

Themed Section: Vascular Endothelium in Health and Disease

REVIEW

Hydrogen sulphide and angiogenesis: mechanisms and applications

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In vascular tissues, hydrogen sulphide (H₂S) is mainly produced from L-cysteine by the cystathionine gamma-lyase (CSE) enzyme. Recent studies show that administration of H₂S to endothelial cells in culture stimulates cell proliferation, migration and tube formation. In addition, administration of H₂S to chicken chorioallantoic membranes stimulates blood vessel growth and branching. Furthermore, *in vivo* administration of H₂S to mice stimulates angiogenesis, as demonstrated in the Matrigel plug assay. Pathways involved in the angiogenic response of H₂S include the PI-3K/Akt pathway, the mitogen activated protein kinase pathway, as well as ATP-sensitive potassium channels. Indirect evidence also suggests that the recently demonstrated role of H₂S as an inhibitor of phosphodiesterases may play an additional role in its pro-angiogenic effect. The endogenous role of H₂S in the angiogenic response has been demonstrated in the chicken chorioallantoic membranes, in endothelial cells *in vitro* and *ex vivo*. Importantly, the pro-angiogenic effect of vascular endothelial growth factor (but not of fibroblast growth factor) involves the endogenous production of H₂S. The pro-angiogenic effects of H₂S are also apparent *in vivo*: in a model of hindlimb ischaemia-induced angiogenesis, H₂S induces a marked pro-angiogenic response; similarly, in a model of coronary ischaemia, H₂S exerts angiogenic effects. Angiogenesis is crucial in the early stage of wound healing. Accordingly, topical administration of H₂S promotes wound healing, whereas genetic ablation of CSE attenuates it. Pharmacological modulation of H₂S-mediated angiogenic pathways may open the door for novel therapeutic approaches.

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Abbreviations

CAM, chicken chorioallantoic membrane; CBS, cystathionine beta-synthase; cGMP, cyclic guanosine monophosphate; CSE, cystathionine gamma-lyase; ERK, extracellular signal regulated kinase; H₂S, hydrogen sulphide; hsp27, heat shock protein 27; HUVEC, human umbilical vein endothelial cells; K_{ATP}, ATP-sensitive potassium channel; MAPK, mitogen activated protein kinase; NO, nitric oxide; PAG, dl-propylargylglycine; PDE, phosphodiesterase; sGC, soluble guanylyl cyclase; VEGF, vascular endothelial growth factor

Hydrogen sulphide (H₂S) is a colourless, flammable, water-soluble gas with the characteristic smell of rotten eggs. Until recently, H₂S was viewed exclusively as a toxic gas and environmental hazard. However, a growing body of data accumulating over the last decade shows that H₂S is also synthesized by mammalian tissues via two pyridoxal-5'-phosphate-dependent enzymes responsible for metabolism of L-cysteine: cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CSE), as well as by a recently identified third pathway

involves the production from L-cysteine of H₂S via the combined action of 3-mercaptopyruvate sulphurtransferase and cysteine aminotransferase. The distribution and regulation of H₂S producing enzymes is complex and is discussed in separate reviews (Wang, 2003; Fiorucci *et al.*, 2006; Szabo, 2007; Kimura, 2010).

The biological roles of endogenous H₂S are multiple and rapidly expanding. These regulatory functions span the central and peripheral nervous system, the regulation

of cellular metabolism, regulation of immunological/inflammatory responses and various aspects of cardiovascular biology. As far as the cardiovascular system, the principal enzyme involved in the formation of H₂S is CSE, expressed in vascular endothelial cells, smooth muscle cells as well as cardiac myocytes. The cardiovascular regulatory roles of H₂S include vasodilatation, vascular protection, regulation of blood pressure and many others (Szabo, 2007; Calvert *et al.*, 2010; Elsey *et al.*, 2010; Gadalla and Snyder, 2010; Predmore and Lefer, 2010).

As a gasotransmitter, H₂S rapidly travels through cell membranes without utilizing specific transporters and exerts a host of biological effects on a variety of biological targets resulting in a variety of biological responses. Similarly to the other two gasotransmitters [nitric oxide (NO) and carbon monoxide], many of the biological responses to H₂S follow a bell-shaped dose-response: the effects of H₂S range from physiological, cytoprotective effects (which occur at low concentrations) to cytotoxic effects (which are generally only apparent at higher concentrations). Depending on the experimental system studied, the molecular mechanisms of the biological actions of H₂S include antioxidant effects, both via direct chemical reactions with various oxidant species, as well as via increased cellular glutathione levels via activation/expression of gamma-glutamylcysteine synthetase; modulation of intracellular caspase and kinase pathways; stimulatory effects on the production of cAMP and modulation of intracellular calcium levels (Wang, 2003; Fiorucci *et al.*, 2006; Szabo, 2007; Calvert *et al.*, 2010; Elsey *et al.*, 2010; Gadalla and Snyder, 2010; Kimura, 2010; Predmore and Lefer, 2010). A distinct pharmacological effect of H₂S relates to the opening of potassium-opened ATP channels (K_{ATP} channels) (Zhao *et al.*, 2001; Cheng *et al.*, 2004; Yang *et al.*, 2005; Siebert *et al.*, 2008). Some of the beneficial therapeutic actions of H₂S – including some of the vasodilatory effect as well as some of the preconditioning and cardiac protective effects – have been attributed to this molecular target of H₂S, as the protective effects of H₂S can be prevented by pretreatment of the

cells, tissues or animals with K_{ATP} channel inhibitors such as glibenclamide.

H₂S simulates endothelial cell proliferation and migration

The phenomenon that H₂S stimulates endothelial cell proliferation and migration (essential, initial components of the angiogenic response) has been reported by two independent groups (Table 1), either in transformed endothelial cells, or in primary endothelial cells (Cai *et al.*, 2007; Papapetropoulos *et al.*, 2009). Additional studies, utilizing the Roche XCelligence system also demonstrate a concentration-dependent increase in cell index (a conductance-based composite measure, reflecting cell number, adherence and growth) (Li *et al.*, 2006; Ozsvári *et al.*, 2010) in response to H₂S in cultured human umbilical vein endothelial cells (HUVEC) *in vitro* (Figure 1). Based on *in vitro* studies with exogenously applied H₂S, it was concluded that the concentrations of H₂S sufficient to induce angiogenesis are in the low micromolar (i.e. the physiological) concentration concentration range (Cai *et al.*, 2007; Papapetropoulos *et al.*, 2009). Nevertheless, it should be pointed out that the absolute ('free' and/or 'bound, biologically available') concentration of H₂S in the blood and physiological fluids is highly dependent on the method of detection used, and there is still a considerable disagreement in the literature on this point (Whitfield *et al.*, 2008; Olson, 2009; Toombs *et al.*, 2010; Wintner *et al.*, 2010). The pro-angiogenic effect of H₂S is further evidenced by the increase in tube-like structure formation in endothelial cells in *in vitro* Matrigel assays, where increases in tube length and increases in the number of branching points were demonstrated (Figure 2) (Cai *et al.*, 2007; Papapetropoulos *et al.*, 2009). The magnitude of the response (% increase in tube formation) induced by H₂S in the Matrigel assay was approximately 50%, an effect which is comparable to what was previously

