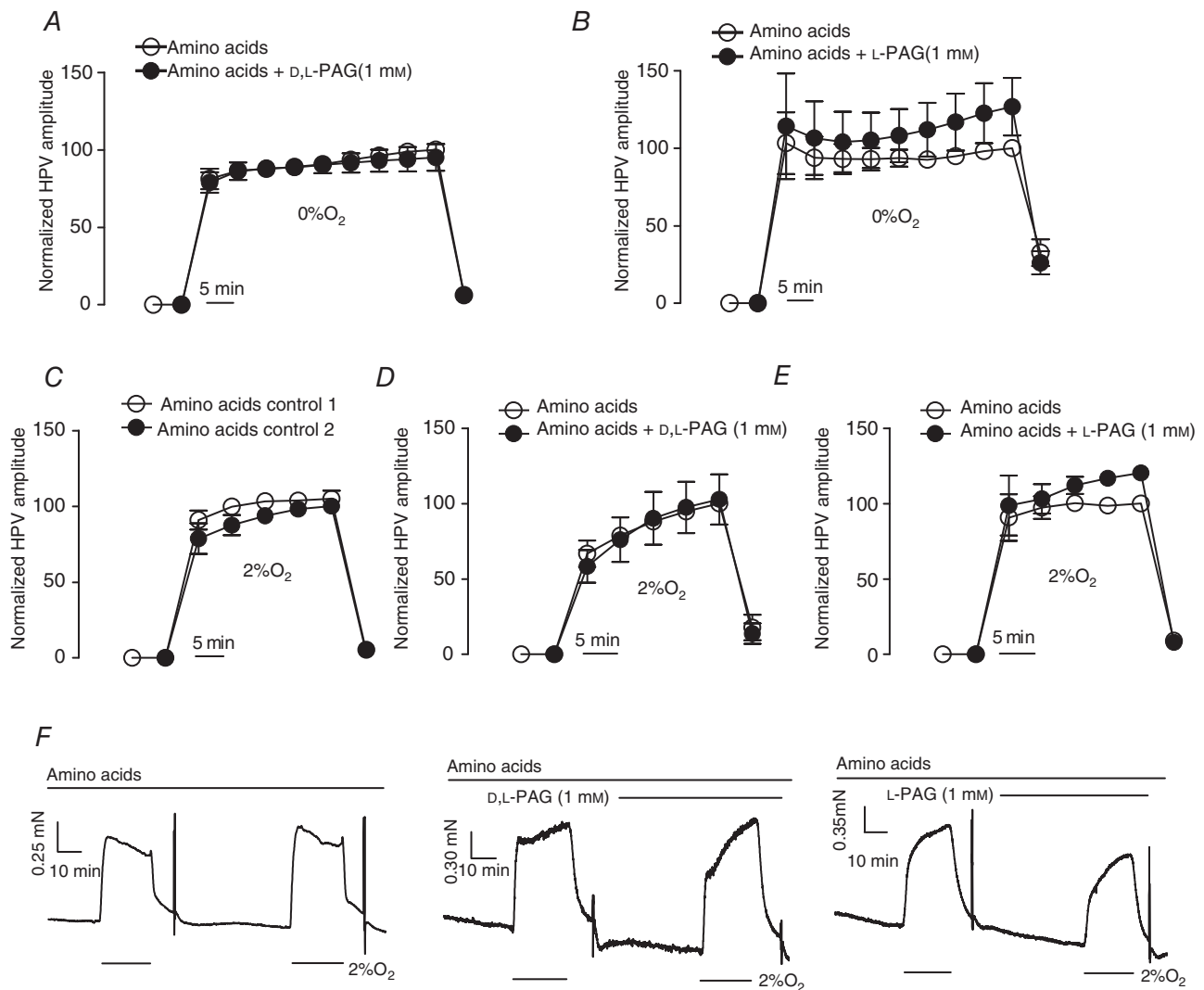


Figure 7. Effects of D,L-PAG or L-PAG on HPV evoked by 0% O₂ in normal PSS, or by 2% O₂ in PSS containing 10 μM L-cysteine, 50 μM L-cystine, and 100 μM L-glutamic acid

