DR VERA RALEVIC (Orcid ID : 0000-0002-5252-280X)



Article type : Regular Paper

# Coronary artery hypoxic vasorelaxation is augmented by perivascular adipose tissue through a mechanism involving hydrogen sulfide and cystathionine-β-synthase

J Donovan, P S Wong, 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 PVAT

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

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/apha.13126

**Abstract**

**Aim**

Hypoxia causes vasodilatation of coronary arteries which protects the heart from ischaemic damage through mechanisms including the generation of hydrogen sulfide ($H_2S$), but the influence of the perivascular adipose tissue (PVAT) and myocardium is incompletely understood. This study aimed to determine whether PVAT and the myocardium modulate the coronary artery hypoxic response, and whether this involves hydrogen sulfide.

**Methods**

Porcine left circumflex coronary arteries were prepared as cleaned segments and with PVAT intact, myocardium intact or both PVAT and myocardium intact, and contractility investigated using isometric recording. Immunoblotting was used to measure levels of $H_2S$ synthesizing enzymes: cystathionine-β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (MPST).

**Results** All three $H_2S$ synthesizing enzymes were detected in the artery and myocardium, but only CBS and MPST were detected in PVAT. Hypoxia elicited a biphasic response in cleaned artery segments consisting of transient contraction followed by prolonged relaxation. In arteries with PVAT intact hypoxic contraction was attenuated and relaxation augmented. In arteries with myocardium intact hypoxic contraction was attenuated, but relaxation was unaffected. In replacement experiments, replacement of dissected PVAT and myocardium attenuated artery contraction and augmented relaxation to hypoxia, mimicking *in situ* PVAT and indicating involvement of a diffusible factor(s). In arteries with intact PVAT, augmentation of hypoxic relaxation was reversed by amino-oxyacetate (CBS inhibitor), but not DL-propargylglycine (CSE inhibitor) or aspartate (inhibits MPST pathway).

**Conclusion**

PVAT augments hypoxic relaxation of coronary arteries through a mechanism involving $H_2S$ and CBS, pointing to an important role in regulation of coronary blood flow during hypoxia.

**Keywords: c**oronary artery; hydrogen sulfide; hypoxia; perivascular adipose tissue; vasorelaxation

## Introduction

In the heart, hypoxia which occurs as a consequence of ischaemia is a dangerous insult which compromises cardiac function. Coronary vasodilatation is an important physiological response to hypoxia that increases blood flow and, therefore, oxygenation to the hypoxic or ischaemic myocardium, but the mechanism is not fully defined. We and others have recently shown that hydrogen sulphide ($H_2S$) (or other thiol-derived product) is a mediator of hypoxic relaxation in porcine coronary arteries,[1,2] consistent with its suggested role as an oxygen sensor/transducer in the vasculature.[3,4] We showed that this involves principally the $H_2S$ synthesizing enzyme cystathionine β-synthase (CBS).[2] This is important because cystathionine γ-lyase (CSE) is widely regarded as the principal $H_2S$ synthesising enzyme in the vasculature,[5] and thus CBS emerges as a novel therapeutic target for treating ischaemic heart disease. These experiments were carried out in porcine coronary arteries that had been cleaned of their adherent perivascular adipose tissue (PVAT) and myocardium and the location of the CBS involved in hypoxic relaxation was identified as the vascular smooth muscle. The influence of the PVAT and myocardium on the hypoxic response of the vasculature is incompletely understood.

PVAT is attracting increasing interest because of its role as a source of vasoactive factors.[6-12] We were interested in its contribution to vasomotor function during hypoxia in the heart because reduced oxygenation in ischemia would be expected to affect the adjacent fat and the myocardium as well. The interactions between the artery wall, PVAT and myocardium during hypoxia are poorly defined. In preliminary studies, we observed that when the PVAT surrounding coronary artery segments was left intact, hypoxic relaxation was augmented.[13] In mouse aorta, PVAT similarly enhanced the hypoxic relaxation response, but the mediator involved was not identified.[14] The objective of the present study was, therefore, to define the role of PVAT and myocardium in the hypoxic response of the coronary artery and to test the hypothesis that this involved $H_2S$, since there is evidence that $H_2S$ can be released from adipocytes as a vasorelaxing factor.[15,16] As a part of this investigation we sought to

specifically define which of the $H_2S$-synthesizing enzymes, CBS, CSE and/or 3-mercaptopyruvate sulfurtransferase (3-MST), was involved in the hypoxic production of $H_2S$ in the coronary artery by PVAT and/or myocardium.

