

## REVIEW

# Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part II. Pathophysiological and therapeutic aspects

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Emerging work demonstrates the dual regulation of mitochondrial function by hydrogen sulfide (H<sub>2</sub>S), including, at lower concentrations, a stimulatory effect as an electron donor, and, at higher concentrations, an inhibitory effect on cytochrome C oxidase. In the current article, we overview the pathophysiological and therapeutic aspects of these processes. During cellular hypoxia/acidosis, the inhibitory effect of H<sub>2</sub>S on complex IV is enhanced, which may shift the balance of H<sub>2</sub>S from protective to deleterious. Several pathophysiological conditions are associated with an overproduction of H<sub>2</sub>S (e.g. sepsis), while in other disease states H<sub>2</sub>S levels and H<sub>2</sub>S bioavailability are reduced and its therapeutic replacement is warranted (e.g. diabetic vascular complications). Moreover, recent studies demonstrate that colorectal cancer cells up-regulate the H<sub>2</sub>S-producing enzyme cystathionine β-synthase (CBS), and utilize its product, H<sub>2</sub>S, as a metabolic fuel and tumour-cell survival factor; pharmacological CBS inhibition or genetic CBS silencing suppresses cancer cell bioenergetics and suppresses cell proliferation and cell chemotaxis. In the last chapter of the current article, we overview the field of H<sub>2</sub>S-induced therapeutic 'suspended animation', a concept in which a temporary pharmacological reduction in cell metabolism is achieved, producing a decreased oxygen demand for the experimental therapy of critical illness and/or organ transplantation.

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### Abbreviations

3-MST, 3-mercaptopyruvate sulfurtransferase; AOAA, aminooxyacetic acid; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; H<sub>2</sub>S, hydrogen sulfide; K<sub>ATP</sub>, ATP-sensitive potassium channel; PAG, propargylglycine; SOU, sulfide-oxidizing unit

### Introduction

H<sub>2</sub>S, a colourless, flammable, water-soluble gas, is gaining increased attention as an endogenous biological mediator. The distribution and regulation of the three H<sub>2</sub>S-producing

enzymes [cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST)], and the wide range of biological effects of H<sub>2</sub>S are discussed in separate reviews (Fiorucci *et al.*, 2006; Szabo, 2007; Calvert *et al.*, 2010; Elsey *et al.*, 2010; Gadalla and

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mitochondrial electron transport; bioenergetics; 3-mercaptopyruvate sulfurtransferase; gasotransmitters; blood vessels; nitric oxide; superoxide; free radicals; cysteine; ischaemia; shock; suspended animation

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Snyder, 2010; Kimura, 2010; 2013; Predmore and Lefer, 2010; Whiteman and Winyard, 2011; Whiteman *et al.*, 2011; Kimura *et al.*, 2012; Wang, 2012). In a recent article, we have overviewed the dual mitochondrial effects of H<sub>2</sub>S, which range from stimulatory effects, occurring at lower concentrations, to the suppression of mitochondrial function, which occurs at higher concentrations (Szabo *et al.*, 2013a). The purpose of the current article is to outline the physiological, pathophysiological and therapeutic aspects of this regulation. Similar to our approach in the companion article (Szabo *et al.*, 2013a), in the current article we use the terms 'sulfide' and H<sub>2</sub>S interchangeably to collectively refer to H<sub>2</sub>S gas as well as its two ionized forms in solution: HS<sup>-</sup> and S<sup>2-</sup>.

## H<sub>2</sub>S as a potential inducer of cytopathic hypoxia in circulatory shock

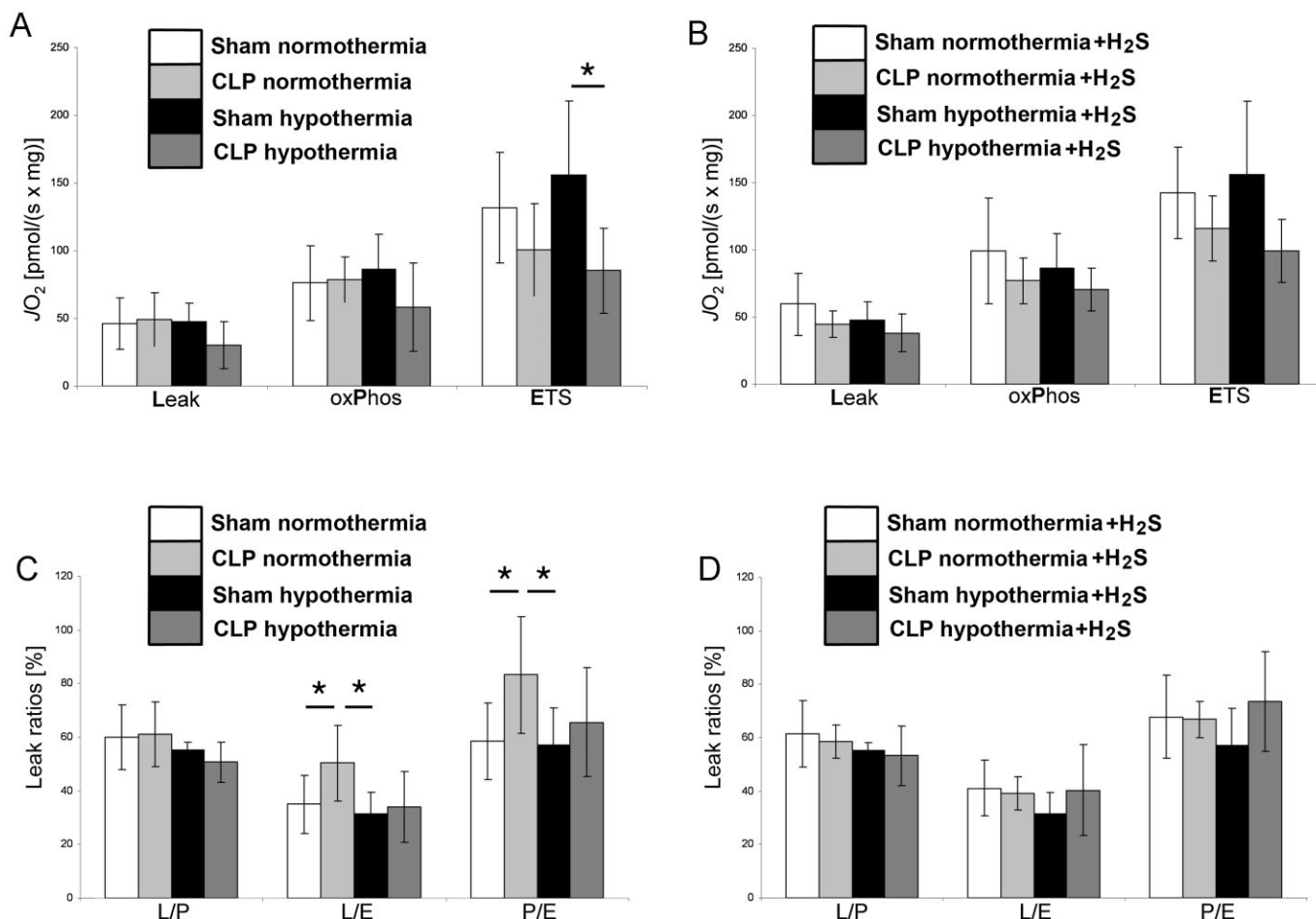
Various forms of circulatory shock lead to an intrinsic impairment of mitochondrial function, a phenomenon often called 'cytopathic hypoxia' (Fink, 2001; Levy, 2007; LaRosa and Opal, 2008). This phenomenon entails the intrinsic impairment of the mitochondria to utilize oxygen, and manifests itself in a narrow arteriovenous oxygen difference due to the inability of the 'poisoned' tissue to 'extract' oxygen during circulatory shock.