HPV to 0% O₂ in the presence of amino acids (10 μM cysteine, 50 μM cystine and 100 μM glutamate) before (open circles) and after (filled circles) incubation of IPAs with D,L-PAG (A, 1 mM, *n* = 8) or L-PAG (B, 1 mM, *n* = 8). Two successive HPVs to 2% O₂ under control conditions in the presence of amino acids (C, *n* = 12). HPV to 2% O₂ in the presence of amino acids before (open circles) and after (filled circles) 30 min incubation of IPAs with D,L-PAG (D, 1 mM, *n* = 8) or L-PAG (E, 1 mM, *n* = 7). F, representative tension trace from a time control experiment (left), an experiment with D,L-PAG (centre) and an experiment with L-PAG (right); in each case HPV was evoked by hypoxic challenge with 2% O₂ in PSS containing cysteine, cysteine and glutamate. The amplitudes of HPV evoked by 2% O₂ in the presence of PSS supplemented with amino acids shown in C–F were typical of those observed under these conditions; for time control experiments the increase in tension observed after 20 min of hypoxia was 0.77 ± 0.05 mN (*n* = 32).

(Asimakopoulou *et al.* 2013). Importantly, however, PAG only blocked the additional increment in HPV caused by the high concentration of cysteine, implying that the underlying response to hypoxia was insensitive to CSE blockade.

The lack of effect of PAG on HPV *per se* implied by this observation was confirmed by examining the effect of PAG on HPV evoked in normal PSS during hypoxic challenge with both 0% and 2% O₂. The rationale for using both levels of hypoxia was based on the observation that sulphide may trigger hypoxic constriction of trout gill by increasing the mitochondrial production of ROS (Skovgaard & Olson, 2012). In this case it seemed possible that sulphide-induced production of ROS, and therefore any contribution of sulphide to HPV, might be greater when hypoxia was imposed using 2% as compared to 0% O₂, as more O₂ would be available to produce ROS. Regardless of whether 0% or 2% O₂ was used for the hypoxic challenge, PAG had no effect on HPV evoked at either degree of hypoxia.

It is possible that lengthy incubation of PA in PSS could deplete cells of cysteine, leaving them unable to synthesize sufficient sulphide to affect O₂ sensing. Although it might be argued that in this case the persistence of HPV would itself rule out a pivotal role for sulphide in O₂ sensing, it is also possible that a sulphide-dependent mechanism might contribute one of several components of O₂ sensing, and that HPV normally observed *in vitro* would lack this component when cysteine becomes depleted. We therefore incubated arteries in a cocktail of quasi-physiological concentrations of cysteine, cystine and glutamate (Brigham *et al.* 1960; Milakofsky *et al.* 1985; Watford, 2002; Iciek *et al.* 2004). The latter two amino acids were added in addition to cysteine to allow cells to synthesize cysteine from cystine taken up by glutamate_{out}/cystine_{in} exchange via the X_C⁻ transporter (Siow *et al.* 1998; Go & Jones, 2011). However, HPV was still unaffected by PAG under these conditions, arguing against the possibility that the lack of effect of PAG on HPV in control PSS arises due to the lack of sufficient