## Results

### Effect of *in situ* PVAT and myocardium on the coronary artery response to hypoxia

In the cleaned segments of porcine coronary artery (PVAT and myocardium removed), hypoxia (95 % $N_2$, 5 % $CO_2$; measured oxygen tension of 30 µM) caused a biphasic response consisting of an initial transient contraction, followed by a prolonged and pronounced vasorelaxation (Figure 1), as observed by us and others previously.[2,17] When both PVAT and myocardium were left attached to the artery segments the hypoxic contraction was abolished and the relaxation was augmented, by more than 100 % (control 71.7 ± 5.6 %, with PVAT and myocardium 160.8 ± 15.3 %, n = 8; $p < 0.001$, paired t test) (Figure 1A-C). These changes were mimicked in vessels in which PVAT alone was left attached to the artery segments; here the hypoxic contraction was reduced and the relaxation was augmented (control 93.5 ± 7.2 %, with PVAT 123.9 ± 8.3 %, n = 8; $p < 0.01$, paired t test) (Figure 1D-F). In vessels in which myocardium alone was left attached to the artery segments, the hypoxic contraction was also blunted, but the relaxation response was not significantly different to that in the controls (control 94.9 ± 12.7 %, with myocardium 96.0 ± 13.9 %, n = 8; $p > 0.05$, paired t test) (Figure 1G-I). These data show that both PVAT and myocardium attenuate the coronary artery hypoxic contraction, but only PVAT augments the hypoxic relaxation.

Data for the effects of the different experimental preparations (PVAT alone, myocardium alone, and myocardium plus PVAT) on contractility to U46619 and KCl, and on the concentration of U46619 used to achieve the required level of contractile tone, under normoxic conditions, are shown in Figure 2. U46619-induced tone was generally well controlled at ~50-60% of the contraction to KCl (Figure 2B), and the contraction to KCl was unaffected by the different experimental conditions (Figure 2C). Interestingly, the concentration of U46619 required to achieve this level of tone was greater in preparations with PVAT (PVAT alone and PVAT plus myocardium), consistent with an anticontractile

effect of PVAT (Figure 2A). Myocardium alone had no significant effect on either the concentration of U46619 or the level of tone. The effect of endothelium removal is shown for reference, and shows that a lower concentration of U46619 is needed to precontract the artery segments in the absence of endothelium (Figure 2A), consistent with the known anticontractile effect of the endothelium. Effects of the endothelium on the hypoxic response are considered in detail in our previous study.[2]

**Effect of replacing dissected PVAT and myocardium on the coronary artery response to hypoxia**

In order to investigate the possible involvement of a diffusible factor, replacement experiments were carried out where dissected PVAT and/or myocardium were placed adjacent to segments of cleaned porcine coronary artery segments (PVAT and myocardium removed) within the organ baths. The presence of both dissected PVAT and myocardium reduced the hypoxic contraction, although not significantly, and augmented the hypoxic relaxation of the coronary artery segments (control 75.5 ± 6.2 %, with added PVAT and myocardium 99.5 ± 4 %, n = 8; $p < 0.001$, paired t test) (Figure 3A,B), thus mimicking the *in situ* effects of PVAT and myocardium on the hypoxic response. The augmentation of the hypoxic relaxation was less pronounced than in the vessels with intact fat and myocardium (~25 % increase with replaced PVAT and myocardium versus >100% increase with *in situ* PVAT and myocardium) (compare Figures 1B and 3B). These data suggest the involvement of a diffusible factor released from PVAT and/or myocardium that can modulate the coronary artery response to hypoxia. Neither replacement of PVAT alone nor myocardium alone, however, mimicked the effects of replacing both PVAT and myocardium; replacement of PVAT alone caused a modest but significant increase in hypoxic contraction and attenuation of relaxation (Figure 3C,D); replacement of myocardium alone had no significant effect on either phase of the hypoxic response (Figure 3E,F).

**Effect of inhibitors of $H_2S$ synthesis on the response to hypoxia in coronary arteries**

The above experiments suggested that PVAT, but not myocardium, modulates the coronary artery hypoxic relaxation response and this involves the actions of a diffusible factor. Accordingly, we used artery segments with PVAT left intact to test the hypothesis that $H_2S$

was involved, using inhibitors of CSE (PPG, 10 μM) and CBS (AOAA, 100 μM), and inhibition of the MPST pathway (aspartate, 1 mM). In arteries with intact PVAT the hypoxic contraction was attenuated and this effect was unaffected by CSE, CBS or MPST, applied alone or in combination (Figure 4A,B). In arteries with intact PVAT the coronary artery hypoxic relaxation was augmented and this effect was reversed by AOAA, which reduced the response ~50 % thus normalising the hypoxic response (control 69.1 ± 5 %, with PVAT 103.5 ± 9.1 %, with PVAT plus AOAA 49.9 ± 6.6 %, n = 7-15; $p < 0.05$, ANOVA) (Figure 4A,C). AOAA was similarly effective when applied alone or in combination with the other $H_2S$ enzyme inhibitors, although for some of the combinations the effect did not reach statistical significance (Figure 4C). In contrast, augmentation by PVAT of hypoxic relaxation was unaffected by either an inhibitor of CSE (PPG) or an inhibitor of the MPST pathway (aspartate), or by a combination of PPG and aspartate (Figure 4C). These data suggest that $H_2S$ or a thiol-derived product is released from PVAT during hypoxia to augment coronary artery hypoxic relaxation.