There are several lines of published studies using shock models induced by bacterial LPS, and by cecal ligation and puncture (CLP, a polymicrobial model of sepsis), showing overproduction of H<sub>2</sub>S and/or the beneficial effect of inhibitors of H<sub>2</sub>S production such as propargylglycine (PAG) (reviewed in Wagner *et al.*, 2009; Coletta and Szabo, 2013). There are also several clinical studies demonstrating the overproduction of sulfide in patients with various forms of circulatory shock (Goslar *et al.*, 2011). It is a logical assumption, therefore, that endogenously produced sulfide may contribute to the pathogenesis of cytopathic hypoxia in circulatory shock, especially in light of the findings showing that acidosis (a common feature of shock) enhances the inhibitory effect of sulfide on complex IV (Groeger *et al.*, 2010; Szabo *et al.*, 2013a). Nevertheless, the question whether endogenously produced sulfide contributes to cytopathic hypoxia has not yet been directly or comprehensively addressed; nor has it been explored how much the specific pattern of perfusion and oxygenation occurring in the tissue during the course of a shock state may modulate this response. For example, in a porcine model of haemorrhagic shock (Bracht *et al.*, 2012b), it was found that a pronounced reduction of mitochondrial respiratory activity occurs in terms of oxygen flux ( $JO_2$ ) per amount of tissue in permeabilized skeletal muscle biopsies after 3 h of shock, when compared with the control before the haemorrhage (control,  $JO_2 = 44 \pm 12$  [pmol/(s\*mg tissue)] vs. 3 h shock,  $JO_2 = 25 \pm 7$  [pmol/(s\*mg tissue)],  $P < 0.001$ ). However, 12 h of reperfusion allowed mitochondrial respiration to almost completely recover ( $JO_2 = 40 \pm 6$  [pmol/(s\*mg tissue)],  $P < 0.001$ ), suggesting that mitochondria highly dynamically adapt to the actual circulatory and oxygenation conditions in this experimental model. Continuous infusion of the sulfide donor Na<sub>2</sub>S did not affect the response to shock and reperfusion (control,  $JO_2 = 35 \pm 13$

[pmol/(s\*mg tissue)] vs. 3 h shock,  $JO_2 = 22 \pm 8$  [pmol/(s\*mg tissue)],  $P < 0.001$ , and 12 h recovery,  $JO_2 = 39 \pm 15$  [pmol/(s\*mg tissue)],  $P < 0.001$ ), suggesting that exogenously administered sulfide does not induce or exacerbate cytopathic hypoxia in this model. It is important to note that the direct answer as to whether endogenous sulfide contributes to the cytopathic hypoxia in circulatory shock would require studies in which H<sub>2</sub>S overproduction in shock is inhibited (by pharmacological tools or by genetically modified animals), followed by metabolic analysis of tissues *ex vivo*. Such studies have not yet been conducted.

Indirect lines of data, nevertheless, indicate that sulfide may have an impact on mitochondrial respiration during circulatory shock. For example, as shown in Figure 1, CLP-induced septic shock reduces the maximum mitochondrial respiratory capacity (ETS capacity) in the liver of mice, this effect being more pronounced under hypothermia than under normothermia (diagram A). Comparison of the respiratory activity compensating for the proton leak at the inner mitochondrial membrane (LEAK respiration) with the OxPhos capacity to the maximum respiration in the uncoupled (ETS) state (L/E and P/E ratios, respectively, diagram C) reveals that the reduction of maximum mitochondrial respiratory capacity is pronounced even under normothermic conditions. Interestingly, treating the animals with sulfide fully compensated for these CLP-related effects, thus maintaining normal mitochondrial activity regardless of body temperature in septic shock (diagrams B and D). These findings would be consistent with a therapeutic effect of exogenous sulfide administration, rather than a potential causative role of sulfide in inducing cytopathic hypoxia in shock. The different effects of sulfide under normo- and hypothermia are clearly explained by the strong temperature-dependent inhibition of mitochondrial respiration by sulfide, which is much weaker at low body temperature (Baumgart *et al.*, 2010).

The study of combined hypothermia and sulfide is interesting because both of these interventions have been previously linked to metabolic suppression, and the combination of them may offer therapeutic possibilities in the context of 'on-demand suspended animation' (see below). Hypothermia not only assumes the importance for H<sub>2</sub>S-induced inhibition of mitochondrial respiration, but also markedly influences substrate utilization and thereby may even improve the activity of the mitochondrial respiratory chain. In anaesthetized and ventilated mice, inhaling 100 p.p.m. H<sub>2</sub>S did not affect endogenous glucose production (as calculated from the rate of appearance of 1,2,3,4,5,6-<sup>13</sup>C<sub>6</sub>-glucose during continuous i.v. isotope infusion), whole body CO<sub>2</sub> production, or direct, aerobic glucose oxidation rate (as derived from VCO<sub>2</sub> and the expiratory <sup>13</sup>CO<sub>2</sub>/<sup>12</sup>CO<sub>2</sub> ratio) during normothermia. However, combining inhaled H<sub>2</sub>S with hypothermia (core temperature 27°C) increased glucose oxidation, suggesting a shift towards preferential carbohydrate utilization (Figure 2) (Baumgart *et al.*, 2010). Such a switch in fuel utilization is associated with an improved yield of oxidative phosphorylation (Korvald *et al.*, 2000): the ATP synthesis/oxygen consumption ratio is higher for glycolysis than for oxidation because NADH (as an electron donor) provides three coupling sites rather than the two that FADH<sub>2</sub> provides (Leverve, 2007). This effect on substrate metabolism coincides with a