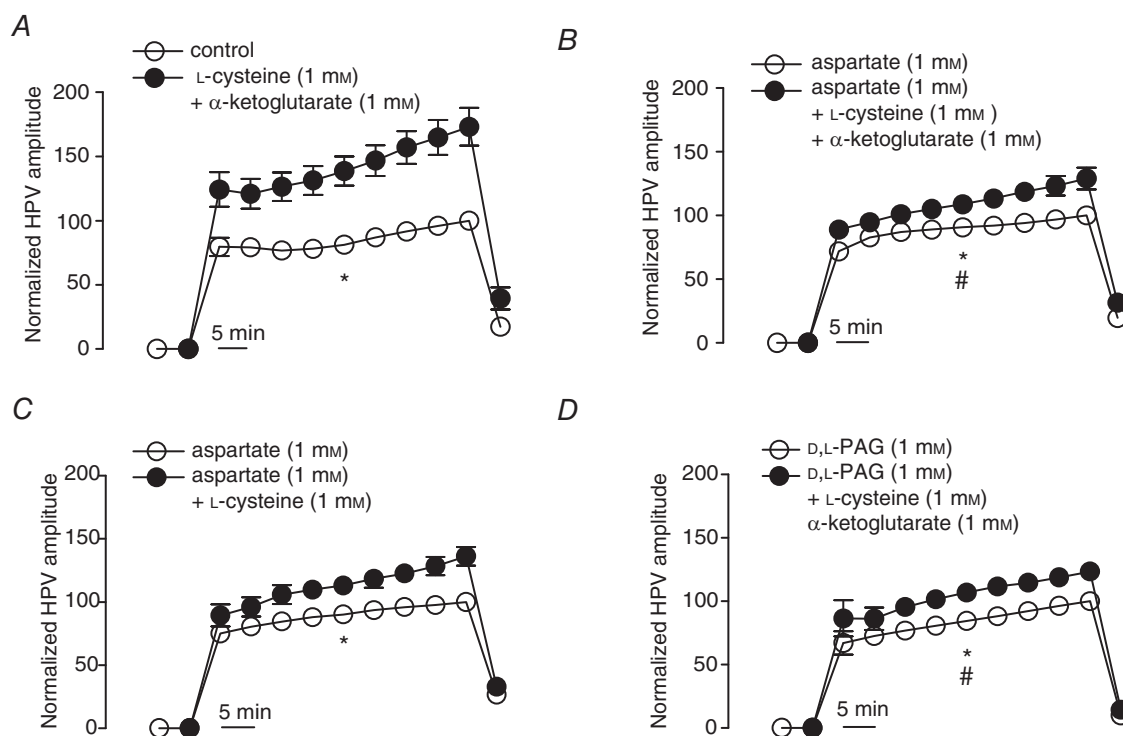


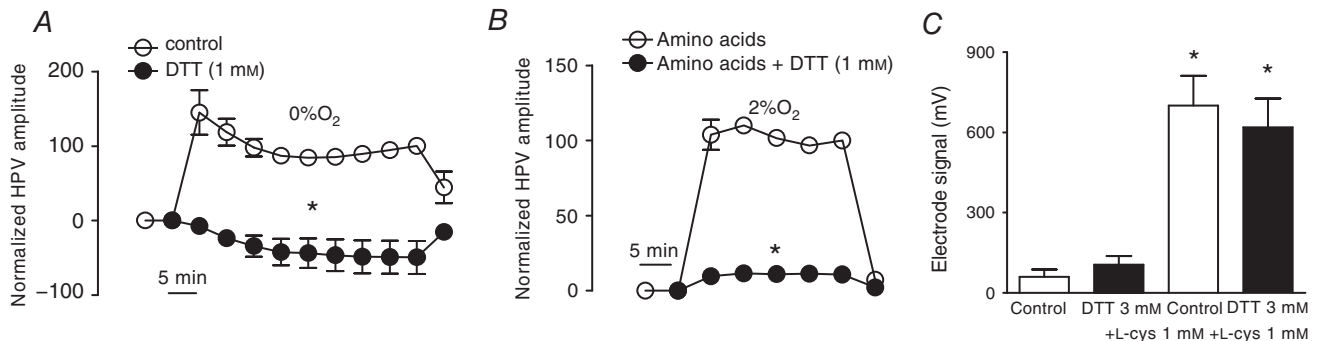
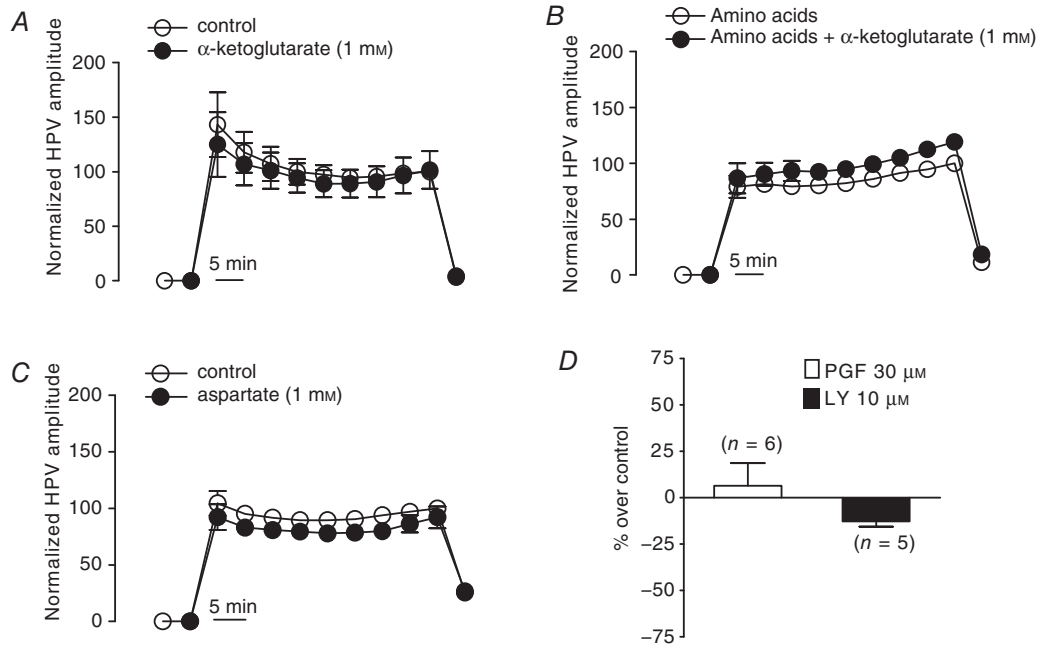
Figure 8. Effects of cysteine and α -ketoglutarate on HPV in the absence or presence of inhibitors of sulphide synthesis