Table 1

Studies demonstrating the effect of H₂S of endothelial cell proliferation, migration, tube formation and angiogenesis *in vitro*

Cell type	H ₂ S concentration (μM)	Time course (h)	Effect	Reference
RF/6A transformed endothelial cell line	1–50	24	An up to 20% increase in proliferation, as assessed by the BrdU method.	Cai <i>et al.</i> , 2007
HUVEC	6–600	48	An up to 100% increase in proliferation, as measured by counting of trypsinized cells in a haemocytometer.	Papapetropoulos <i>et al.</i> , 2009.
RF/6A transformed endothelial cell line	1–50	24	An up to 30% increase in migration, as assessed by the Transwell method.	Cai <i>et al.</i> , 2007
HUVEC	6–600	48	An up to sixfold increase in migration, as assessed by the Transwell method.	Papapetropoulos <i>et al.</i> , 2009.

H₂S, hydrogen sulphide; HUVEC, human umbilical vein endothelial cells.

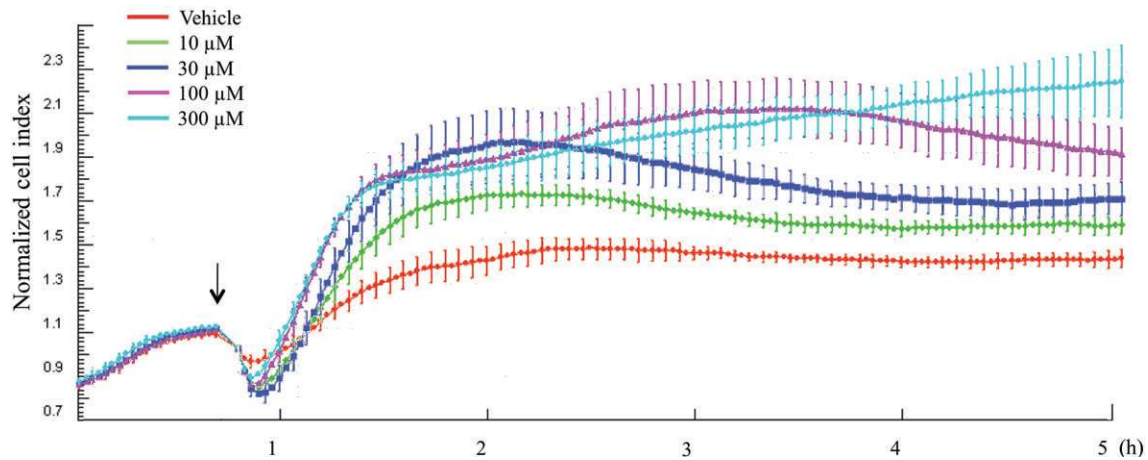


Figure 1

Concentration-dependent increase in the cell index by hydrogen sulphide (H₂S) in human umbilical vein endothelial cells, as measured by the XCelligence cell-microelectronic sensing technique method. Cells were cultured in gelatin pretreated 96-well E-plates. Cells were attached and grown overnight and subjected to vehicle or various concentrations of H₂S (10–300 μM) and cell index was continuously recorded over 5 h. Please note the concentration-dependent increase in cell index in response to H₂S (10–300 μM). At a higher concentration tested (1000 μM) (not shown), the stimulatory effect of H₂S was less pronounced than with 300 μM, consistent with the type of bell-shaped concentration-response curve often seen with H₂S. Responses shown are the mean ± SEM of *n* = 6 wells for each condition.

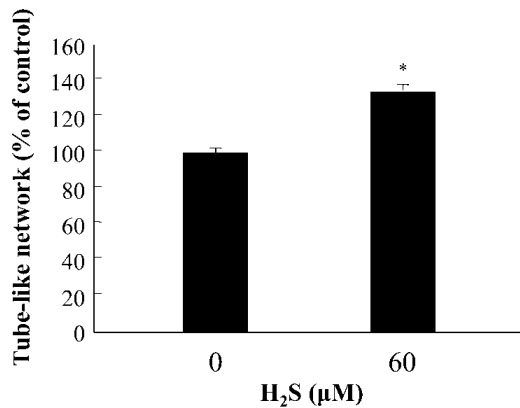


Figure 2

Increase by hydrogen sulphide (H₂S) of tube formation in the Matrigel assay in cultured human umbilical vein endothelial cells *in vitro*. Cells were plated at a density of 15 000 cells/well in 96-well plates, precoated with 50 μL of growth factor-reduced Matrigel in the presence of H₂S (60 μM) or vehicle, in complete medium. Following a 24 h-incubation, tube formation was quantified by image analysis software (Scion Image Release Beta 4.0.2) and expressed as a percentage of control. H₂S treatment significantly (*P* < 0.05) increased tube formation.

reported with NO donors, activators of soluble guanylyl cyclase (sGC), or with the phosphodiesterase (PDE) 5 inhibitor sildenafil in the same assay (Shimizu *et al.*, 2004; Pyriochou *et al.*, 2006; 2007a,b). Additional *in vitro* data relevant in the context of angiogenesis include data demonstrating that in the RF/6A cells H₂S also increases adhesion (by approximately 20% at the maximum of the dose-response) (Cai *et al.*, 2007). In addition to direct pro-angiogenic effects, H₂S has also been shown to induce angiogenesis indirectly: via release

of vascular endothelial growth factor (VEGF) from hypoxic smooth muscle cells, and subsequent VEGF-mediated responses in the endothelial cells (Liu *et al.*, 2010a).

H₂S simulates angiogenesis *in vivo*

The chicken chorioallantoic membrane provides a simple and predictive model for investigating the regulatory processes of angiogenesis. Using this method, we have observed that H₂S induces a potent and concentration-dependent increase in the length and complexity of the vascular network (Figure 3) (Papapetropoulos *et al.*, 2009). The Matrigel plug assay provides another, rather reliable and predictable method to assess the angiogenic potential of test compounds. Moore and colleagues have used this assay in a mouse model with success, and demonstrated that intraperitoneal administration of NaHS (a H₂S donor), for 7 days at 10–50 μmol·kg⁻¹·day⁻¹ increased neovascularization, cellular infiltration and haemoglobin content, demonstrating a pro-angiogenic effect in the mouse *in vivo* (Cai *et al.*, 2007). It is noteworthy that at higher doses, the H₂S donor failed to promote angiogenesis in the same assay (Cai *et al.*, 2007), underlining the general theme already mentioned in the Introduction section with respect to the bell-shaped biological dose-responses frequently noted with H₂S.

