Potentiation of Hypoxic Pulmonary Vasoconstriction by Hydrogen Sulfide Precursors 3-Mercaptopyruvate and D-Cysteine Is Blocked by the Cystathionine γ Lyase Inhibitor Propargylglycine

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Abstract

Although the gasotransmitter hydrogen sulfide (H_2S) generally dilates systemic arteries in mammals, it causes constriction of pulmonary arteries. In isolated rat pulmonary arteries, we have shown that the H_2S precursor cysteine enhances both hypoxic pulmonary vasoconstriction and tension development caused by the agonist prostaglandin $F_{2\alpha}$ under normoxic conditions. These effects were blocked by propargylglycine (PAG), a blocker of the enzyme cystathionine γ lyase which metabolises cysteine to sulfide. In the present study, we evaluated whether 3-mercaptopyruvate (3-MP), a sulfide precursor which is thought to give rise to sulfide when it is metabolised by the enzyme mercaptopyruvate sulfurtransferase, also enhanced contraction. Application of 3-MP prior to hypoxic challenge caused a marked enhancement of HPV which was completely blocked by both L- and D,L-PAG (both 1 mM). Cumulative application of 3–1,000 μM 3-MP during an ongoing contraction to $PGF_{2\alpha}$ under normoxic conditions also caused a marked increase in tension. Application of D-cysteine (1 mM) also enhanced HPV, and this effect was prevented by both the D-amino acid oxidase inhibitor sodium benzoate (500 μM) and 1 mM L-PAG.

Keywords

Hypoxic pulmonary vasoconstriction • 3-mercaptopyruvate • D-cysteine

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10.1 Introduction

The gasotransmitter H$_2$S (hereafter 'sulfide') is produced in cells *via* both enzymatic and non-enzymatic pathways (*e.g.* mobilization by reductants of sulfane sulphur bound to proteins). Three enzymes, cystathionine γ lyase (CSE), cystathionine β synthase (CBS), and mercaptopyruvate sulfurtransferase (MST), which acts in concert with cysteine amino-transferase(CAT), produce sulfide from cysteine. Of these, CSE is thought to be the most important enzymatic source of sulfide in the cardiovascular system (Wang 2012).

We reported that application of cysteine to rat pulmonary arteries (PA) causes further tension development during an ongoing vasoconstriction to the agonist PGF$_{2\alpha}$, and also potentiates hypoxic pulmonary vasoconstriction (HPV), the unique response of pulmonary arteries which functions to match pulmonary perfusion to ventilation during hypoxia (Prieto-Lloret et al. 2014). Both effects were blocked by the CSE antagonist PAG, implying that cysteine induced potentiation of contraction was due to the production of sulfide, which causes contraction in these arteries (Olson et al. 2006).

We have recently observed that these effects of L-cysteine are mimicked by exogenously applied 3-mercaptopyruvate (3-MP), a substance which is produced when cysteine is metabolised by CAT, and which can then be further processed by MST to form sulfide. However, 3-MP binds to MST with a rather low affinity (Km=~1.5 mM), whereas we find that application of as little 10 μM exogenous 3-MP causes tension development.

However, CAT is a reversible enzyme (Singh and Banerjee 2011), and the possibility therefore exists that it can use 3-MP to generate L-cysteine which is then metabolised to sulfide by CSE. In the current study we explored this possibility by examining the effect of 3-MP on HPV in the presence and absence of PAG. Similar experiments were carried using D-cysteine, which can be metabolised to 3-MP via the enzyme D-amino acid oxidase (DAO).

10.2 Methods

10.2.1 Ethical Approval and Myography

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) were killed by lethal injection (*i.p.*) of sodium thiopental. The heart and lungs were excised and placed in cold physiological salt solution (PSS) containing (in mmol/L): 118 NaCl, 24 NaHCO$_3$, 1 MgSO$_4$, 0.435 NaH$_2$PO$_4$, 5.56 glucose, 1.8 CaCl$_2$, and 4 KCl. Rings of small PA (inner diameter 0.5–1.0 mm) were cleaned of adventitia and parenchyma under a dissection microscope, mounted on an isometric small vessel wire myograph, and stretched to 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 KCl (80KPSS; isotonic replacement of NaCl by KCl). The pH of the solution was maintained at 7.4 by gassing with 20 %O$_2$, 5 %CO$_2$, balance N$_2$.