**Measurement of endogenous $H_2S$ levels and release in coronary arteries, PVAT and myocardium**

$H_2S$ levels in the extracellular space above homogenates from cleaned coronary arteries (without PVAT or myocardium), PVAT alone and myocardium alone, contained within sealed containers, was measured using a zinc trap method during conditions of hypoxia (95% $N_2$, 5% $CO_2$) and during gassing with oxygen (95% $O_2$, 5% $CO_2$). Gaseous $H_2S$ levels were, unexpectedly, lower under conditions of hypoxia than during gassing with oxygen, for both the porcine coronary arteries and PVAT (Figure 5B). Only for myocardium was the gaseous $H_2S$ greater under conditions of hypoxia than during gassing with oxygen (Figure 5B). Analysis of control samples (absence of tissue) showed an increase in $H_2S$ detected during gassing with oxygen compared to hypoxia (Figure 5C). Measurement of $H_2S$ levels in homogenates of porcine coronary arteries alone, PVAT alone and myocardium alone showed that the tissue levels were unchanged by the different gassing conditions (Figure 5A).

**Expression of CSE, CBS and MPST in the porcine coronary artery and in its adjacent muscle and fat**

Using antibodies we have previously characterized for cross-reactivity with porcine enzymes,[18] immunoblot analysis showed that all three enzymes CSE (44 kDa) (Figure 6), CBS (63 kDa) (Figure 7) and MPST (33 kDa) (Figure 8) are present within the porcine coronary artery (using actin (45 kDa) as a control to normalise for protein level variation) (n=4). All three enzymes were also present in the adjacent myocardium; expression levels of CSE in the myocardium (Figure 6) were lower than those of CBS and MPST (Figure 7 and 8). CBS and MPST, but not CSE, were detected in the PVAT (Figure 6-8). These data are in line with our previous observations in the cleaned porcine coronary artery[2] and extend the data to coronary artery PVAT and myocardium.

**Discussion**

The main finding of this study is that PVAT augments the hypoxic relaxation response of porcine coronary arteries through a mechanism which involves $H_2S$. Contractility studies using inhibitors of the enzymatic synthesis of $H_2S$ showed that the most important enzyme responsible for $H_2S$ generation from coronary artery PVAT during hypoxia is CBS, and this correlates with the immunoblotting data which show that CBS and MPST, but not CSE, are the principal $H_2S$ synthesising enzymes expressed in coronary artery PVAT.

The porcine coronary artery response to hypoxia consists of an initial transient contraction followed by a prolonged relaxation; the hypoxic contraction is endothelium dependent and involves NO and the relaxation is endothelium-independent.[2,17] We have recently provided evidence that hypoxic relaxation of the porcine coronary artery involves $H_2S$ generated predominantly by CBS; in that study the PVAT and myocardium were dissected away and the source of the $H_2S$ mediating hypoxic relaxation was identified as the vascular smooth muscle.[2] In the present study, we investigated the effect of leaving intact the adjacent PVAT and myocardium to mimic more closely the conditions experienced by the coronary artery *in vivo*. When PVAT and myocardium were both left intact, this abolished the hypoxic contraction and greatly augmented hypoxic relaxation of the coronary artery, by more than 100 %. Leaving intact either PVAT, or myocardium, also attenuated the hypoxic contraction,

but only PVAT augmented the hypoxic relaxation. These data indicate that the coronary artery relaxation response to hypoxia can be profoundly increased by the adjacent PVAT. This is not simply due to an anticontractile effect of PVAT on hypoxic contraction, and consequent inhibition of the functional antagonism that contraction may have on hypoxic relaxation, because both PVAT and myocardium alone reduced the hypoxic contraction, but only PVAT augmented relaxation.

In order to investigate whether these effects involved a diffusible factor we carried out replacement experiments where dissected PVAT and myocardium, separately or combined, were placed adjacent to the coronary artery in the organ baths. We found that replacement of PVAT and myocardium together mimicked the effect of leaving PVAT intact, and PVAT and myocardium together intact, on the hypoxic response; the hypoxic vasorelaxation was augmented and the hypoxic contraction was attenuated. This indicates the involvement of vasorelaxant/anticontractile factor(s) released from PVAT and/or myocardium during hypoxia. The changes seen with the replacement experiments, for both hypoxic contraction and relaxation, were smaller than those observed when PVAT and myocardium were left *in situ*, which is consistent with the involvement of a diffusible factor achieving different local concentrations at the artery under the two experimental conditions. Replacement experiments involving PVAT alone or myocardium alone did not mimic their *in situ* effects, or the effect or replacing PVAT together with myocardium, for reasons which are unclear.