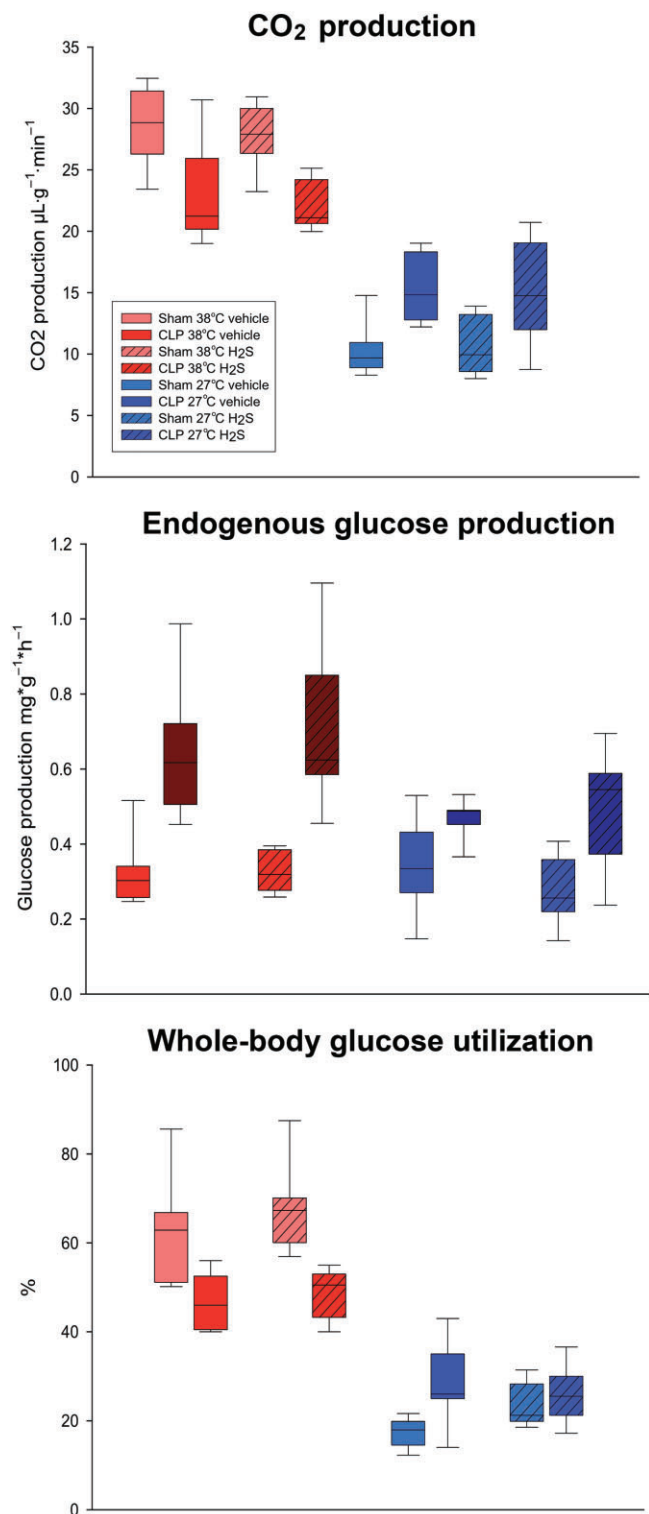


**Figure 1**

Effect of H<sub>2</sub>S and hypothermia on mitochondrial respiration in mechanically permeabilized small murine liver biopsies. Eight groups of anaesthetized and mechanically ventilated mice were studied in order to combine all the treatments of interest, namely sham operation versus CLP (closed white and black bars vs. light and grey bars), normothermia (38°C, white and light grey bars) versus hypothermia (27°C, black and dark grey bars) and i.v. application of the sulfide donor Na<sub>2</sub>S (diagrams B and D) or placebo (diagrams A and C). The study design as well as the methods used to measure mitochondrial respiratory activity in the tissue samples is described in Baumgart *et al.* (2010). Briefly, mitochondrial respiration was measured in terms of oxygen flux ( $J_{O_2}$ ) per mg wet tissue under maximum stimulation by complex I + II substrates and ADP in the coupled (OxPhos) and FCCP-induced uncoupled (ETS) conditions. The Leak state (Leak) was obtained in the OxPhos state by inhibiting the ATP synthesis by oligomycin, and represents the respiratory activity necessary to compensate for the proton leakage, slipping and cations exchange along the inner mitochondrial membrane. Relating the Leak state to the OxPhos state and to the ETS state yields the L/P and L/E ratios, respectively, the P/E ratio is the ratio OxPhos to ETS state (see diagrams C and D). Data are shown as mean  $\pm$  SEM of  $n = 6$  determinations, \* $P < 0.05$ .

significantly attenuated responsiveness of hepatic mitochondrial respiration to stimulation with exogenous cytochrome C oxidase, that is, an improved maintenance of the outer mitochondrial membrane (Kuznetsov *et al.*, 2004). It should be noted, however, that this protective effect of inhaled H<sub>2</sub>S partially disappeared during CLP-induced septic shock: not only did inhaled H<sub>2</sub>S not affect the sepsis-induced metabolic acidosis (Wagner *et al.*, 2011). Finally, during sepsis, inhaled H<sub>2</sub>S did not improve the responsiveness to stimulation with exogenous cytochrome-c-oxidase either. It remains open whether the lacking effects of H<sub>2</sub>S on substrate utilization were due to the sepsis *per se* and/or the ongoing noradrenaline infusion needed to maintain target haemodynamics: noradrenaline causes oxidative stress and may inhibit mitochondrial respiration (Bracht *et al.*, 2012a).

Taken together, the above data, as well as multiple prior studies with sulfide donation or sulfide inhibition (reviewed in Wagner *et al.*, 2009; Coletta and Szabo, 2013), reveal a complex, and only partially understood, regulation of sulfide in circulatory shock. While in some experimental models sulfide donation is clearly beneficial, in other models, sulfide donation actually exacerbates the outcome. In yet other models, however, pharmacological inhibitors of sulfide biosynthesis are therapeutically effective. These findings may reflect the diversity of the roles of sulfide in various models, forms and stages of shock (ranging from sulfide deficiency to sulfide overproduction), and may also relate to tissue/organ-specific differences or species-specific differences, as well as may be related to the complex, biphasic or bell-shaped biological effects of sulfide. Clearly, additional work needs to be



conducted in this area, and it needs to be directly tested whether endogenously produced sulfide contributes to cytopathic hypoxia in certain models. In addition, further work needs to be conducted to test whether metabolic effects or mitochondrial protective mechanisms contribute to the therapeutic effects of sulfide donation in those models where sulfide administration was found beneficial.

**Figure 2**

Glucose utilization in anaesthetized and ventilated mice and inhaling 100 p.p.m. gaseous H<sub>2</sub>S or the carrier gas N<sub>2</sub> (vehicle) during deliberate normothermia (38°C) or hypothermia (27°C) with CLP-induced septic shock or after sham operation. All data are median (quartiles),  $n = 8-11$  per group. # $P < 0.05$  shows significant difference versus respective normothermic vehicle-treated animals, § $P < 0.05$  shows significant difference between vehicle and respective H<sub>2</sub>S-treated mice, \$ $P < 0.05$  shows significant difference versus respective sham-operated group. Data in sham-operated mice were adapted from a prior set of studies by Baumgart *et al.* (2010).