10.2.2 HPV Experimental Protocol, Data Representation and Statistical Analysis

PA were challenged with hypoxia to evoke HPV three times, with each 45 min hypoxic episode being separated from the others by ≥35 min. Hypoxia was applied by gassing the myograph solution with 0 % O$_2$, which caused a rapid fall in pO$_2$ in the solution to a stable level of 6–9 mmHg. Tension was measured just before hypoxia was imposed, every 5 min during the hypoxic periods, and then 5 min after re-oxygenation when HPV had almost completely subsided.

As described in Connolly et al. (2013) we used the 2nd HPV as the control response and experimental interventions (*e.g.* applying 3-MP)

were made 30 min prior to the 3rd challenge with hypoxia. When we examined the effect on HPV of a combination of two agents (e.g. PAG and 3-MP), the first was applied 30 min before the 2nd hypoxic challenge and remained present during the 3rd hypoxic challenge and the second was then applied after the 2nd HPV, 30 min prior to the 3rd hypoxic challenge. For calculating effects of drugs on HPV, 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 The amplitudes of the control and intervention HPVs were compared every 5 min using repeated measures ANOVA followed by a Bonferroni posthoc test in order to determine whether the intervention had significantly changed the response.

In other experiments, 3-MP or D-cysteine were applied cumulatively in ascending concentrations during an ongoing stable contraction to 5 μM $PGF_{2\alpha}$. The resulting increase in force was expressed as a percent of the maximal (80KPSS) contraction in that artery. When used to study this response, inhibitors were applied 30 min before $PGF_{2\alpha}$; their effects were assessed using Student's unpaired t-test. The threshold for statistical significance for all comparisons was set at $P < 0.05$.

10.3 Results

As shown in Fig. 10.1a, a low concentration of 3-MP (30 μM) markedly enhanced HPV. This response was abolished by the CSE blocker PAG (1 mM) in both its L and racemic forms (Fig. 10.1b, c).
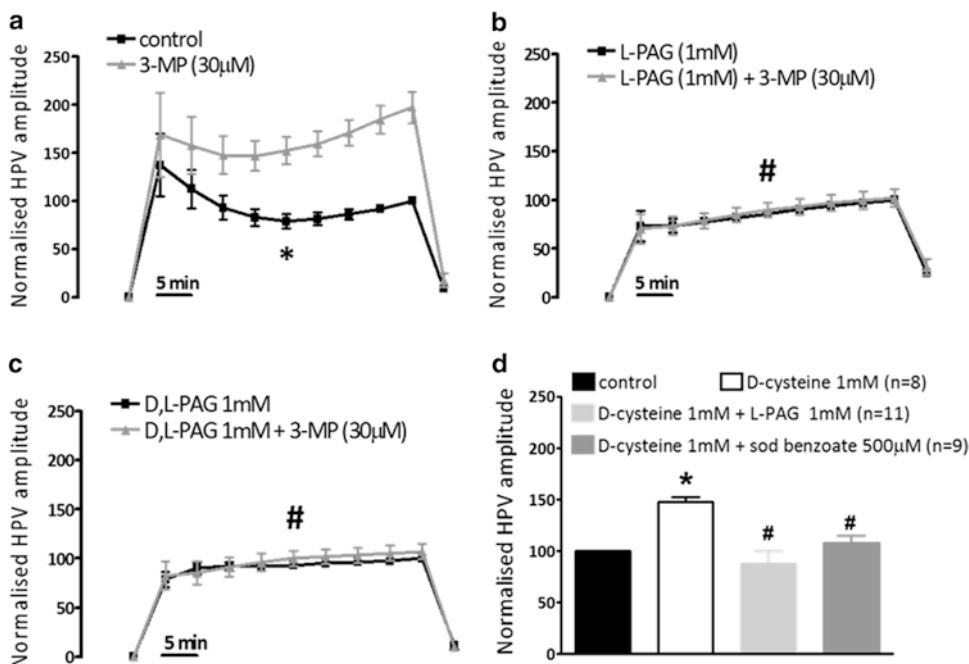

Fig. 10.1 3-MP – induced enhancement of HPV is blocked by PAG. HPV to 6–9 mmHg O_2 before (*black squares*) and after (*grey triangles*) incubation of PAs with 30 μM 3-MP (**a**, n=11), 30 μM 3-MP in the additional presence of 1 mM L-PAG (**b**, n=8) and 30 μM 3-MP in the additional presence of D,L-PAG (**c**, n=9). Results are shown as mean±SEM; * indicates a significant (p<0.05) effect of 3-MP on HPV, # indicates a significant block of the effect of 3-MP by PAG. (**d**) Effect of 1 mM D-cysteine on HPV, measured after 45 min of hypoxia, under control conditions and in the presence of 1 mM PAG or 500 μM sodium benzoate. D-cysteine significantly (p<0.05) increased HPV amplitude (*), and this effect was significantly suppressed by PAG and sodium benzoate (#)