We and others have shown that $H_2S$ is an important mediator of the hypoxic vasorelaxant response of the coronary artery, in artery segments with their PVAT removed.[1,2] In the present study, further studies were carried out to test its possible involvement as a diffusible factor released from PVAT. In vessels with intact PVAT, the augmentation of the coronary artery hypoxic relaxation was reversed by AOAA, normalising the hypoxic response and pointing to CBS as the enzyme involved in the generation of $H_2S$ by PVAT. AOAA was similarly effective when applied alone or in combination with the other inhibitors of the enzymatic synthesis of $H_2S$ (PPG and aspartate). In contrast, augmentation of hypoxic relaxation by PVAT was unaffected by an inhibitor of CSE (PPG) and inhibition of the MPST pathway (aspartate), or by their combination. As in PVAT, CBS also appears to be the most important enzyme involved in hypoxic generation of $H_2S$ by the coronary artery (in the

absence of PVAT and myocardium).[2] It is unlikely that reversal by AOAA of the PVAT-mediated augmented hypoxic relaxation is simply due to its attenuation of $H_2S$ produced by the coronary artery smooth muscle (and not $H_2S$ produced by PVAT), since AOAA at 100 μM was ineffective against hypoxic relaxation of the cleaned coronary artery (absence of PVAT and myocardium),[2] whereas in the present study it reduced the hypoxic relaxation in arteries with intact PVAT by ~50 % (Figure 4C).

The hypoxic contraction of the porcine coronary artery without PVAT and myocardium is endothelium-dependent and a principal mechanism involved is the NO pathway.[2,17] The present *in situ* experiments suggest that both PVAT and myocardium can attenuate the hypoxic contraction, and the replacement experiments identify the involvement of a diffusible factor. In arteries with PVAT intact the attenuated contraction was, interestingly, unaffected by inhibitors of CSE (PPG), CBS (AOAA) or of the MPST pathway (aspartate), applied alone or in combination (Figure 3B). The simplest explanation is that an anticontractile factor that is not $H_2S$ is involved in PVAT-mediated attenuation of hypoxic contraction of the coronary artery. Evaluation of its identity needs further experimentation and there are a number of possible candidates.[6-12] In rat coronary arteries, cardiomyocyte-rich perivascular tissue had an anticontractile effect on agonist-induced contractions, which involved a reduction in $Ca^{2+}$ sensitivity and was unaffected by the CSE inhibitor PPG.[19,20] We found no anticontractile effect of myocardium on responses to U46619 under normoxic conditions, suggesting that there is no ongoing basal release of anticontractile factors from myocardium adjacent to the porcine coronary artery. This is in contrast to PVAT and endothelium, the presence of both of which was associated with an anticontractile effect as evidenced by the requirement of a lower concentration of U46619 to induce contractile tone. However, our data do suggest that hypoxia is a stimulus for the release of non-$H_2S$ anticontractile factors from myocardium.

The concentrations of the inhibitors that we used (PPG, 10 μM; AOAA, 100 μM) are in line with their relative potencies at CSE and CBS respectively. AOAA has limited selectivity for CBS,[21,22] however, since PPG had no effect on the hypoxic response this is unlikely to involve CSE. In addition, we have previously shown that AOAA, but not PPG, blocks pyridoxal phosphate-dependent generation of $H_2S$ from the porcine coronary artery.[2] The

inhibitors were shown to have no effect on pinacidil induced relaxations of the porcine coronary artery.[2] The augmented hypoxic relaxation that we have observed is thus likely to involve CBS-mediated generation of $H_2S$ by PVAT under hypoxic conditions, increasing the levels of $H_2S$ overall. The $H_2S$ then causes relaxation through a mechanism which may involve potassium channels and $Cl^-/HCO_3^-$ exchange.[1,2,14,23] Maenhaut et al. similarly suggested an involvement of potassium channels in the enhanced hypoxic vasorelaxation of mouse aortas with intact adipose tissue.[14] Activation of potassium channels has been suggested to release $H_2S$ from PVAT as a relaxing factor in rodent mesenteric artery and aorta.[16]

We investigated the effect of hypoxia on $H_2S$ levels measured in the space immediately above homogenates of porcine coronary arteries, PVAT and myocardium, in sealed Erlenmeyer flasks, using a zinc trap method.[24] We detected an increase in gaseous $H_2S$ during hypoxia only for the myocardium. However, $H_2S$ levels measured in controls in the absence of tissue showed $H_2S$ levels to be greater during gassing with oxygen than during hypoxia for reasons which are not clear. This may have masked changes in $H_2S$ levels in the experiments using tissues. Our data suggest that $H_2S$ generated within the tissue during hypoxia does not accumulate in the extracellular space at levels sufficient for detection by the zinc trap method. Measurement of $H_2S$ levels in tissue homogenates showed no differences in $H_2S$ levels with gassing condition in the porcine coronary arteries, PVAT and myocardium.

Immunoblotting was carried out to investigate which $H_2S$ synthesizing enzymes are involved in the hypoxic generation of $H_2S$; this showed the presence of all three $H_2S$-synthesizing enzymes, CBS, CSE and MPST, in the coronary artery, consistent with our previous immunoblotting and immunohistochemical studies.[2] CBS, CSE and MPST were all also found to be expressed in the myocardium. However, only CBS and MPST were expressed in PVAT, in line with our functional studies pointing to an involvement of CBS in augmentation of hypoxic relaxation of the coronary artery by PVAT. In contrast, CSE is the main $H_2S$-synthesising enzyme in rat aortic PVAT and adipocytes of epididymal fat pads.[15,25] Hypoxic relaxation of the rat thoracic aorta was essentially abolished by PPG,[3] pointing to a predominant involvement of CSE and suggesting that there are pronounced tissue and/or species differences in the enzymes involved in hypoxic synthesis of $H_2S$. Although the size

and arterial system of the porcine heart are very similar to that of humans,[26] further investigations are warranted to understand whether the $H_2S$ system within the coronary vasculature and its PVAT is a therapeutic target in man.