## H<sub>2</sub>S as a mitochondrial protectant in diabetes

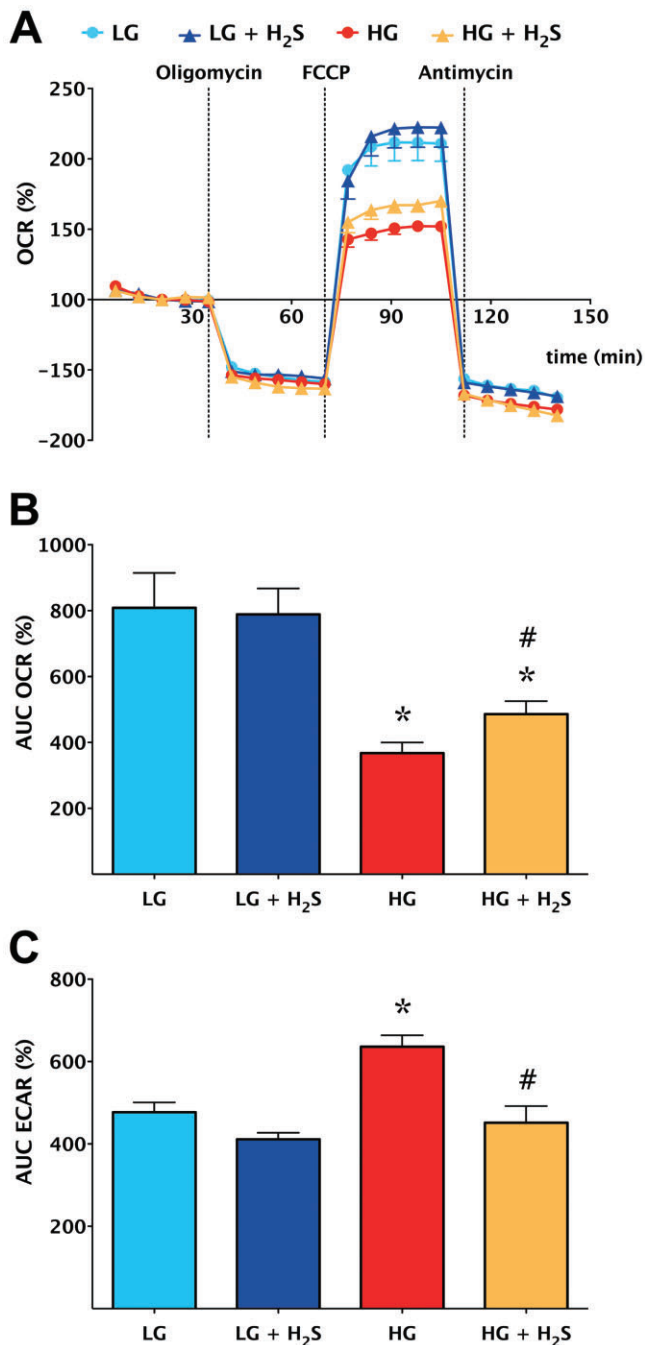
Chronic or intermittent elevations in circulating glucose concentration damage the blood vessels, especially the innermost cell layer, the endothelium. Diabetic endothelial dysfunction is now recognized as a central pathophysiological event in the development of many, if not all, complications of diabetes, ranging from vascular events (peripheral vascular disease, coronary artery disease, diabetic retinopathy, diabetic neuropathy) to other diabetic complications such as impaired wound healing, cardiomyopathy and male erectile dysfunction (Szabo, 2005; 2009; Pacher and Szabo, 2006; Horváth *et al.*, 2009; Rosenson *et al.*, 2011; Shin *et al.*, 2011; Stitt *et al.*, 2013).

The mechanisms and experimental therapy of diabetic/hyperglycaemic endothelial dysfunction have been characterized in significant detail. Several lines of studies show that elevated extracellular glucose induces mitochondrial dysfunction in endothelial cells (Nishikawa *et al.*, 2000; Du *et al.*, 2003; Giacco and Brownlee, 2010; Gerö *et al.*, 2013). On one hand, this entails the suppression of cellular bioenergetics through the impairment of the mitochondrial electron transport/ATP generation (Sivitz and Yorek, 2010; Pangare and Makino, 2012). On the other hand, intracellular production of reactive oxygen and nitrogen species from the dysfunctional mitochondria initiates multiple pathways of pro-inflammatory signalling (Nishikawa *et al.*, 2000; Du *et al.*, 2003; Giacco and Brownlee, 2010), as well as deleterious, self-amplifying cycles of cell injury such as the activation of the nuclear enzyme PARP (García Soriano *et al.*, 2001; Horváth *et al.*, 2009).

Several lines of studies demonstrate that hydrogen sulfide exerts protective effects against the development of diabetic complications (reviewed in Szabo, 2012), at least in part by protecting the diabetic mitochondria. Circulating sulfide levels are decreased in diabetes, in part due to an increased consumption of sulfide by reactive oxygen species (ROS) production and in part due to the down-regulation of CSE, the principal H<sub>2</sub>S-producing enzyme in endothelial cells (Brancaleone *et al.*, 2008; Jain *et al.*, 2010; Whiteman *et al.*, 2010; Suzuki *et al.*, 2011; Kundu *et al.*, 2013; Yamamoto *et al.*, 2013). These events are paralleled with impaired cellular bioenergetics as well as reduced endothelium-dependent relaxant responses and increased pro-inflammatory signalling events, ultimately leading to cell death. Silencing of CSE further exacerbates the hyperglycaemic ROS production,

while supplementation of CBS (by pharmacological tools or by overexpression of H<sub>2</sub>S-producing enzymes) suppresses it (Suzuki *et al.*, 2011). Pharmacological supplementation or genetic expression of H<sub>2</sub>S-producing enzymes in cells subjected to hyperglycaemia reduces mitochondrial ROS formation (Suzuki *et al.*, 2011; Guan *et al.*, 2012; Zhong *et al.*, 2012; Zhou and Lu, 2013) and exerts cytoprotective effects (Suzuki *et al.*, 2011), including a partial normalization of mitochondrial bioenergetics (restoration of oxidative phosphorylation and inhibition of glycolysis, which is elevated in hyperglycaemic endothelial cells as a compensatory mechanism) (Suzuki *et al.*, 2011) (Figure 3). Sulfide also protects against the activation of pro-inflammatory signalling pathways in

hyperglycaemic endothelial cells (e.g. NF- $\kappa$ B activation, inflammatory cytokine production) (Guan *et al.*, 2013; Manna and Jain, 2013; Si *et al.*, 2013), reduction in matrix protein synthesis/protection against remodelling (Yuan *et al.*, 2011; Lee *et al.*, 2012; Kundu *et al.*, 2013), as well as against the activation of PARP (Suzuki *et al.*, 2011). The beneficial effects of sulfide supplementation have also been demonstrated in multiple models of diabetes *in vivo*, resulting in improved cardiac contractility, improved endothelium-dependent relaxations, and improved retinal and renal function and histopathology (Gao *et al.*, 2011; Suzuki *et al.*, 2011; Yang *et al.*, 2011; Ahmad *et al.*, 2012; Zhong *et al.*, 2012; Si *et al.*, 2013; Yamamoto *et al.*, 2013). Given the fact that the direct reaction of H<sub>2</sub>S with various oxidant species is relatively slow (Carballal *et al.*, 2011) and is outcompeted by many other reactions in the mitochondrion, it is likely that indirect antioxidant effects (e.g. via the regulation of signalling processes and/or via up-regulation of various antioxidant pathways) are also involved in the observed mitochondrial protective effects of sulfide. The beneficial effects on bioenergetic/mitochondrial processes are likely to play a role in the therapeutic effect of H<sub>2</sub>S in diabetic complication models. However, it is likely that additional effects of H<sub>2</sub>S (on signal transduction, redox balance and, perhaps, vasodilatory/haemodynamic effects) also contribute.