Similarly, D-cysteine (1 mM) caused a significant increase in the amplitude of HPV (Fig. 10.1d; the amplitude of HPV measured after 45 of hypoxia is shown). This effect of D-cysteine was abolished by L-PAG, and also by the DAO inhibitor sodium benzoate (500 μM). Neither of these blockers had any effect on HPV in the absence of D-cysteine (not shown).

Under normoxic conditions, 3-MP (3–1,000 μM) caused a concentration-dependent increase in tension when applied during a stable ongoing contraction to 5 μM $PGF_{2\alpha}$ (Fig. 10.2a, b). 1 mM 3-MP applied on its own also caused a contraction, and this was reversed by the reducing agent dithiothreitol (DTT, 1 mM) (Fig. 10.2c). Like 3-MP, D-cysteine also caused an increase in tension when applied during a contraction to

5 μM $PGF_{2\alpha}$, although this was smaller than that induced by 3-MP (Fig. 10.2d) and reached a maximum level before appearing to fall at higher concentrations. Like its effect on HPV, the increase in the $PGF_{2\alpha}$ contraction caused by D-cysteine was significantly attenuated by sodium benzoate (Fig. 10.2d).

10.4 Discussion

The key novel finding of these experiments is that the putative sulfide precursor 3-MP enhances both HPV and the contraction to $PGF_{2\alpha}$ under normoxic conditions. D-cysteine, which can be converted to 3-MP by D-amino acid oxidase, also caused a PAG-sensitive enhancement of HPV.
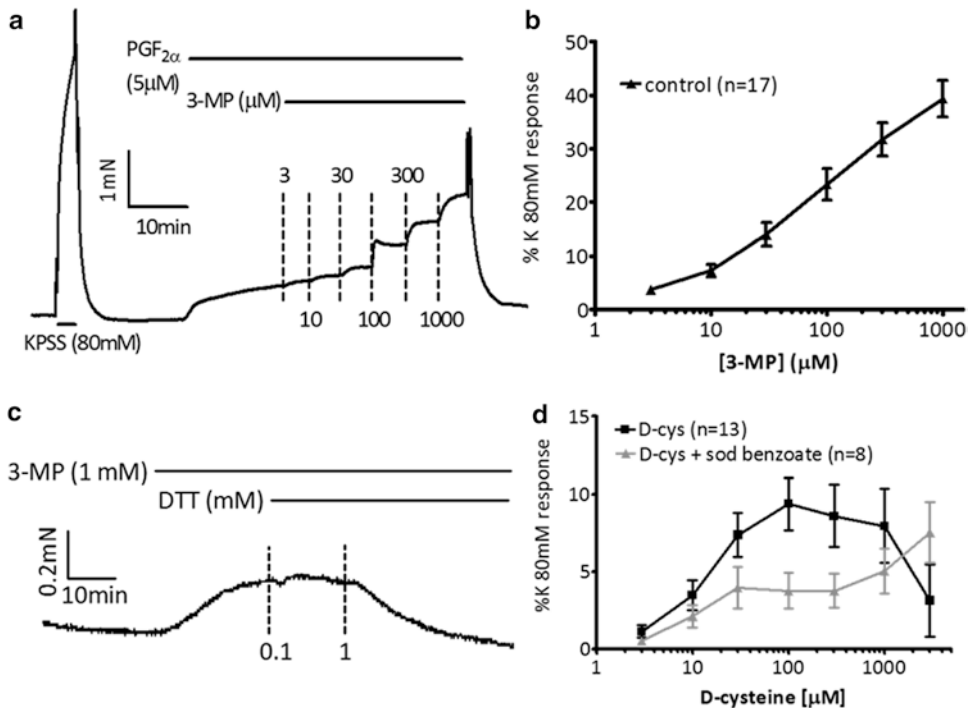