In conclusion, the present study has shown for the first time that the coronary artery hypoxic response is augmented by PVAT and this involves $H_2S$ generated by CBS. Our finding that $H_2S$ is an important endogenous mediator released during a hypoxic insult in the coronary vasculature is consistent with evidence in other blood vessels (rat aorta, lamprey and hagfish dorsal aorta, rat and bovine pulmonary arteries, perfused rat lung) that $H_2S$ acts as an oxygen sensor and transducer of the hypoxic response.[3,4,27,28] We have recently suggested that CBS may be a novel therapeutic target for the treatment of heart failure;[2] the present study reinforces this and identifies the cellular location of this target (CBS) as PVAT as well as the vascular smooth muscle.

## Materials and methods

### Tissue preparation

Pig hearts (either sex, age less than 6 months, weighing ~50 kg) were obtained on ice from a local abattoir (G Wood & Sons Ltd, Mansfield, UK). The left circumflex coronary artery was crudely dissected and stored overnight at 4 °C in gassed (95 % $O_2$, 5 % $CO_2$) Krebs-Henseleit solution as previously described.[2,29] After allowing time for equilibration to room temperature, coronary arteries were either: a) finely dissected to remove surrounding tissue, b) dissected with fat attached to the vessel, c) dissected with muscle attached to the vessel, or d) dissected with both fat and muscle attached to the vessel. Arteries were then cut into rings of ~5 mm (internal diameter ~2-3 mm) and suspended between two wires before transfer to organ baths containing gassed (95% $O_2$, 5% $CO_2$) Krebs'-Henseleit solution. In some experiments, PVAT, myocardium, or both PVAT and myocardium were finely dissected out from the porcine coronary arteries and then were threaded onto the same wires used to suspend the porcine coronary artery segments, to investigate the possible involvement of a diffusible factor. The endothelium was left intact in all artery segments except for some of the data in Figure 2, where indicated; here the endothelium was removed by gentle rubbing as previously described.[2]

**Responses in the porcine isolated coronary artery**

Arterial rings were mounted onto wires in 20 ml tissue baths. Each ring was attached to an isometric force transducer (ADInstruments, Sydney, Australia) which was connected to a Powerlab (ADInstruments). Tension changes were captured by a computer running LabChart software (ADInstruments). The arterial rings were tensioned to ~10 g and allowed to equilibrate for 60 min. The viability of the tissue was assessed using 60 mM KCl. Once a reproducible response to KCl was achieved (2-3 additions) and the tissue had returned to baseline tension, the thromboxane $A_2$ analogue U46619 (11α,9α-epoxymethano-$PGH_2$) was added in a cumulative manner to contract the vessels to ~50-60 % of the final KCl response.

**Experimental protocols**

Once an appropriate level of U46619 induced contraction had been achieved, the vessels were exposed to hypoxia by switching the gas to 95% $N_2$/ 5% $CO_2$ for 30 min. The roles of PVAT and myocardium as modulators of the contraction and relaxation response of porcine coronary arteries under hypoxic conditions were examined in the presence or absence of PVAT and myocardium as described above. To define if $H_2S$-synthesizing enzymes were involved in the hypoxic production of $H_2S$ in the porcine coronary arteries by PVAT and/or myocardium, the effects of the CSE inhibitor DL-propargylglycine (PPG, 10 μM), CBS inhibitor amino-oxyacetate (AOAA, 100 μM) and MPST pathway inhibitor (1 mM aspartate; inhibits cysteine aminotransferase activity) alone or in combination were examined. All inhibitors were added before the addition of U46619, and thus were in contact with the tissue for at least 1 hour prior to hypoxia.

**Immunoblotting**

Coronary arteries were fine dissected and then segments were immediately frozen on dry ice. The 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). Once homogenised, the protein concentration in each of the samples was measured using the Bradford method and normalised. Samples, with solubilization buffer 6 × SB (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 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) for 60 min at room temperature. Blots were incubated overnight at 4 °C with primary antibody (1 μl per 500 ml MPST, 1.5 μl CBS/CSE in 500 ml) diluted in blocking solution. We used antibodies previously characterised for cross-reactivity with porcine enzymes with further validation using omission of primary antibody.[2,18] After washing in Tris-buffered saline containing 0.1% (v/v) Tween 20, the blots were incubated with an appropriate IRDye®-conjugated secondary antibody (Li-Cor Biosciences, Biotechnology, Lincoln, NE, USA). Proteins were visualized using the Li-Cor/Odyssey infrared imaging system.