## H<sub>2</sub>S as endogenously produced metabolic 'fuel' in cancer cells

Cellular bioenergetics is essential for invasive tumour growth and metastasis and constitutes a key point in the control of cancer progression (Pathania *et al.*, 2009; VanderHeiden *et al.*, 2009; Ramsay *et al.*, 2011; Bailey *et al.*, 2012). Tumour cells rely both on oxidative phosphorylation (mitochondrial activity) and on increased glycolytic activity ('aerobic glycolysis' to produce ATP), which is used to support tumour cell growth, division cell movement. Tumour cell bioenergetics is intricately intertwined with pro-inflammatory and

### Figure 3

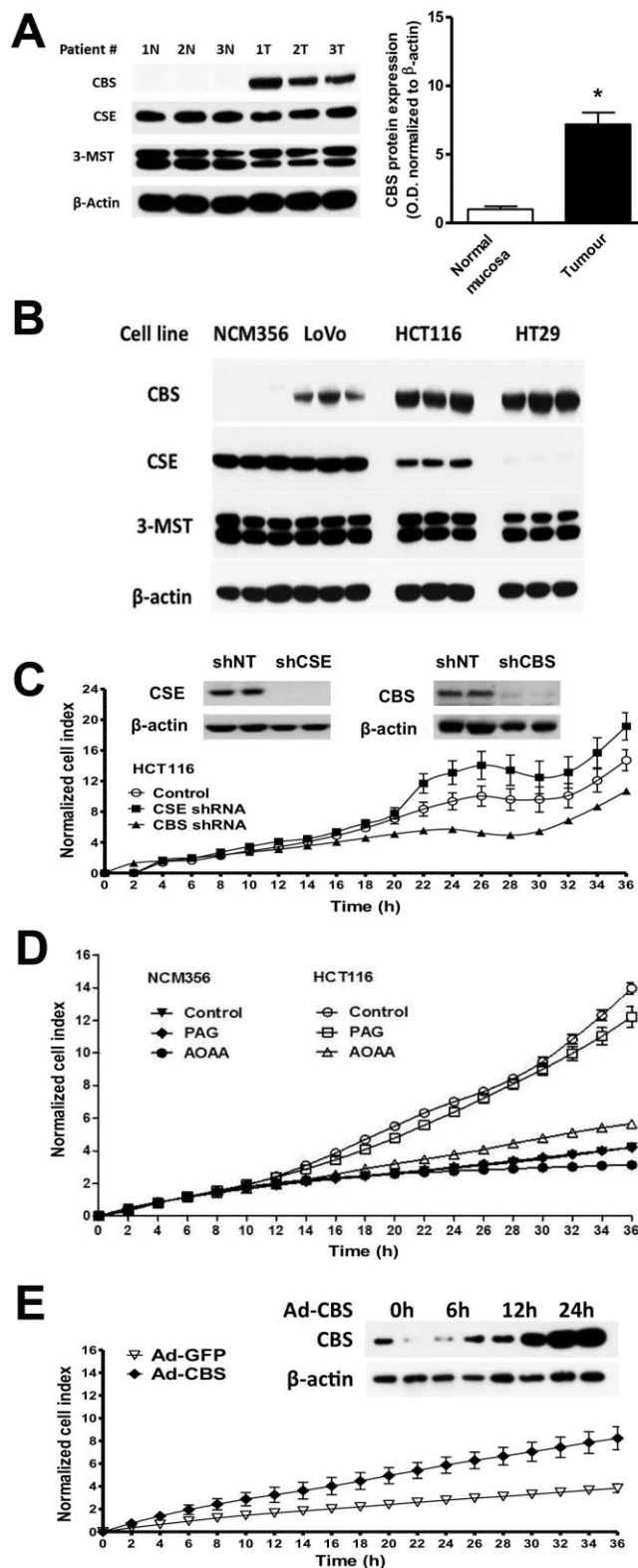
H<sub>2</sub>S reduces the degree of the bioenergetic derangements in endothelial cells placed in high extracellular glucose. Bioenergetic analysis of bEnd3 murine microvascular endothelial placed in low (5.5 mM, LG) or high (40 mM, HG) glucose conditions for 7 days in the absence or presence of H<sub>2</sub>S treatment (300  $\mu$ M every 8 h) was performed using the Seahorse XF24 Analyzer as described (Suzuki *et al.*, 2011). (A) A time course for measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) under basal conditions, followed by the sequential addition of oligomycin (1  $\mu$ g·mL<sup>-1</sup>), FCCP (0.3  $\mu$ M) and antimycin A (2  $\mu$ g·mL<sup>-1</sup>). (B) OCR and (C) ECAR values, representing indices of mitochondrial oxidative phosphorylation and glycolysis respectively. Increased extracellular glucose induced a suppression of oxidative phosphorylation and an increase in glycolysis (presumably a compensatory response), as compared with normal glucose (\**P* < 0.05). Treatment with H<sub>2</sub>S partially restored these changes (#*P* < 0.05). Data represent mean  $\pm$  SEM of *n* = 15 wells from three experiments performed on different experimental days. Reproduced with permission from Suzuki *et al.* (2011).

pro-growth tumour cell signalling processes (Cairns *et al.*, 2011; Schulze and Harris, 2012). Therapeutic targeting of tumour cell energetics – especially using approaches that confer selectivity to the tumour cell – is an emerging concept in contemporary cancer therapy (Ramsay *et al.*, 2011; Bailey *et al.*, 2012).

Recent data from a colorectal cancer models indicate that cancer cells can utilize H<sub>2</sub>S as an endogenous bioenergetic ‘fuel’ (Szabo *et al.*, 2013b). A comparison of human colon cancer specimens with patient-matched normal mucosa tissue revealed the selective up-regulation of CBS in the cancers. In contrast, the expression of the other two H<sub>2</sub>S-producing enzymes (CSE and 3-MST) remained unchanged. Similar to colon tumours, colon adenocarcinoma-derived cell lines (HCT116, HT-29, LoVo) exhibit selective up-regulation of CBS when compared with NCM356 cells (a non-tumorigenic control human epithelial cell line) (Figure 4A,B). Homogenates of the patient-derived colon tumour specimens, as well as homogenates of the colon cancer-derived cell lines, exhibit an increased rate of H<sub>2</sub>S production, which is blocked by the CBS inhibitor aminooxyacetic acid (AOAA), a widely used CBS inhibitor (Abe and Kimura, 1996; Julian *et al.*, 2002; Austgen *et al.*, 2011; Han *et al.*, 2011; Asimakopoulou *et al.*, 2013; Rashid *et al.*, 2013; Wang *et al.*, 2013). However, H<sub>2</sub>S production was not significantly inhibited by the CSE inhibitor PAG. (For additional discussion on the limitations of pharmacological inhibitors of CBS and CSE, see the final section of the current article.)