Signalling pathways involved in the pro-angiogenic effect of H₂S

The cellular signalling pathways involved in the angiogenic effect of H₂S have been studied, in some detail, in endothelial cell models *in vitro*. Exposure of endothelial cells to donors of

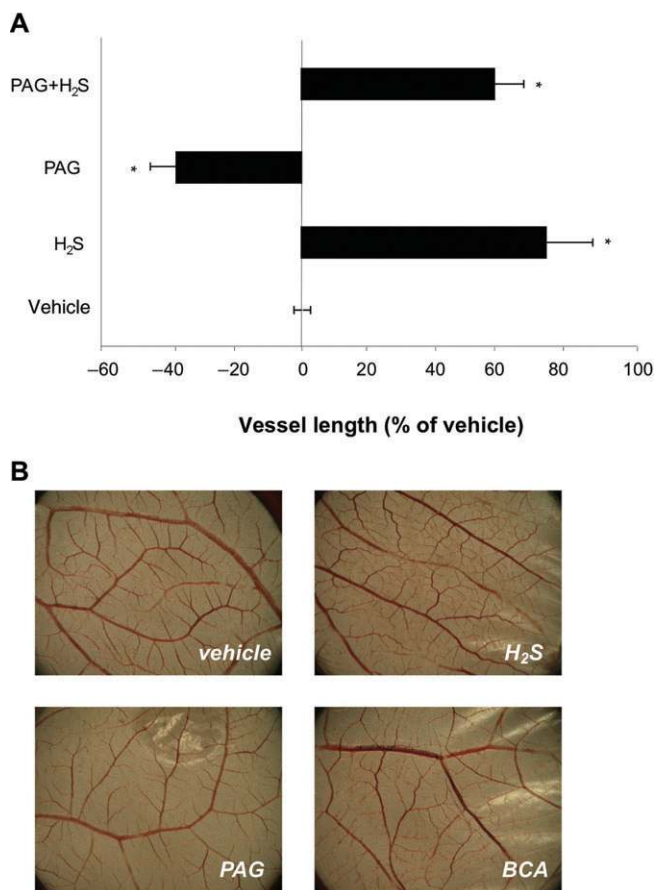


Figure 3

Exogenously applied and endogenously produced hydrogen sulphide (H₂S) promotes angiogenesis in the chicken chorioallantoic membrane. (A) Membranes were treated with H₂S (240 pmol·egg⁻¹) or the H₂S synthesis inhibitor dl-propylargylglycine (PAG) (300 mmol·egg⁻¹) or the combination of the two agents for 48 h and vascular network length and branching were determined. Note the enhancement of angiogenesis in response to H₂S, the inhibition of angiogenesis by PAG, and the lack of effect of PAG on the pro-angiogenic effect of H₂S. *n* = 36–45; **P* < 0.05 versus vehicle. Panel (B) shows representative photomicrographs. BCA, beta-cyano-L-alanine.

H₂S has been shown to activate multiple signalling pathways with established roles in neovascularization. NaHS administration activates the PI-3K/Akt axis (Cai *et al.*, 2007; Papapetropoulos *et al.*, 2009) while exposure to Na₂S enhances the phosphorylation of members of the mitogen activated protein kinase (MAPK) pathway [extracellular signal regulated kinase (ERK)1/2 and p38] (Papapetropoulos *et al.*, 2009), with time courses that are different (Figure 4). Activation of PI-3K/Akt has been shown to regulate tube-like structure formation in RF/6A cells (Cai *et al.*, 2007), while activation of MAPK was shown to play a key role in migration in HUVEC (Papapetropoulos *et al.*, 2009).

The K_{ATP} channels mediate, at least in part, the cellular action of H₂S (Zhao *et al.*, 2001). It was recently shown that H₂S promotes K_{ATP} channel opening through interaction with Cys 6 and Cys26, both of which reside on the N-terminal region of the SUR subunit of the K_{ATP} channel complex (Jiang

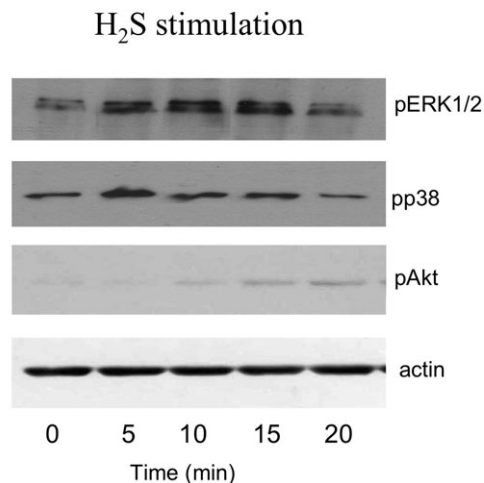


Figure 4

Time-course showing the effect of hydrogen sulphide (H₂S) (60 μM) on a number of signalling pathways involved in the pro-angiogenic effect of H₂S in vascular endothelial cells. Please note the differential time-courses of the response. ERK, extracellular signal regulated kinase.

et al., 2010). To determine whether activation of K_{ATP} channels contributes to the angiogenic actions of H₂S, we pretreated endothelial cells with sulphonylurea-type inhibitors and monitored their ability to migrate. In these experiments glibenclamide-treated cultures exhibited a markedly reduced migratory response to H₂S (Papapetropoulos *et al.*, 2009). The observation that glibenclamide blocked p38 phosphorylation indicates that K_{ATP} channels are upstream of MAPK pathways in the H₂S-triggered angiogenic pathway. However, it is still unclear whether K_{ATP} channel opening is required for PI-3K/Akt activation by H₂S. Furthermore, although H₂S induces hsp27 phosphorylation (Papapetropoulos *et al.*, 2009); siRNA inactivation of this protein inhibits the pro-angiogenic effect of H₂S (Papapetropoulos *et al.*, 2009). Cells pretreated with the p38 MAP kinase inhibitor SB203580 showed reduced H₂S-induced hsp27 phosphorylation, suggesting that H₂S enhances endothelial cells (EC) migration through a K_{ATP} channel/p38/hsp27 pathway. An interesting side observation of the above-mentioned series of studies was that K_{ATP} channel activation, on its own, is able to stimulate angiogenesis in HUVEC: incubation of cells with the K_{ATP} channel opener SG209, induced a concentration-dependent migratory response, indicating that K⁺ efflux *per se* can drive EC motility (Papapetropoulos *et al.*, 2009).