**Endogenous $H_2S$ Production Assays**

The enzyme activity of endogenous $H_2S$ production in porcine coronary arteries, PVAT and myocardium was determine using the methylene blue method adapted from.[24] Segments of finely dissected porcine coronary arteries (10% wt/vol), PVAT (14% wt/vol) or myocardium (14% wt/vol) were homogenized in ice-cold Tris-EDTA buffer (0.1 M, 1 mM, pH 7.4) using Precellys 24 homogenizer (Bertin Technologies, Stretton, Derbyshire) for porcine coronary arteries and Ultra-turrax homogenizer (Janke & Kunkel IKA-Werke GmbH & Co, Staufen, Germany) for PVAT and myocardium. Homogenate was then centrifuged at 3000 rpm for 5 min at 4°C. To examine the $H_2S$ production in the tissue homogenates, the supernatant layer was removed and incubated with 100 mM L-cysteine and 100 μM pyridoxal phosphate. 2.5 ml of the supernatant was then placed in a 50 ml Erlenmeyer flask and was flushed with either 95% $O_2$/5% $CO_2$ or 95% $N_2$/5% $CO_2$ for 10 min. A piece of filter paper (1.5 x 1.5 cm) soaked with zinc acetate was suspended on top of the sealed flask using a string. The flasks were then incubated at 37°C for 90 min with gentle shaking in a water bath to allow trapping of the $H_2S$ released. The presence of $H_2S$ produced was detected using colorimetric change method (0.1% (w/v) dimethylphenylene-diamine sulphate (DMPD) in 5 M HCl and 50 mM $FeCl_3$). To examine the amount of gaseous $H_2S$ produced by the supernatant, the filter paper was placed into a 1.5 ml Eppendorf incubated with 200 μl of zinc acetate for 15 min. 500 μl of DMPD/ $FeCl_3$ was then added into the Eppendorf and left for a further 20 min. In

triplicates, 200 µl of the mixtures were pipette into a 96-well plate. To assess the amount of $H_2S$ generated in the homogenate, again in triplicates, 10 µl of homogenate was incubated with 40 µl of zinc acetate and 150 µl of DMPD/ $FeCl_3$ in a 96-well plate for 20 min. Samples were then analysed at the absorbance of 670 nm on a spectrometer. Concentrations of the $H_2S$ generated were extrapolated from a standard curve obtained using $Na_2S$ (0 – 80 µM). Data are expressed as picomoles per mg of protein.

**Data analysis**

Hypoxic responses were measured in grams (g) and plotted against time from which area under the curve data were generated. The contractile and relaxation phases of the hypoxic response were expressed as a percentage of the U46619 response. Values for all figures refer to mean ± SEM and data were assessed for statistical significance via Student's *t*-test or one-way ANOVA with Tukey's post-test (GraphPad Prism 6, San Diego, CA, USA). Differences were considered to be significant when the P-value was $<0.05$, where n is the number of animals.

**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 stock concentration, then further diluted to 10 µM in distilled water. Stock solutions of PPG, AOAA and aspartate (Sigma, Poole Dorset) were prepared in distilled water. Primary antibodies for Western blotting were purchased from; CSE, AbCam (Cambridge, UK), CBS, Abnova (Jhongli, Taiwan), MPST and actin, Sigma (Dorset, UK).

**Funding**

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

**Conflict of interest**

None

**References**

1. Hedegaard ER, Nielsen BD, Kun A, Hughes AD, Krøigaard C, Mogensen S, Matchkov VV, Fröbert O, Simonsen U. KV7 channels are involved in hypoxia-induced vasodilatation of porcine coronary arteries. Br J Pharmacol, 171: 69-82, 2014.

2. Donovan J, Wong PS, Roberts R, Garle MJ, Alexander SPH, Dunn WR, Ralevic V. A critical role for cystathionine-β-synthase in hydrogen sulfide-mediated hypoxic relaxation of the coronary artery. Vascular Pharmacol, 93-95: 20-32, 2017.

3. Olson KR, Dombkowski RA, Russell MJ, Doellman MM, Head SK, Whitfield NL, Madden JA. Hydrogen sulfide as an oxygen sensor/transducer in vertebrate hypoxic vasoconstriction and hypoxic vasodilation. J Exp Biol, 209(20): 4011-4023, 2006.

4. Olson KR. Hydrogen sulfide as an oxygen sensor. Antioxid Redox Signal, 22: 377-397, 2015.

5. Dunn WR, Alexander SPH, Ralevic V, Roberts R. Effects of hydrogen sulfide in smooth muscle. Pharmacology and Therapeutics, 158: 101-13, 2016.

6. Gao YJ. Dual modulation of vascular function by perivascular adipose tissue and its potential correlation with adiposity/lipoatrophy-related vascular dysfunction. Curr Pharm Res, 13(21): 2185-2192, 2007.

7. Maenhaut N, van de Voorde J. Regulation of vascular tone by adipocytes. BMC Mecicine, 9: 25, 2011.

8. Aghamohammadzadeh R, Withers S, Lynch F, Greenstein A, Malik R, Heagerty A. Perivascular adipose tissue from human systemic and coronary vessels: the emergence of a new pharmacotherapeutic target. Br J Pharmacol, 165: 670-682, 2012.