To investigate the functional consequence of CBS up-regulation, gene-specific shRNA sequences in lentiviral vectors were used to suppress the expression of either CBS or CSE in HCT116 cells. Densitometric analyses of Western blots revealed an approximately 50% decrease in CBS expression with comparable reductions in both cell proliferation and H<sub>2</sub>S production (Figure 4C). In contrast to CBS, silencing of CSE or its inhibition by PAG did not significantly affect either HCT116 cell proliferation or H<sub>2</sub>S production (Szabo *et al.*, 2013b). To further define the function of CBS in colon cancer cells, both NCM356 cells (which express CSE, but only low levels of CBS) were treated and HCT116 cells (which abundantly express CBS) with the pharmacological CBS inhibitor, AOAA. Consistent with the CBS knock-down experiments, AOAA has also been utilized. AOAA inhibited the growth of

HCT116 colon cancer cells, but did not affect the proliferation of the slower growing non-malignant NCM356 cell line (Szabo *et al.*, 2013b) (Figure 4D). Conversely, forced overexpression of CBS in NCM356 cells significantly increased their basal rate of proliferation (Figure 4E). AOAA treatment also



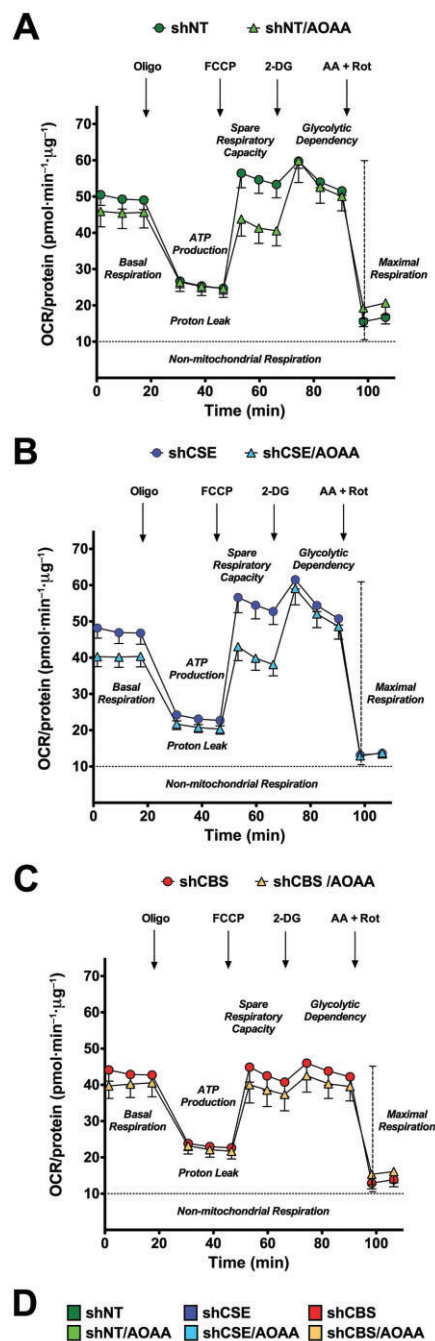
**Figure 4**

CBS is up-regulated in colon cancer cells and supports cancer cell proliferation. (A) High levels of CBS expression in human colon cancer tissues as compared with non-cancerous margin tissue. (B) High levels of CBS expression in various human colon cancer lines. Please note that the non-transformed colonic epithelial cell NCM356 did not show high CBS expression. Furthermore, please note that 3-MST expression was uniform among all tissues, while CSE expression was variable. (C) Lentiviral stable silencing of CBS, but not of CSE attenuates the growth of HCT116 colon cancer cells. Inset shows the efficiency of (D) pharmacological inhibition of CBS with AOAA (1 mM) but not of CSE with PAG (3 mM) attenuates the growth of HCT116 colon cancer cells. (E) Forced adenoviral expression of CBS in NCM356 cells increases their rate of proliferation. Inset shows the adenoviral up-regulation of CBS over time. Data represent mean ± SEM; reproduced with permission from Szabo *et al.* (2013b).

suppressed the migration and invasion of HCT116 cells. Finally, inhibition of CBS with AOAA reduced endothelial cell migration in colon cancer/endothelial cell co-cultures (Szabo *et al.*, 2013b).

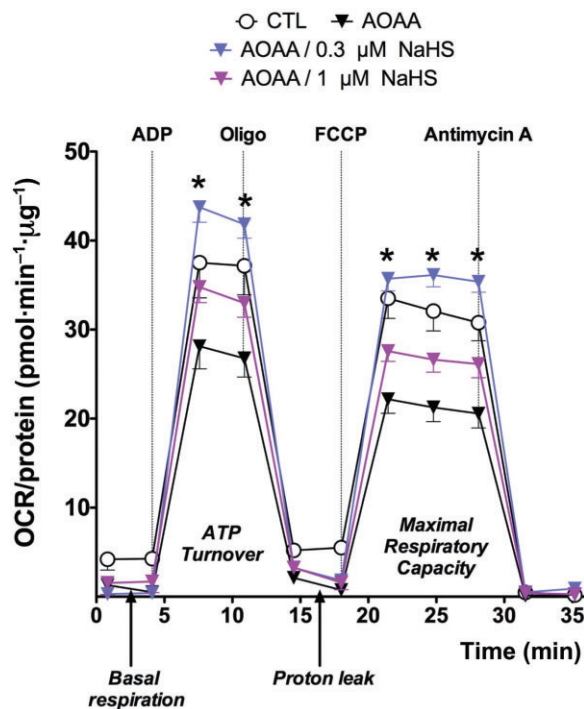
The above data demonstrated that CBS and CBS-derived H<sub>2</sub>S play an important role in promoting tumour cell proliferation. In the context of the current article, the most relevant question is the following: What role, if any, do H<sub>2</sub>S-associated energetic alterations play in these actions? In order to answer this question, HCT116 colonic cancer cells, and NCM356 cells as non-transformed controls, were subjected to bioenergetic measurements using the Extracellular Flux Analysis (Szabo *et al.*, 2013b). In agreement with the physiological role of endogenous H<sub>2</sub>S in promoting cellular bioenergetics in various cell types including colonocytes (reviewed in Szabo *et al.*, 2013a), low concentrations of H<sub>2</sub>S caused a stimulation of mitochondrial function in mitochondria isolated from HCT116 cells. Moreover, shRNA-mediated silencing of CBS, or CBS inhibition with AOAA, reduced basal cellular respiration, suppressed the calculated ATP synthesis and attenuated the spare respiratory capacity (Figure 5A–C). CBS silencing or CBS inhibition also reduced glycolytic functions, while CSE silencing was without significant effect (Figure 5D). This latter effect can be attributed, at least in part, to inhibition of GAPDH activity, as H<sub>2</sub>S is known to be an activator of GAPDH via sulfhydration (Mustafa *et al.*, 2009; Paul and Snyder, 2012). In mitochondria prepared from HCT116 cells, L-cysteine stimulated mitochondrial electron transport; this effect was markedly attenuated in mitochondria prepared from shCBS cell expression (Szabo *et al.*, 2013b). In AOAA-treated mitochondria (which showed suppressed electron transport), sulfide addition improved and restored mitochondrial function (Figure 6). This mitochondrion-autonomous response was consistent with the results of cell fractionation studies, showing that a significant portion of the total amount of cellular CBS was associated with the mitochondria in HCT116 cells. CBS was primarily associated with the outer mitochondrial membrane (Szabo *et al.*, 2013b).