HPV to 0% O₂ before (open circles) and after (filled circles) incubation of IPAs with L-cysteine and α -ketoglutarate (A, both 1 mM, $n = 24$) and in the additional presence of aspartate (B, 1 mM, $n = 18$). HPV to 0% O₂ in the presence of aspartate before (open circles) and after (filled circles) incubation of IPAs with L-cysteine (C, both 1 mM, $n = 11$). HPV to 0% O₂ in the presence of D,L-PAG before (open circles) and after (filled circles) incubation of IPAs with L-cysteine and α -ketoglutarate (both 1 mM, $n = 10$). For panels A, B and D : *significant ($P < 0.05$) effect of cysteine and α -ketoglutarate; #the response to L-cysteine and α -ketoglutarate was significantly inhibited ($P < 0.05$) at all the time points during hypoxia compared to the response in A. For panel C: *significant ($P < 0.05$) effect of cysteine; #the response to L-cysteine was significantly inhibited ($P < 0.05$) at all the time points during hypoxia compared to the response in Fig. 4B.

intracellular cysteine to support sulphide synthesis and a consequent enhancement of HPV.

It seemed unlikely that CBS would contribute to sulphide synthesis in rat PAs, as we detected no mRNA for this enzyme in these arteries and (Olson *et al.* 2010) did not detect CBS protein in PASMCS from bovine

PAs (although it was present in the endothelium). Also, homocysteine, which is metabolized to cysteine via CBS (Wang, 2012), did not enhance either HPV or the PGF_{2α} contraction (data not shown), implying that any CBS present would be insufficient to generate enough sulphide to affect tension development.



Effects on HPV of co-application of cysteine and α -ketoglutarate on HPV

The CAT/MST pathway provides an alternative route for sulphide synthesis, the involvement of which we also evaluated in light of our observation that MST is expressed in rat IPAs and also was found in bovine PAs (Olson *et al.* 2010) and rat. The combination of cysteine and α -ketoglutarate enhanced HPV, and this effect appeared to be slightly larger than that caused by cysteine alone. Although this difference was not statistically significant, we found that the CAT antagonist aspartate, although it did not affect the cysteine-induced increase in HPV, strongly attenuated the effect of cysteine + α -ketoglutarate on HPV. PAG also partially blocked the increase in HPV caused by cysteine and α -ketoglutarate, implying that both CSE and CAT/MST were contributing to sulphide synthesis under these conditions.