The exact modes of interactions of the above-mentioned effectors of H₂S-induced angiogenesis remains to be delineated in future studies. In particular, it remains unknown what the downstream effectors of MAPK and PI-3K pathways are that contribute to angiogenesis. It has been proposed that part of the downstream effectors include survivin and integrin α₂ and integrin β₁ (Cai *et al.*, 2007). In addition, the up-regulation of integrin α₂ and integrin β₁ was also shown in the same experimental system. However, the causative role of these changes in the pro-angiogenic effect of H₂S has not yet been definitively tested. When considering potential

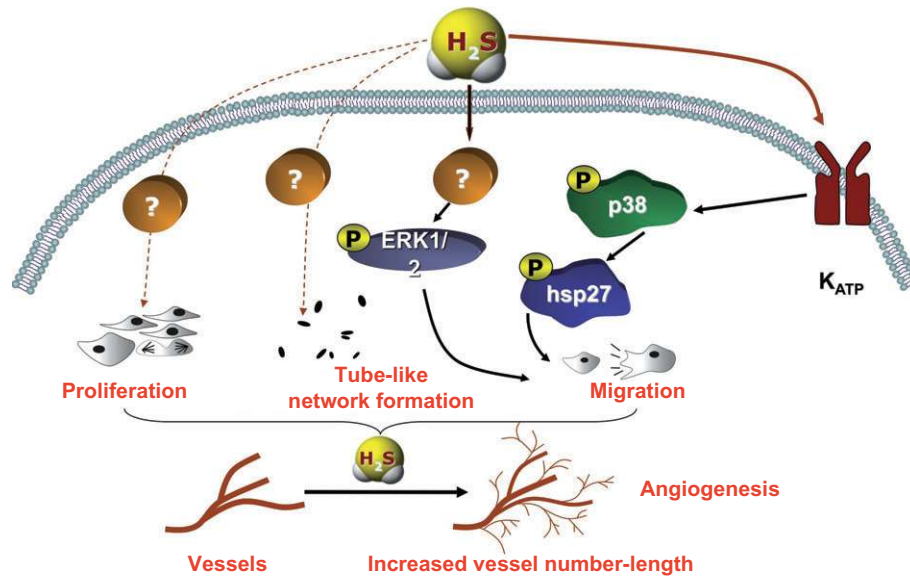


Figure 5

Signalling pathways involved in the pro-angiogenic effect of hydrogen sulphide (H₂S) in vascular endothelial cells. ERK, extracellular signal regulated kinase; K_{ATP}, ATP-sensitive potassium channel.

downstream effector pathways, it should also be noted that hypoxia inducible factor-1 (a transcription factor with a central role in the angiogenic response) was also shown to be activated by H₂S (Budde and Roth, 2010; Liu *et al.*, 2010b). A cartoon summarizing our current knowledge of the H₂S-induced pro-angiogenic pathways and some of the potential interactions between them is shown in Figure 5.

Role of endogenous H₂S in angiogenesis

We recently demonstrated that exposure of endothelial cells to VEGF increases the production of H₂S and that endogenously produced H₂S participates in the angiogenic signalling of VEGF (Papapetropoulos *et al.*, 2009). The VEGF-induced and the H₂S-induced endothelial cell migration response are not additive (Figure 6). This may be consistent with the notion that the two responses, at least in part, involve similar downstream mechanisms. Indeed, subsequent studies showed that endogenously produced H₂S is a key mediator of VEGF-induced angiogenesis (Papapetropoulos *et al.*, 2009). This conclusion is based on the finding that pharmacological inhibition, genetic deletion or silencing of CSE, a major H₂S-producing enzyme in the endothelium, reduces migration and sprouting of endothelial cells *in vitro* (Figure 7). Further evidence for the importance of this pathway in VEGF-induced angiogenesis comes from the *ex vivo* mouse aorta sprouting assay, where the VEGF-induced angiogenesis was markedly suppressed in aortic rings of the CSE^{-/-} mice (Figure 8) (Papapetropoulos *et al.*, 2009).

H₂S is not a universal requirement for all angiogenic growth factor signalling; for example, inhibition of CSE did not affect fibroblast growth factor-induced migration

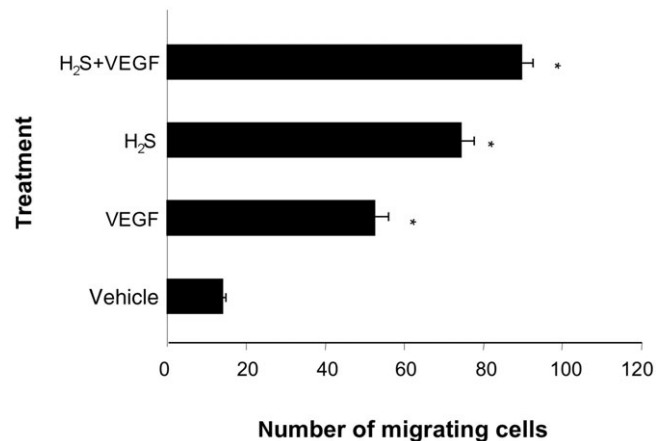


Figure 6

Incubation with vascular endothelial growth factor (VEGF) and hydrogen sulphide (H₂S) does not produce an additive effect on endothelial migration. Endothelial cells were allowed to migrate for 4 h in response to VEGF (20 ng·mL⁻¹), H₂S (60 μM) or a combination of the two. *n* = 5; **P* < 0.05 versus vehicle.

(Figure 7). The dependence of VEGF signalling on H₂S might be due to the fact that VEGF increases intracellular calcium levels (Papapetropoulos *et al.*, 1997) that will, in turn, promote a calmodulin-dependent increase in CSE activity (Yang *et al.*, 2008). As inhibition of endogenously produced H₂S reduces p38 and ERK1/2 phosphorylation, it is most likely that H₂S production in response to VEGF lies upstream and is required for MAPK activation. Similar to the situation with exogenously applied H₂S, the pro-angiogenic VEGF/H₂S response also involves the activation of K_{ATP} channels: inhibition of these channels suppresses VEGF-induced