These findings indicate that endogenous sulfide, produced from CBS, stimulates tumour cell bioenergetics (both



## Figure 5

CBS supports colon cancer bioenergetics. Effect of the CBS inhibitor AOAA on cellular bioenergetics is shown in (A) HCT116 cells transfected with non-targeting vector (shNT), in (B) cells transfected with a CSE-silencing vector (shCSE) and (C) in cells transfected with a CBS-silencing vector (shCBS). Part (D) shows a comparison of the various bioenergetic responses. Please note that HCT116 cells with CBS silencing show a suppression basal bioenergetic function. In addition, the FCCP-induced increase in oxygen consumption is markedly attenuated in CBS-silenced cells. AOAA causes a comparable suppression of the bioenergetic responses in the shNT and the shCSE cells, while in the shCBS cells (in which the bioenergetic response is already suppressed), AOAA only exerts a slight degree of additional effect. (\* $P < 0.05$  shows significantly lower bioenergetic parameters in shCBS cells as compared with shNT cells; # $P < 0.05$  shows significant effect of AOAA in either the shNT, shCBS or shCSE cells, when compared with the respective response in the absence of AOAA.) Data represent mean  $\pm$  SEM of  $n = 3$  determinations. Reproduced with permission from Szabo *et al.* (2013b).



**Figure 6**

Restoration of mitochondrial function by sulfide in colon cancer cells treated with AOAA. Mitochondria were prepared from vehicle-treated HCT116 cells or from cells treated with AOAA (1 mM, 90 min) and subjected to bioenergetic analysis in the presence or absence of sulfide administration (applied as NaHS) at 0.3 and 1  $\mu$ M, as described (Szabo *et al.*, 2013b). Please note that AOAA suppressed mitochondrial OCR, while sulfide restored the functionality of the mitochondria. Data represent mean  $\pm$  SEM of  $n = 3$  determinations; \* $P < 0.05$  shows the significant stimulatory effect of sulfide.

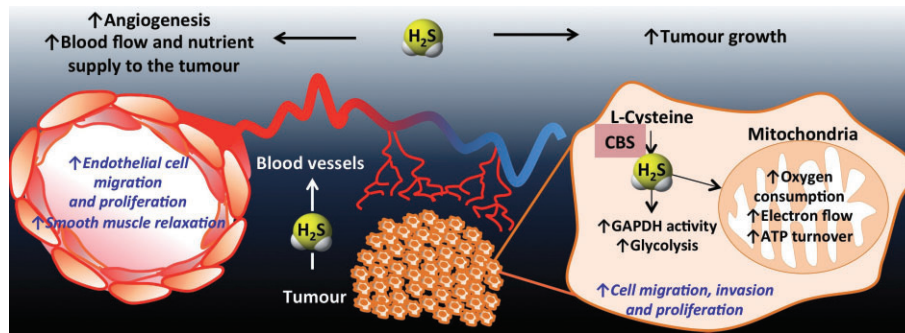
oxidative phosphorylation and glycolysis). The effect CBS silencing or CBS inhibition has on tumour progression *in vivo* has also been investigated by utilizing nude mice subjected to heterotopic transplantation of either HCT116 cells or patient-derived colon cancer xenografts (Szabo *et al.*, 2013b). Consistent with the *in vitro* findings showing that CBS inhibition suppresses tumour cell proliferation, shRNA-mediated knock-down of CBS expression (shCBS) or treatment of the mice with AOAA significantly reduced the growth rate and size (i.e. volume) of HCT116 tumour xenografts (Szabo *et al.*, 2013b). In contrast, silencing of CSE (shCSE) or pharmacological inhibition of CSE with PAG did not affect tumour growth. CBS suppression caused a significant reduction in the density of CD31-positive blood vessels within the tumour tissue as well as the prevalence of larger blood vessels and the extent of vessel branching, consistent with the hypothesis that CBS-derived H<sub>2</sub>S acts locally in a paracrine manner to stimulate tumour angiogenesis (Szabo *et al.*, 2013b); the pro-angiogenic role of H<sub>2</sub>S is supported by multiple lines of *in vitro* and *in vivo* studies (Cai *et al.*, 2007; Papapetropoulos *et al.*, 2009; Szabo and Papapetropoulos, 2011; Coletta *et al.*, 2012). Finally, in a series of translational studies, AOAA treatment showed a reduced rate of the growth of patient-derived tumour transplants (Szabo *et al.*, 2013b).

Taken together, these data support the hypothesis that CBS-derived endogenously produced H<sub>2</sub>S plays a key role in cancer cell bioenergetics and cell proliferation via autocrine mechanisms. Moreover, via paracrine mechanisms, H<sub>2</sub>S also supports tumour angiogenesis and acts as a local vasodilator, thereby stimulating the supply of the tumour with nutrients via the bloodstream (Figure 7). Several independent lines of studies show that H<sub>2</sub>S can produce an activation of kinase pathways and inhibition of phosphatase pathways (Cai *et al.*, 2007; 2010; Hu *et al.*, 2008; 2011; Krishnan *et al.*, 2011; Manna and Jain, 2011), leading to the regulation of the cell cycle (Deplancke and Gaskins, 2003; Cai *et al.*, 2010). Because these pathways are crucial contributors to tumour cell proliferation, it is conceivable that such effects may also contribute to the proliferative effects of the CBS/H<sub>2</sub>S axis in colorectal tumours (and possibly other tumours as well).

To put the above findings in the context of prior findings in the literature, it is interesting to note that a number of papers have previously reported that CBS expression is increased in various tumours (Goss, 1986; Zhang *et al.*, 2005; Guo *et al.*, 2012a). Moreover, in one prior study, CBS expression has been associated with increased cell proliferation (MacLean *et al.*, 2002) (even though none of these prior investigations raised the potential involvement of H<sub>2</sub>S). In addition, several studies demonstrated increased H<sub>2</sub>S production in the exhaled air and flatus of cancer patients (Kumar *et al.*, 2012; Yamagishi *et al.*, 2012; Altomare *et al.*, 2013) and increased urinary excretion of thiosulfate, the stable breakdown product of H<sub>2</sub>S (Chwatko *et al.*, 2013) (even though these investigations have not identified the enzymatic source). Finally, several recent studies noted the inhibitory effect of AOAA on cancer cell proliferation *in vitro* and/or *in vivo* (Thornburg *et al.*, 2008; Weinberg *et al.*, 2010; Anso *et al.*, 2013; Son *et al.*, 2013) (even though these investigators attributed the effects of AOAA to pharmacological actions other than CBS inhibition).