The increase in HPV elicited by the combination of cysteine and α -ketoglutarate was not significantly larger

than that caused by cysteine alone, implying that the CAT/MST pathway was not providing any additional increment in [sulphide] that would promote HPV over and above that due to the metabolism of cysteine by CSE. Nevertheless, aspartate attenuated the effect of cysteine + α -ketoglutarate on HPV. We speculate that a possible explanation for these apparently inconsistent results may be that the concentration of cysteine in cells is likely to be below its K_M values for both CAT and CSE, even if the solution has been supplemented with 1 mM cysteine (Singh & Banerjee, 2011). In this case, because elevating the α -ketoglutarate concentration in PSMCs should by the law of mass action increase the utilization of the relatively limited pool of cellular cysteine by CAT, the availability of cysteine for metabolism by CSE would be reduced. Thus, adding α -ketoglutarate could activate an aspartate-sensitive component of sulphide synthesis but at the same time diminish PAG-sensitive sulphide synthesis, such that overall sulphide synthesis might not change significantly.

Crucially, application of α -ketoglutarate on its own did not enhance HPV, either in normal PSS or in PSS supplemented with physiological concentrations of cysteine, cystine and glutamate. This implies that the CAT/MST pathway is not operative unless an artificially high concentration of cysteine is present. Likewise, aspartate itself had no effect on HPV under control conditions. These observations are consistent with the properties of CAT, which is thought to function mainly as an aspartate aminotransferase as its K_M for cysteine (22 mM) is much higher than that for aspartate (1.6 mM), whereas the intracellular concentration of aspartate (e.g. 730 μ M in mouse liver) is higher than that of cysteine (130 μ M) (Akagi, 1982; Singh & Banerjee, 2011).

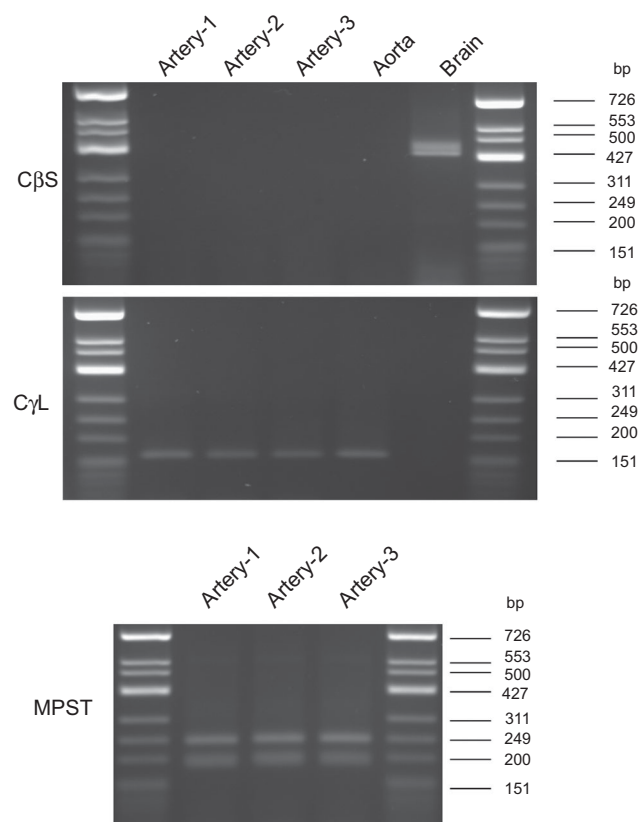


Figure 11. mRNA expression of C γ L, C β S and MST by RT-PCR on rat IPA, aorta and brain

The expected product sizes are 427 bp and 469 bp for cystathionine β -synthase (two alternative splice variants) (top), 160 bp for cystathionine γ -lyase (middle) and 243 bp for MST. An extra band at 200 bp with MST is likely to be the product of an alternative splice variant.