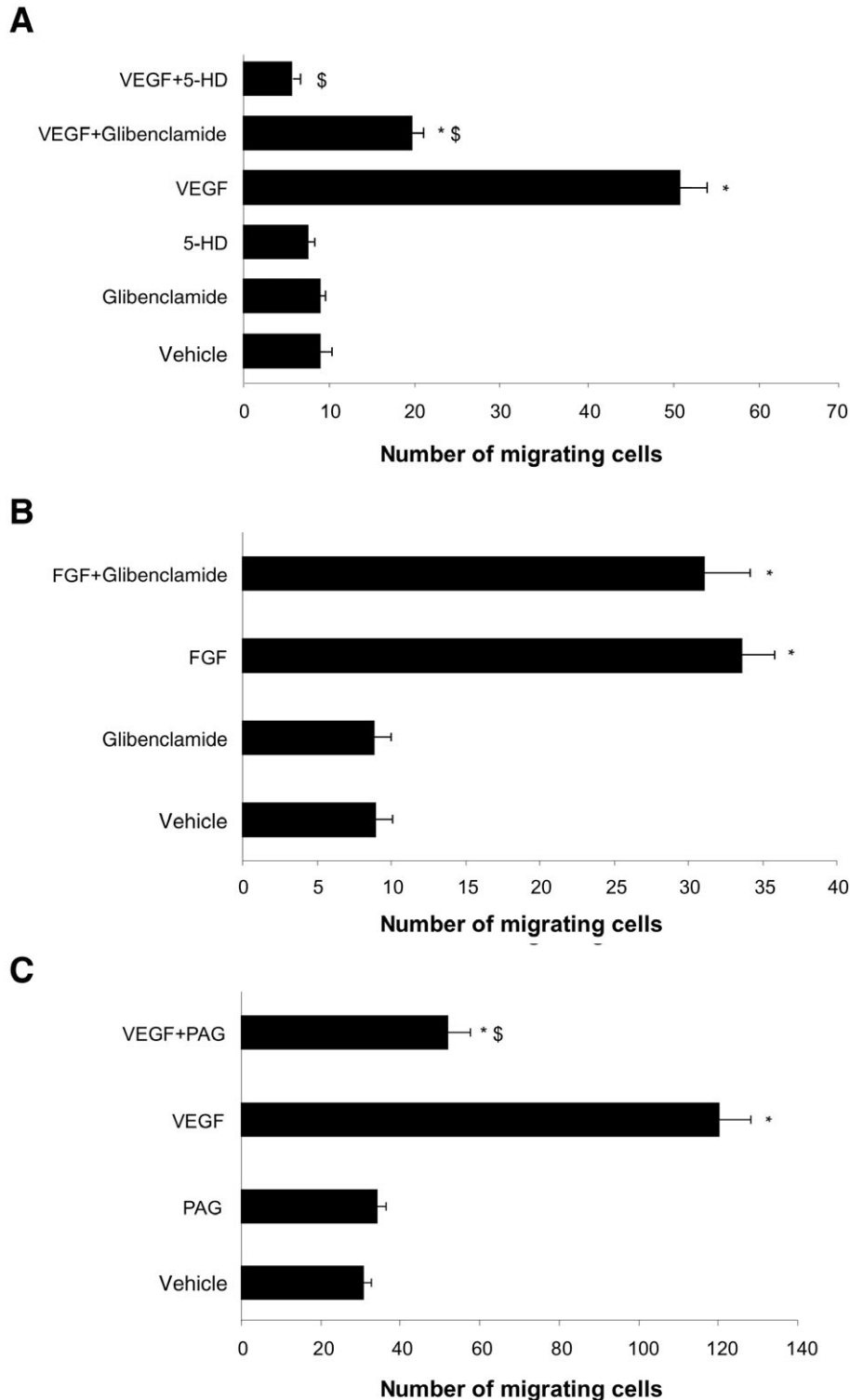


Figure 7

H_2S is required for vascular endothelial growth factor (VEGF)-stimulated, but not fibroblast growth factor (FGF)-stimulated endothelial cells (EC) migration; role of K_{ATP} channels in the response. The EC were serum starved overnight. Cells were then treated with the K_{ATP} channel inhibitors glibenclamide ($10 \mu M$) or 5-HD ($100 \mu M$) for 30 min. Cells were then trypsinized, placed in transwells and allowed to migrate for 4 h in the presence of vehicle, VEGF ($20 \text{ ng}\cdot\text{mL}^{-1}$) (A) or FGF-2 ($10 \text{ ng}\cdot\text{mL}^{-1}$) (B). $n = 5$; $*P < 0.05$ versus vehicle and $\#P < 0.05$ versus VEGF. In the experiments shown in (C), EC were treated with the cystathionine gamma-lyase inhibitor dl-propylargylglycine (PAG) (3 mM) for 30 min. Cells were then placed in transwells and allowed to migrate for 4 h in the presence of vehicle or VEGF ($20 \text{ ng}\cdot\text{mL}^{-1}$). $n = 5$; $*P < 0.05$ versus vehicle and $\$P < 0.05$ versus VEGF.

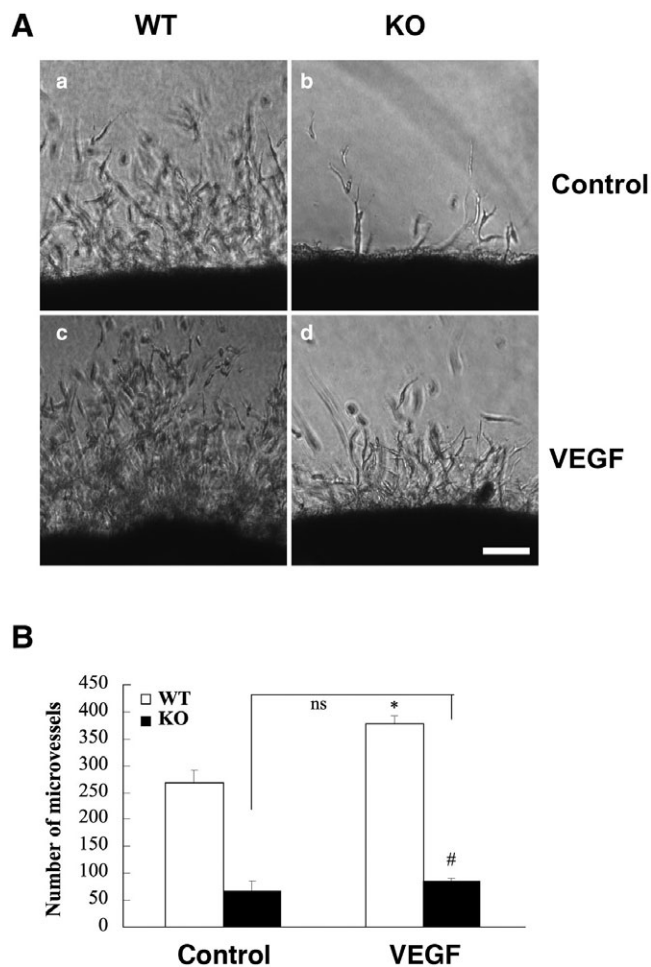


Figure 8

The angiogenic actions of vascular endothelial growth factor (VEGF) are regulated by endogenously produced hydrogen sulphide (H_2S) in the *in vitro* aortic ring angiogenesis assay. Aortic ring explants from cystathionine gamma-lyase (CSE) wild-type (WT) or knockout (KO) mice were incubated in the presence or absence of VEGF (20 ng mL^{-1}). Representative photomicrographs are presented in (A), and quantification of the response is shown in the right panel (B). Please note the substantially lower number of new microvessels in the CSE-deficient groups. From Papapetropoulos *et al.* 2009, reproduced with permission.

angiogenesis (whereas FGF-induced angiogenesis does not involve K_{ATP} channels) (Figure 7) (Papapetropoulos *et al.*, 2009).