9. Gollasch M. Vasodilator signals from perivascular adipose tissue. Br J Pharmacol, 165, 633-642, 2012.

10. Gollasch M. Adipose-vascular coupling and potential therapeutics. Ann Rev Pharmacol Toxicol, 57: 417–436, 2017.

11. Brown NK, Zhou Z, Zhang J, Zeng R, Wu J, Witzman DT, Chen YE, Chang L. Perivascular adipose tissue in vascular function and disease. Arterioscler Thromb Vasc Biol, 34: 1621-1630, 2014.

12. Xia N, Li H. The role of perivascular adipose tissue in obesity-induced vascular dysfunction. Br J Pharmacol, 174(20): 3425-3442, 2017.

13. Donovan J, Garle M, Alexander S, Dunn W, Ralevic V. Perivascular adipose tissue derived hydrogen sulphide contributes to hypoxic relaxation of the porcine coronary artery. Nitric Oxide, 47: S27, 2015.

14. Maenhaut N, Boydens C, van de Voorde J. Hypoxia enhances the relaxing influence of perivascular adipose tissue in isolated mice aorta. Eur J Pharmacol, 641: 207-212, 2010.

15. Fang L, Jing Z, Yu C, Tiemin M, Guoheng X, Chaoshu T, Xinmin L, Bin G.

Hydrogen sulfide derived from periadventitial adipose tissue is a vasodilator. J Hypertension, 27(11): 2174-2185, 2009.

16. Schleifenbaum J, Köhn C, Voblova N, Dubrovska G, Zavarirskaya O, Gloe T, Crean CS, Luft FC, Huang Y, Schubert R, Gollasch M. Systemic peripheral artery relaxation by KCNQ channel openers and hydrogen sulphide. J Hypertension, 28(9): 1875-1882, 2010.

17. Mellemkjaer S, Nielsen-Kudsk JE. Glibenclamide inhibits hypoxic relaxation of isolated porcine coronary arteries under conditions of impaired glycolysis. Eur J Pharmacol, 270: 307-312, 1994.

18. Rashid S, Heer JK, Garle MJ, Alexander SPH, Roberts RE. Hydrogen sulphide-induced relaxation of porcine peripheral bronchioles. Br J Pharmacol, 168: 1902-1910, 2013.

19. Aalbaek F, Bonde L, Kim S, Boedtkjer E. Perivascular tissue inhibits rho-kinase-dependent smooth muscle Ca(2+) sensitivity and endothelium-dependent H2S signalling in rat coronary arteries. J Physiol, 593(21): 4747-4764, 2015.

20. Bonde L, Shokouh P, Jeppesen PB, Boedtkjer E. Crosstalk between cardiomyocyte-rich perivascular tissue and coronary arteries is reduced in the Zucker Diabetic Fatty rat model of type 2 diabetes mellitus. Acta Physiol, 219(1): 227-238, 2017.

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

22. Papapetropoulos A, Whiteman M, Cirino G. Pharmacological tools for hydrogen sulphide research: a brief, introductory guide for beginners. Br J Pharmacol, 172: 1633-1637, 2015.

23. Morales-Cano D, Moreno L, Barreira B, Pandolfi R, Chamorro V, Jimenez R, Villamor E, Duarte J, Perez-Vizcaino F, Cogolludo A. Kv7 channels critically determine coronary artery reactivity: left-right differences and down-regulation by hyperglycaemia. Cardiovasc Res, 106(1): 98-108 2015.

24. 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. Hypoxic regulation of the cerebral microcirculation is mediated by a carbon monoxide-sensitive hydrogen sulfide pathway. Proc Natl Acad Sci USA, 109: 1293-1298, 2012.

25. Feng X, Chen Y, Zhao J, Tang C, Jiang Z, Geng B. Hydrogen sulfide from adipose tissue is a novel insulin resistance regulator. Biochem Biophys Res Commun, 380: 153-159, 2009.

26. Swindle MM, Makin A, Herron AJ, Clubb FJ, Frazier KS. Swine as models in biochemical research and toxicology testing. Vet Pathol, 49(2): 344-356, 2011.

27. Olson KR, Forgan LG, Dombkowski RA, Forster ME. Oxygen dependency of hydrogen sulfide-mediated vasoconstriction in cyclostome aortas. J Exp Biol, 211: 2205-2213, 2008.

28. Madden JA, Ahlf SB, Dantuma MW, Olson KR, and Roerig DL. Precursors and inhibitors of hydrogen sulfide synthesis affect acute hypoxic pulmonary vasoconstriction in the intact lung. J Appl Physiol, 112: 411–418, 2012.

29. Rayment SJ, Latif ML, Ralevic V, Alexander SPH. Evidence for the expression of multiple uracil nucleotide-stimulated P2 receptors coupled to smooth muscle contraction in porcine isolated arteries. Br J Pharmacol, 150: 604-612, 2007.