HPV and thiosulphate

Olson *et al.* (2013) have recently suggested that mitochondrial thiosulphate provides a reservoir of sulphide which is mobilized during HPV by a reducing shift in the mitochondrial redox state, with evidence for the latter provided by Waypa *et al.* (2010). This proposal was supported by the observation that HPV in bovine PAs was increased by incubation of tissues with the reducing agent DTT at concentrations of 100 μ M and above, and by evidence that DTT increased the production of sulphide induced by thiosulphate in homogenates of several tissues, including liver (Olson *et al.* 2013). These experiments were conducted under anoxic conditions in sealed vials to avoid sulphide oxidation and loss to the atmosphere. We conducted similar experiments, using pieces of intact liver, and recorded the release of sulphide into the solution

over 30 min in control PSS and also in PSS supplemented with 1 mM cysteine to promote the formation of sulphane sulphur, which would be predicted to give rise to sulphide when acted on by a reducing agent. Sulphide production was small and variable in control PSS, but was substantial in the presence of cysteine. In neither case was the release

of sulphide into the solution increased in the presence of 3 mM DTT. Moreover, rather than increasing HPV as described for bovine PAs, 1 mM DTT virtually abolished it, a finding consistent with reports from many laboratories that antioxidants tend to attenuate HPV (e.g. Connolly *et al.* 2013).