There have been significant advances in the pharmacological modulation and therapeutic exploitation of angiogenesis, both in the field of tumour biology. In both cases, inhibition of angiogenesis is of therapeutic significance, as it induces, respectively, tumour hypoxia (Folkman, 2006; Ruegg and Mutter, 2007; Niu and Chen, 2010; Reynolds, 2010) and inhibition of unwanted overproliferation of blood vessels (Frank, 2009; Nicholson and Schachat, 2010). Based on the current results one can speculate that pharmacological inhibition of H_2S biosynthesis may have the potential of affecting

the same responses, either on its own, or in combination with other angiogenesis inhibitory approaches.

H_2S/NO interactions in angiogenesis

NO, the 'original' gasotransmitter is a well-known pro-angiogenic mediator that exerts its effects primarily via the sGC/cyclic GMP (cGMP) pathway (Papapetropoulos *et al.*, 1997; Morbidelli *et al.*, 2003; Pyriochou *et al.*, 2006; 2007a,b; Morbidelli *et al.*, 2010). Over the last decade, a multitude of studies have investigated the complex relationship between NO and H_2S in various experimental models. The interactions of these two gasotransmitters depend on the experimental system used, but include direct interactions, followed by the formation of nitrosothiols (Whiteman *et al.*, 2006), release of NO from nitrite or nitrosothiols by H_2S (Grossi, 2009; Tomaskova *et al.*, 2009) synergistic vasorelaxant effects (Hosoki *et al.*, 1997) and many others (Liu and Bian, 2010; Yong *et al.*, 2010).

It should be noted that in addition to H_2S , NO has also been shown to lie upstream of ERK1/2 activation in VEGF-treated cells (Parenti *et al.*, 1998) and to contribute to many of its angiogenic properties (Zachary and Gliki, 2001). Careful analysis of the results in the literature reveals that simultaneous production of both H_2S and NO may be required to drive the migratory response to VEGF; generation of either NO or H_2S alone is not sufficient for the angiogenic response. For example, inhibition of NO production blunts the VEGF-stimulated migration in cultured EC (Ziche *et al.*, 1997; Dimmeler *et al.*, 2000) and so does CSE inhibition by dl-propylalanyl-glycine (Papapetropoulos *et al.*, 2009). These observations, when considered together, suggest that the two gasotransmitters may act in a concerted manner targeting the same pathway, and not additively through parallel pathways, to promote migration. One possible mechanism for such an orchestrated action requiring both H_2S and NO to enhance VEGF migration might relate to the ability of H_2S to inhibit PDE activity. We recently observed that H_2S increases cGMP levels in smooth muscle cells by inhibiting cGMP-degrading PDE (Bucci *et al.*, 2010). If a similar mechanism operates in EC, increased production of NO after VEGF exposure would stimulate sGC to produce cGMP; at the same time increased production of H_2S would inhibit PDE activity allowing for a transient increase in cGMP levels that triggers the activation of downstream pathways (Figure 9). Indeed, H_2S -stimulated migration can be blocked by cGMP-dependent protein kinase inhibition (A. Papapetropoulos and C. Szabó, unpubl. obs.). The interactions of NO and H_2S in the context of angiogenesis remain to be studied in further detail. According to conflicting reports in the literature, this latter pathway may (Parenti *et al.*, 1998) or may not (Breslin *et al.*, 2003) be stimulated by NO in endothelial cells.

An alternative, but not mutually exclusive mode of H_2S and NO interaction might involve intracellular calcium. Exposure to H_2S was reported to increase endothelial calcium levels (Bauer *et al.*, 2010), which would lead to eNOS activation and increased NO release. In line with the above mentioned interactions between NO and H_2S , the protective

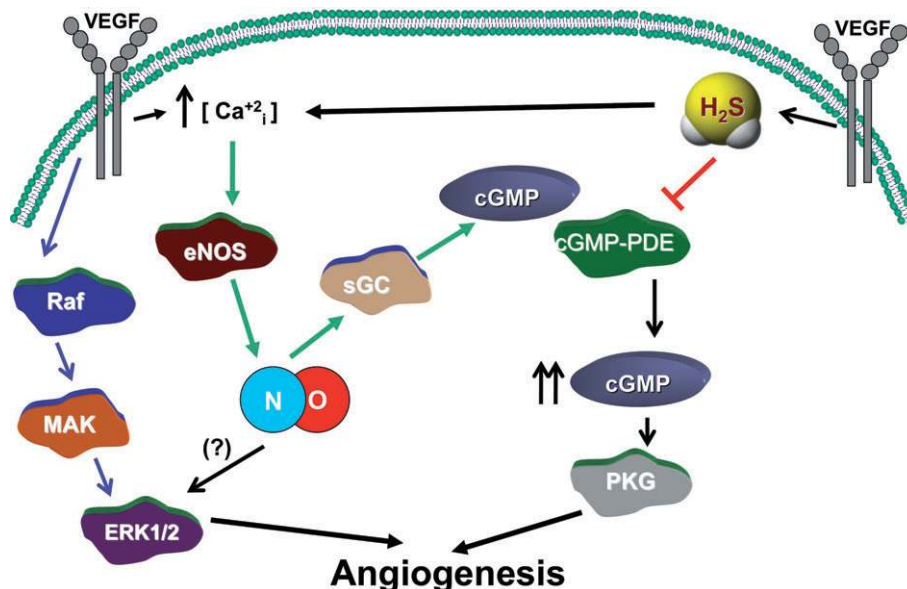


Figure 9

A proposed pathway of the interaction between nitric oxide (NO) and hydrogen sulphide (H₂S) in endothelial cells during angiogenesis. Vascular endothelial growth factor (VEGF) receptor activation increases NO production, leading to soluble guanylyl cyclase activation and increased cGMP synthesis. At the same time VEGF receptor activation triggers the generation of increased amounts of H₂S; H₂S inhibits cGMP-degrading phosphodiesterases leading to a further elevation of cGMP that in turn activates cGMP-dependent protein kinase and promotes angiogenesis. Because H₂S increases the levels of intracellular calcium, VEGF-stimulated H₂S production might enhance NO production. The relationship between these pathways and the canonical pathway of VEGF receptor activation-induced stimulation of the intracellular signalling axis of Raf, MAK and extracellular signal regulated kinase (ERK)1/2 remains to be delineated in future studies. PDE, phosphodiesterase; sGC, soluble guanylyl cyclase.

actions of H₂S in cardiac arrest and in intestinal injury is lost in eNOS knockout mice (Minamishima *et al.*, 2009; Yusof *et al.* 2009).