**Figure legends**

**Figure 1**

Porcine coronary artery biphasic response to hypoxia and the effect of leaving intact the perivascular adipose tissue (PVAT) and myocardium. Coronary arteries were prepared with PVAT and myocardium removed (control), with PVAT and myocardium left intact (A-C), with PVAT intact (D-F), and with myocardium intact (G-I) and the effect of hypoxia (95 % $N_2$, 5 % $CO_2$) was investigated after pre-contraction of the arteries with the thromboxane $A_2$ receptor agonist U46619. Hypoxia elicited a biphasic response: contractions are shown in A, D and G; relaxations are shown in B, E and H; representative traces are shown in C, F and I. Hypoxic contraction was attenuated under all experimental conditions (when both myocardium and PVAT were left intact, and when PVAT and myocardium alone were left intact). Hypoxic relaxation was augmented when both myocardium plus PVAT were left intact, and with PVAT intact, but not when myocardium alone was left intact. N = 8. Data shown are mean ± s.e.mean. * $P<0.05$, paired t test.

**Figure 2**

Effect of different preparations of porcine coronary artery segments on contractions to U46619 and KCl (60 mM) and on the concentration of U46619 used to achieve the required contractile tone (of about 50-60% of the KCl response). Coronary artery segments were prepared with perivascular adipose tissue (PVAT) alone, myocardium alone, with both myocardium and PVAT, and without endothelium. A, Concentration of U46619; B, Contractile tone achieved; C, Contraction to KCl. Data were analysed using Student's *t* test, where data from the different preparations are compared to their respective controls (n = 8). Data shown are mean ± s.e.mean. * $P < 0.05$.

**Figure 3**

Porcine coronary artery biphasic response to hypoxia and the effect of replacing dissected perivascular adipose tissue (PVAT) and myocardium. Coronary arteries were prepared with PVAT and myocardium removed (control), with replacement of PVAT and myocardium (A, B), with replacement of PVAT (C, D), and with replacement of myocardium (E, F) and the effect of hypoxia (95 % $N_2$, 5 % $CO_2$) was investigated after pre-contraction of the arteries with the thromboxane $A_2$ receptor agonist U46619. The replacement of both myocardium and PVAT augmented the hypoxic relaxation, but effects of replacement of either myocardium alone or PVAT alone had little or no effect (n = 8). Data shown are mean ± s.e.mean. * $P<0.05$, paired t test.

**Figure 4**

Porcine coronary artery biphasic response to hypoxia and the effect of leaving intact the perivascular adipose tissue (PVAT) and inhibition of hydrogen sulfide synthesis. Coronary arteries were prepared with PVAT and myocardium removed (control) and with PVAT intact and the effect of hypoxia (95 % $N_2$, 5 % $CO_2$) was investigated after pre-contraction of the arteries with the thromboxane $A_2$ receptor agonist U46619. (A) Representative trace showing that the contractile response to hypoxia is attenuated and the relaxant response augmented in arteries with intact PVAT, and the reversal of the PVAT-mediated augmentation of relaxation by AOAA (100 µM; CBS inhibitor). (B) The attenuated contraction was unaffected by inhibitors of the enzymatic synthesis of $H_2S$ (PPG, 10 µM, AOAA, 100 µM, aspartate, 1 mM), alone or in combination. (C) The augmented relaxant response to hypoxia in arteries with intact PVAT was reversed by the CBS inhibitor inhibitor AOAA, applied alone or in combination with other inhibitors of the enzymatic synthesis of $H_2S$, but was unaffected by PPG and aspartate, inhibitors of CSE and the MPST pathway, respectively. Data shown are mean ± s.e.mean. n = 7-15. * $P<0.05$, ANOVA.

**Figure 5**

Measurement of endogenous $H_2S$ levels and release in porcine coronary arteries, PVAT and myocardium. (A) Measurement of $H_2S$ levels in homogenates of porcine coronary arteries, PVAT and myocardium (n = 5-6). (B) $H_2S$ levels in the extracellular space above cleaned coronary arteries, PVAT alone and myocardium alone, measured using a zinc trap method during conditions of hypoxia (95% $N_2$, 5% $CO_2$) and during gassing with oxygen (95% $O_2$, 5% $CO_2$) (n = 4-5). (C) Analysis of $H_2S$ levels in control samples (absence of tissue) during gassing with oxygen and hypoxia (n = 4).

**Figure 6**

Immunoblot analysis of porcine coronary artery, myocardium and perivascular adipose tissue (PVAT) (n=4) shows expression of CSE (44 kDa) within the porcine coronary artery and myocardium, but not in PVAT. Samples were the same as used for CBS immunoblotting, with the same loading; actin (45 kDa) levels used to indicate the level of protein are from Figure 7.

**Figure 7**

Immunoblot analysis of porcine coronary artery, myocardium and perivascular adipose tissue (PVAT) (n=4) shows expression of CBS (63 kDa) within the porcine coronary artery, myocardium and PVAT. Actin (45 kDa) was used to indicate the level of protein.

**Figure 8**

Immunoblot analysis of porcine coronary artery, myocardium and perivascular adipose tissue (PVAT) (n=4) shows expression of MPST (33 kDa) within the porcine coronary artery, myocardium and PVAT. Actin (45 kDa) was used to indicate the level of protein. Lane 5 in each blot is a liver sample.