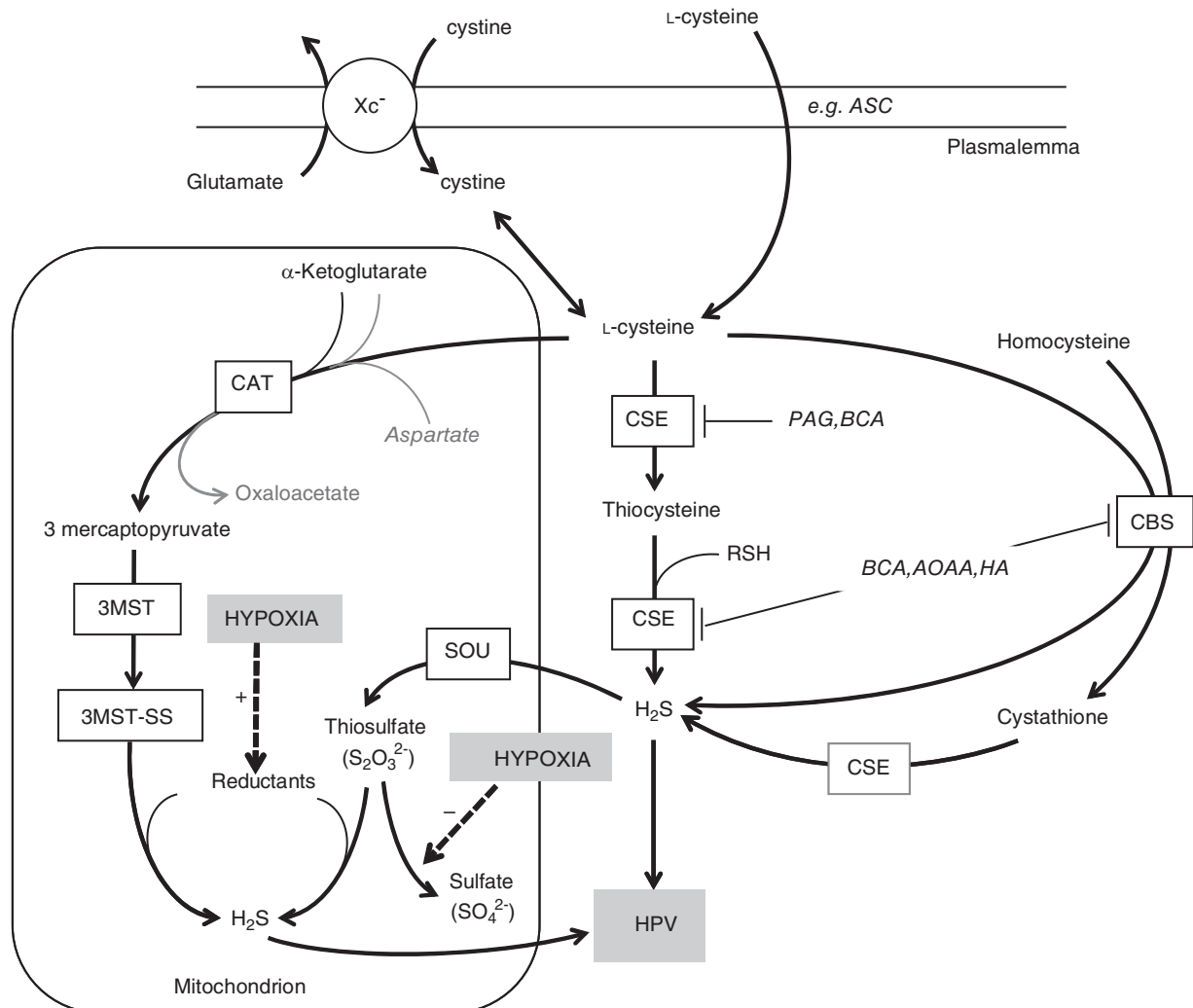


Figure 12. Pathways and inhibitors of sulphide synthesis relevant to the study

Sulphide is synthesized enzymatically from cysteine by cystathione γ -lyase (CSE) and cystathionine β -synthase (CBS); both are expressed in vascular cells but the former is thought to be more important. CSE is blocked by PAG, BCA, AOA and HA; the latter three drugs also block CBS. Sulphide can also be produced from 3 mercaptopyruvate (3-MP) by the mitochondrial enzyme 3 mercaptopyruvate sulphurtransferase (MST), a process which involves the addition of sulphane sulphur to MST to form MST-SS, and its subsequent release by reductants to yield sulphide. 3-MP is synthesized by cysteine aminotransferase (CAT) from cysteine and α -ketoglutarate. CAT also produces oxaloacetate from aspartate and α -ketoglutarate, such that aspartate competitively inhibits the production of 3-MP by CAT; the affinity of CAT for aspartate is much higher than that for cysteine (Singh & Banarjee, 2011). Cysteine can enter cells via various pathways, either as cystine through an exchange with glutamate mediated by the Xc⁻ transporter or directly through the alanine–serine–cysteine (ASC) or other transporters (Go & Jones, 2011). Sulphide is oxidized to thiosulphate by a mitochondrial ‘sulphide oxidation unit’ (SOU) comprising sulphide quinone oxoreductase (SQR), a dioxygenase (ETHE1) and a sulphur transferase (Szabo *et al.* 2014); thiosulphate is then oxidized further, ultimately to sulphate. Hypoxia is proposed to increase the sulphide concentration by attenuating its mitochondrial oxidation and also by increasing the concentration of mitochondrial reductants, which would promote the reduction of thiosulphate and the release of MST-bound sulphane sulphur (Olson *et al.* 2013).

Conclusions

Although our experiments cannot rule out the possibility that hypoxia might trigger HPV by raising the intracellular [sulphide] in PSMCs via pathways we did not investigate, the observations described here suggest strongly that HPV occurs without the need for sulphide synthesis by CSE or CAT/MST, and also do not support a role for mitochondrial thiosulphate in this response. Although it is remarkable that sulphide and hypoxia have similar effects in a multitude of vascular preparations, this could be explained if they cause contractions that share a common mechanism (e.g. increased production of ROS via mitochondrial complex 3, as we have previously suggested in preliminary reports; Connolly *et al.* 2010; Prieto-Lloret *et al.* 2011; Aaronson *et al.* 2012). Additional insights into the relationship between sulphide and HPV are likely to emerge as the mechanisms by which sulphide causes contraction in PAs are better defined.

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Additional information

Competing interests

None.

Author contributions

J.P.-L.: conception and design of the experiments, collection, analysis and interpretation of data, drafting the article or revising it critically for important intellectual content. Y.S.: conception and design of experiments, collection, analysis and interpretation of data. J.P.T.W.: conception and design of the experiments, drafting the article or revising it critically for important intellectual content. P.I.A.: conception and design of the experiments, collection, analysis and interpretation of data, drafting the article or revising it critically for important intellectual content.

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