H₂S as a promoter of wound healing and therapeutic angiogenesis

In a model of cutaneous burn injury and wound healing we have demonstrated that topical administration of a H₂S-saturated physiological solution significantly enhances the closure of the wound (Figure 10) (Papapetropoulos *et al.*, 2009). Consistent with these observations, the wound healing response in CSE^{-/-} mice is significantly delayed, when compared to the response in wild-type animals (Papapetropoulos *et al.*, 2009). These observations may lay the groundwork for therapeutic approaches for the topical delivery of H₂S in order to promote wound healing in burns or in other conditions. Since the topical delivery of gaseous H₂S is challenging, one may envision approaches involving H₂S donors or precursors and/or possibly approaches around the therapeutic local overexpression of H₂S-producing enzymes. Polysulphides (including the ones that are present in garlic, such as diallyl disulphide and diallyl trisulphide) have recently been shown to produce H₂S in cells via a reaction that involves glutathione (Benavides *et al.*, 2007). Although such compounds may be interesting as potential therapeutic stimulators of H₂S-mediated angiogenesis, it must be noted

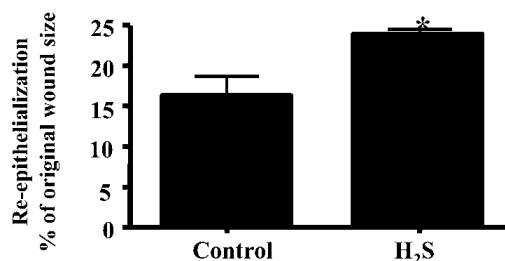


Figure 10

Hydrogen sulphide (H₂S) promotes re-epithelialization in burn wounds. Rats received a 30% total body surface area dorsal full-thickness scald burn under deep anaesthesia. Animals were treated daily with subcutaneous injections of vehicle, or H₂S (50 µg·cm⁻²·day⁻¹) at four equally spaced sites in the transition zone between burn eschar and healthy tissue. Planimetric measurement of the wound surface and re-epithelialization, as well as the ratio of wound contraction was performed. *n* = 6; **P* < 0.05 versus control.

that several publications have reported that compounds of this class do not stimulate angiogenesis, but in fact, they inhibit it (Xiao *et al.*, 2006; Thejass and Kuttan, 2007). The reason for this unexpected response remains to be investigated in future studies.

The healing of gastric ulcers is another process in which angiogenesis plays an important role (Szabo *et al.*, 1999; Tarnawski, 2005). Wallace has demonstrated in a rat model of

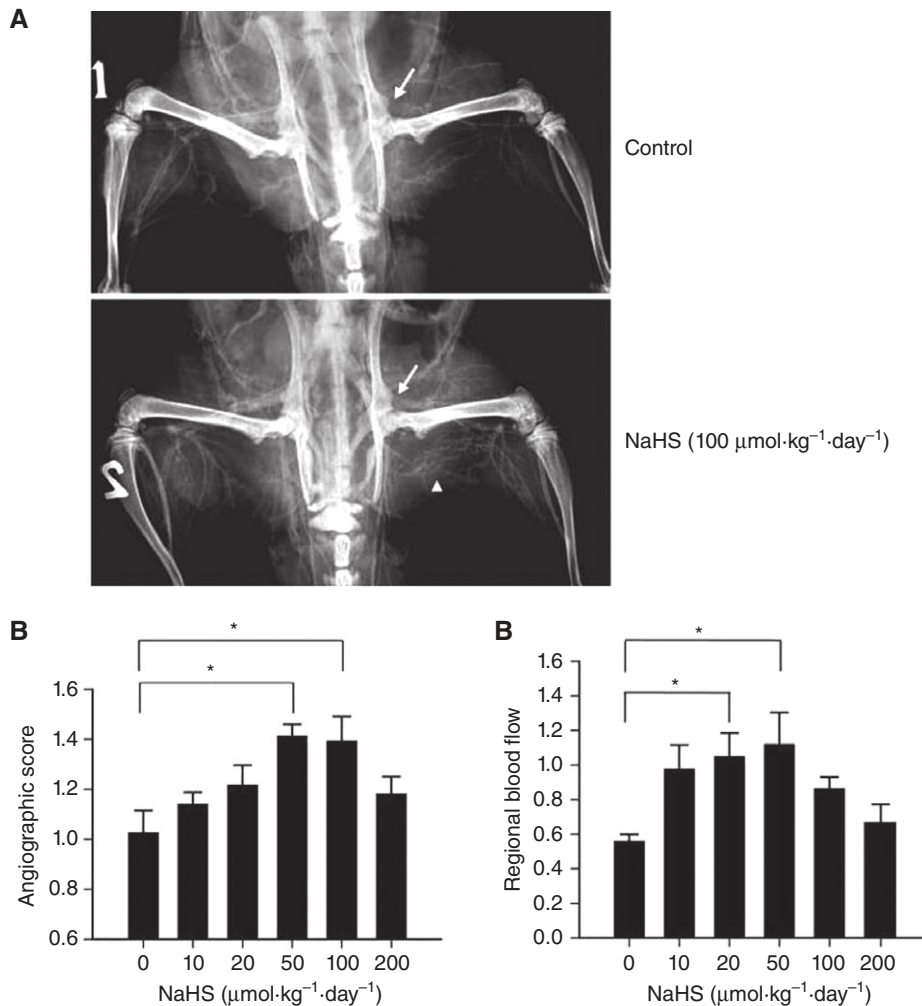


Figure 11

Hydrogen sulphide (H_2S) promotes collateral vessel formation and regional blood flow after femoral artery occlusion in the rat hind limb ischaemia model. (A) Representative post-mortem angiograms obtained 4 weeks after surgery. There was more collateral vessel formation in the ischaemic left hindlimb of the rats treated with NaHS at a dose of $100 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$. Arrow denotes the site of ligation at the femoral artery. The arrowhead indicates the typical 'corkscrew' appearance of collateral vessels. (B) Quantitative analysis of collateral vessel development was performed by measuring the total length of the contrast-opacified vessels. The angiographic score was significantly greater in the rats receiving NaHS (50 and $100 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) than in the control animals ($*P < 0.05$). Data represent the mean \pm SEM of three to five experiments in each group. (C) Blood flow measured with microsphere assay. The regional blood flow in ischaemic limb was standardized to tissue weight and is represented as the ratio of fluorescence intensity in the ischaemic hind limb to that of the contralateral nonischaemic hind limb in each animal. NaHS treatment (20 and $50 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) significantly improved regional blood flow in the ischaemic limb ($*P < 0.05$). Data represent the mean \pm SEM of seven to nine experiments (from Wang *et al.*, 2010; reproduced with permission).