Fig. 10.2 3-MP and D-cysteine enhance, and DTT inhibits, the contraction induced by 5 μM $PGF_{2\alpha}$ under normoxic conditions. (**a**) Typical experiment showing that cumulative application of 3-MP (3–1,000 μM) during an ongoing contraction to 5 μM $PGF_{2\alpha}$ caused a progressive further increase in tension. (**b**) Mean ± SEM increase in tension caused by 3-MP in 17 experiments using the protocol described for panel A (*solid triangles*). Tension is expressed as a percent of that evoked by 80KPSS in that artery, which represents the maximal contraction. (**c**) Effect of 0.1 and 1 mM DTT on the contraction evoked by 1 mM 3-MP. Trace shown is representative of 5 similar experiments. (**d**) Effect of on tension cumulative application of D-cysteine (3–3,000 μM) during an ongoing contraction to 5 μM $PGF_{2\alpha}$ in the absence (*black squares*) and presence (*grey triangles*) of 500 μM sodium benzoate

Sulfide can be synthesised from L-cysteine by enzymatic pathways involving CBS, CSE, and CAT/MST. CSE, which is strongly expressed in rat PA (Prieto-Lloret et al. 2014), is generally thought of as being the important sulfide-synthesising pathway in the vasculature (Liu et al. 2012). MST is also expressed in rat pulmonary arteries (Olson et al. 2010; Prieto-Lloret et al. 2014), whereas CBS is not (Prieto-Lloret et al. 2014).
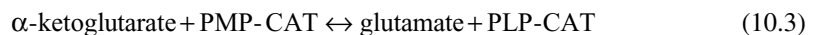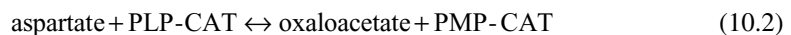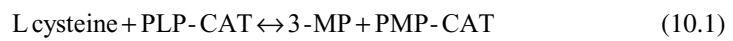
Although it is unclear whether D-cysteine is produced in the body, a significant proportion of L-cysteine is converted to the D form during food processing and can enter the body that way (Shibuya et al. 2013). D-cysteine can then be converted to 3-MP by D-amino acid oxidase, which is strongly expressed in the kidney and parts of the brain (Kimura 2014).

PA are unusual in that they are constricted by sulfide, which generally dilates systemic arteries (Olson and Whitfield 2010). We have reported that pre-incubation of isolated rat pulmonary artery segments in 1 mM L-cysteine increased the magnitude of both HPV and the contraction to PGF_{2a} under normoxic conditions. These effects were reversed by blocking CSE with PAG but not by the CAT blocker aspartate, implying that they were most likely due to an increased production of sulfide via CSE (Prieto-Lloret et al. 2014).

On the other hand, application of 1 mM α-ketoglutarate together with 1 mM cysteine also caused an enhancement of HPV which was markedly attenuated by aspartate as well as PAG, leading us to speculate that in the presence of exogenous α-ketoglutarate, which is required for the metabolism of cysteine by CAT, cysteine might also yield sulfide via the CAT/MST (Prieto-Lloret et al. 2014).

CAT, which is found both in the mitochondria and the cytoplasm, is also referred to as aspartate aminotransferase (AAT). It catalyses the following reversible reactions,

$$L\text{ cysteine} + PLP\text{-}CAT \leftrightarrow 3\text{-}MP + PMP\text{-}CAT \tag{10.1}$$

$$aspartate + PLP\text{-}CAT \leftrightarrow oxaloacetate + PMP\text{-}CAT \tag{10.2}$$

$$\alpha\text{-ketoglutarate} + PMP\text{-}CAT \leftrightarrow glutamate + PLP\text{-}CAT \tag{10.3}$$

in which pyridoxal-5′-phosphate (PLP) acts as a co-factor which accepts an amino acid from cysteine or aspartate, becoming pyridoxamine-5′-phosphate (PMP), and then donates it to α-ketoglutarate to form glutamate (Singh and Banerjee 2011).

MST then metabolises 3-MP, resulting in the transfer of sulfane sulphur to form MST-SSH. This then gives rise to sulfide owing to the action of reductants such as dihydrolipoic acid (Olson et al. 2013).

Notably, however, the K_m for the metabolism of 3-MP by MST is high (>1 mM) even under the highly alkaline conditions which favour this reaction (Singh and Banerjee 2011). The extent to which the CAT/MST pathway synthesises sulfide in cells of the vasculature at normal intracellular concentrations of cysteine is therefore questionable (Kimura 2014).