gastric ulceration (induced experimentally by topical administration of acetic acid) that H_2S donors of two different chemical classes (as well as the H_2S precursor L-cysteine) improved the resolution of the ulcers (Wallace *et al.*, 2009). Although angiogenic responses were not measured in this study, it is conceivable that a pro-angiogenic effect of H_2S may have contributed to its therapeutic effects. It is also interesting to note that there was an up-regulation of endogenous H_2S production during the healing response (Wallace *et al.*, 2009); this endogenous H_2S may serve as a potential mechanism to accelerate wound healing. We speculate that some of the benefit of H_2S donors seen in enhancing the

resolution of colitis in rat models (Wallace *et al.*, 2009) may also have involved, among others, a pro-angiogenic response.

Therapeutic angiogenesis is also of substantial importance in the context of organ ischaemia, or the reperfusion of previously ischaemic organs. Several *in vivo* studies began characterizing the effects of H_2S administration in this context. In a rat model of chronic hindlimb ischaemia induced by unilateral ligation of the femoral artery the therapeutic intraperitoneal administration of the H_2S donor NaHS (at $10\text{--}200 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ for 28 days) was tested (Wang *et al.*, 2010). The results (Figure 11) showed that at the lower doses used, H_2S therapy significantly improved capillary

density, angiographic scores, and these effects correlated with an improvement in hindlimb blood flow (Wang *et al.*, 2010). Interestingly (and, again, underlying the bell-shaped dose-responses to H₂S), higher doses of the H₂S donor were not found to be effective in this model. Part of the response to H₂S has been attributed to an up-regulation of VEGF production and an increase in the phosphorylation of the VEGF receptor (Wang *et al.*, 2010). These findings are somewhat in line with the *in vitro* model of hypoxic angiogenesis discussed earlier, where conditioned media of smooth muscle cells treated with H₂S contained significantly increased levels of VEGF, which, in turn, stimulated angiogenesis in endothelial cells (Liu *et al.*, 2010a).

Consistent with the effects of H₂S donation in hindlimb ischaemia, a recent study reported on the pro-angiogenic effect of H₂S supplementation in the ischaemic rat heart. Permanent ligation of the left anterior descending artery was performed; H₂S was administered as NaHS once a day at 56 µmol·kg⁻¹ intravenously. Functional and histological evaluations, performed 42 days later, revealed that H₂S therapy improved cardiac function (assessed by electrocardiography), and these alterations were associated with a significant increase in capillary density in the myocardium (Liu *et al.*, 2010a).

Limitations, conclusions and future directions

A substantial limitation of the current state-of-the-art with respect to the role of H₂S in angiogenesis is that it is primarily based on *in vitro* studies in reductionist models, such as endothelial cell proliferation, migration and tube formation. While these models do have substantial validity, they clearly do not mimic the complex phenomenon of angiogenesis *in vivo*. There are also important distinctions that can be made between the processes of vasculogenesis, angiogenesis and arteriogenesis. These complex phenomena are tightly regulated in a spatial and temporal manner, and involve the degradation of extracellular matrix, the proliferation and migration of endothelial cells towards angiogenic cues and the establishment of tubular structures, the basis of new blood vessels (Silvestre *et al.*, 2008; De Val and Black, 2009; Eble and Niland, 2009). Much additional work needs to be done to delineate the exact role of H₂S in each of these processes. While there are no published data on the potential effects of H₂S on matrix degradation, we hypothesize (based on *in vitro* findings) that H₂S may stimulate proliferation, migration and tube formation of endothelial cells *in vivo*. However, this remains to be proven in carefully designed future *in vivo* studies.

Another limitation of the current state-of-the-art is that the role of K_{ATP} channels in angiogenesis is based on pharmacological studies alone. In fact, most of the work investigating the role of endothelial cell K_{ATP} channels is based on pharmacological inhibitors of limited selectivity and specificity (e.g. Broadhead *et al.*, 2004; Tajima *et al.*, 2008; Figura *et al.*, 2009; Grossini *et al.*, 2009), as opposed to studies knocking down or inactivating specific channel subunits. Therefore, the relative importance of sarcolemmal versus mitochondrial channels in

the observed effects remains to be established. Indeed, one must point out that the type of K_{ATP} channel subunits present in endothelial cells have not yet been characterized in full detail (Yoshida *et al.*, 2004; Morrissey *et al.*, 2005; Malester *et al.*, 2007) and there are only a few reports that provide electrophysiological evidence of K_{ATP} channels in endothelial cells (Adams and Hill, 2004; Jackson, 2005) and some of the endothelial responses to K_{ATP} channel openers have been attributed to indirect, gap junction-transmitted hyperpolarization from the underlying smooth muscle cells (White and Hiley, 2000; Takano *et al.*, 2004). Because in the current studies, endothelial cells in culture were used, such transmission is not possible, but nevertheless the exact localization and nature of the K_{ATP} channels involved will require substantial additional work. Likewise, the potential contribution to the angiogenic responses of other types of potassium channels activated by H₂S (Cheang *et al.*, 2010; Tang *et al.*, 2010; Zuidema *et al.*, 2010) remains to be elucidated in further studies.

In conclusion, recently emerging work demonstrates the pro-angiogenic role of exogenously applied and endogenously produced H₂S. Some of the intracellular signalling pathways have been delineated, and additional pathways are likely to be identified in the future. It is noteworthy that recent studies suggest a potential interaction of H₂S with the NO pathway in the context of angiogenesis. Both pharmacological supplementation/stimulation of H₂S and pharmacological inhibition of H₂S may have potential therapeutic applications. Experimental data support the view that H₂S donation may be of therapeutic relevance for wound healing and post-ischaemic therapy. In addition, one can hypothesize that H₂S inhibition may be potentially applicable to inhibition of tumour blood supply and/or the hyperproliferative response of the blood vessels in the diabetic eye. However, these latter possibilities are, at the present time, are only based on *in vitro* findings, and remain to be tested *in vivo*.

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Conflict of interest

C. S. is a shareholder of Ikaria Inc, a for-profit organization involved in therapeutic applications of H₂S.

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