To further explore whether the CAT/MST pathway plays any role in promoting contraction in pulmonary arteries, we examined whether 3-MP was able to mimic L-cysteine in enhancing both HPV and the contraction to $PGF_{2\alpha}$ evoked under normoxic conditions, and found that 30 μM 3-MP had an effect similar to, and indeed somewhat larger than, L-cysteine (see Prieto-Lloret et al. 2014 for the effect of cysteine).

There are currently no well-characterised pharmacological antagonists of MST with which to assess the effects on cells of its metabolism of 3-MP. However, given that the production of sulfide by the CAT/MST pathway is facilitated by
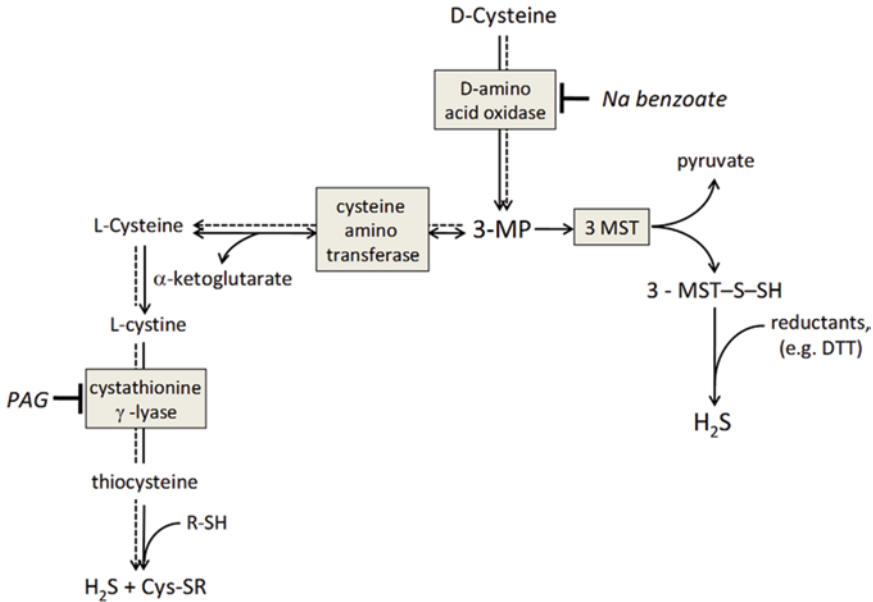
Fig. 10.3 Enzymatic pathways by which sulfide may be synthesized from 3-MP and D-cysteine. The scheme illustrated is based on information presented in Wang (2012), Olson et al. (2013) and Kimura (2014). The *dashed line* shows the proposed pathway by which sulfide may be synthesized from D-cysteine and 3-MP under hypoxic conditions

reductants (Olson et al. 2013), it might be predicted that the reductant dithiothreitol (DTT) would increase the contractile effect of 3-MP. However, as shown in Fig. 10.2c, 1 mM DTT inhibited rather than increased the contraction caused 1 mM 3-MP. Although this was not a conclusive experiment, as DTT might cause relaxation through effects independent of sulfide, it suggested that 3-MP might give rise to sulfide via another pathway, i.e. the production of L-cysteine by the reversal of CAT and its subsequent metabolism by CSE to sulfide.

In this case, it would be predicted that PAG should block the enhancement of HPV and of the $PGF_{2\alpha}$ – induced contraction by 3-MP. The results shown in Fig. 10.1 are in accord with this prediction, since 1 mM PAG, which would not be expected to block MST, abolished the potentiating effect of 30 μM 3-MP on HPV.

Since 3-MP can also be produced in cells through the metabolism of D-cysteine by DAO, it would be expected that D-cysteine should mimic the effects of 3-MP, and that these effects should be inhibited by blocking DAO or PAG. As shown in Fig. 10.1d, both PAG and the DAO blocker sodium benzoate prevented the enhancement of HPV induced by 1 mM D-cysteine. D-cysteine also increased tension in the presence of $PGF_{2\alpha}$ and this was attenuated by sodium benzoate (Fig. 10.2d).

In summary, both 3-MP and D-cysteine promote HPV in rat PA, and this effect is blocked by PAG. Since both can give rise to L-cysteine, which also enhances HPV in a PAG-sensitive manner, we speculate that under hypoxic conditions sulfide can be produced by the reversal of CAT and the pathway illustrated by the dashed line in Fig. 10.3, at least when cells are supplied with sufficient D-cysteine or 3-MP. However, the extent to which this pathway is operative in these arteries under physiological conditions remains to be determined.

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