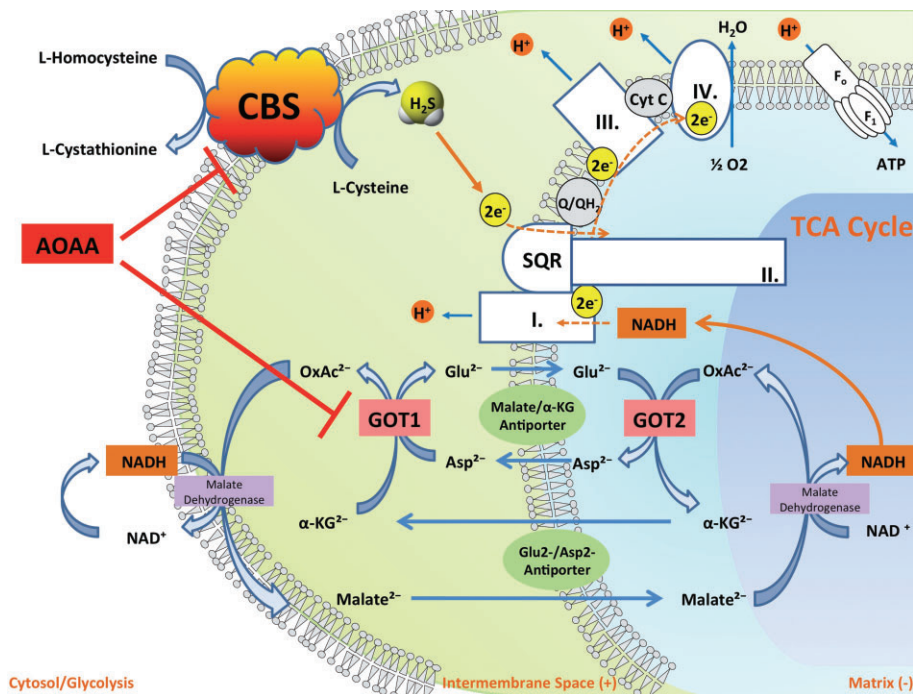
The pharmacology of AOAA is, indeed, complex. This compound is generally known as a non-specific inhibitor of transaminases. It inhibits the activity of these enzymes because it irreversibly binds to the prosthetic group pyridoxal phosphate, which is commonly found in the active site of transaminases. In the context of recent studies, its inhibitory effect on GOT1 [an essential component of the malate/aspartate shuttle, a mechanism that links glycolysis to the transfer of electron donors into the mitochondria of cancer cells (Greenhouse and Lehninger, 1976; 1977; Dang *et al.*, 2011)] has been linked to antitumor actions (Thornburg *et al.*, 2008). In addition, the function of GOT1 is linked to glutaminolysis, a metabolic pathway that is at least partially selective to cancer cells (and, hence, may be therapeutically targetable) (Le *et al.*, 2012; Son *et al.*, 2013); AOAA has been shown to inhibit the metabolic activity of cancer cells by interfering with glutaminolysis (Son *et al.*, 2013). The studies discussed above show that CBS/H<sub>2</sub>S production can also be considered as a partially cancer-selective metabolic pathway. It can be argued that with AOAA, one can simultaneously block the energetic 'fuelling' of the cancer cell via the malate/aspartate shuttle and the energetic 'fuelling' of the cancer cell via the CBS/sulfide route (Figure 8), thereby inducing 'synthetic lethality' with two independent pharmacological effects of a single compound. In order to compare the effects





**Figure 7**

Multiple mechanisms of the cancer-cell-survival function of the CBS/H<sub>2</sub>S axis *in vivo*. As a result of the CBS overexpression, H<sub>2</sub>S is overproduced in colon cancer cells. H<sub>2</sub>S serves as an inorganic electron donor, stimulating mitochondrial electron transport, increasing ATP turnover. In addition, it increases the glycolytic activity of the tumour cell by activating GAPDH. Via these autocrine bioenergetic effects, H<sub>2</sub>S stimulates cancer cell proliferation, migration and invasion. In addition, H<sub>2</sub>S diffuses into the surrounding cells and tissues, stimulating angiogenesis, as well as acting as a vascular relaxant. Via these paracrine effects, CBS-derived H<sub>2</sub>S promotes the supply of blood and nutrients to the tumour. Reproduced with permission from Szabo *et al.* (2013b).

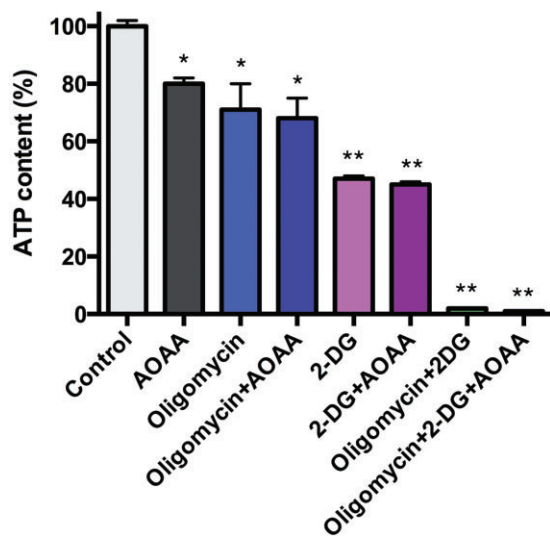


**Figure 8**

By simultaneously inhibiting CBS activity and inhibiting GOT1, a key enzyme of the malate/aspartate shuttle, AOAA acts as an inducer of 'synthetic lethality' in cancer cells. CBS-derived H<sub>2</sub>S supports mitochondrial electron transport and cancer cell bioenergetics by donating electrons at complex II. By inhibiting CBS, AOAA suppresses this bioenergetic pathway. The malate-aspartate shuttle translocates electrons that are produced in glycolysis across the semipermeable inner membrane of the mitochondrion in order to support oxidative phosphorylation. These electrons enter the electron transport chain at complex I. The shuttle system is required because the mitochondrial inner membrane is impermeable to NADH (a primary reducing equivalent of the electron transport chain). In humans, the cytoplasmic enzyme (GOT1) is one of the key enzymes in the malate shuttle: it functions to catalyse the interconversion of aspartate and  $\alpha$ -ketoglutarate to oxaloacetate and glutamate using pyridoxal phosphate as a cofactor. By inhibiting GOT1, AOAA reduces the transfer of electron donors to the mitochondria, thereby suppressing cancer cell bioenergetics. By the simultaneous inhibition of CBS and GOT1, AOAA interferes with two key pathways of cancer cell mitochondrial function.

of AOAA with the effects of glycolysis inhibition and inhibition of ATP synthase, we have evaluated the effect of the glycolysis inhibitor 2-deoxyglucose, the ATP synthase inhibitor oligomycin, and AOAA (alone and in various combina-

tions) on ATP levels in HCT116 tumour cells. AOAA reduced ATP levels to 80% of control, whereas oligomycin reduced them to 71%. Addition of AOAA to oligomycin did not exert a substantial further inhibitory effect. Likewise, addition of



**Figure 9**

Relative importance of oxidative phosphorylation and glycolysis in the maintenance of ATP levels in HCT-116 cells. HCT-116 cells were cultured for 8 h in the presence of the ATP synthase inhibitor oligomycin ( $1 \mu\text{g}\cdot\text{mL}^{-1}$ ), the glycolysis inhibitor 2-deoxyglucose (100 mM), the CBS/GOT1 inhibitor AOAA (1 mM) and their various combinations, followed by the measurement of cellular ATP levels, using a bioluminescent kit Sigma (St. Louis, MO, USA) as described (Módis *et al.*, 2013a). Data represent mean  $\pm$  SEM of  $n = 3$  determinations; \* $P < 0.05$  and \*\* $P < 0.01$  show significant inhibitory effect of the various pharmacological inhibitors compared with the vehicle control group.

AOAA to 2-deoxyglucose (which, on its own, reduced ATP levels to 45%) did not further inhibit cellular ATP levels (Figure 9). These findings point to a coordinated interaction between sulfide-dependent modulation of both oxidative phosphorylation and glycolysis; in both cases more likely in a role as an enhancer/potentiator, rather than an independent activator. It must also be emphasized that the effects of sulfide in cancer cells are likely to be more complex and are likely to include additional effects beyond mitochondrial function (such as effects on glycolysis) as well as pharmacological effects beyond bioenergetics (such as effects on kinase pathways and cell cycle). While it is clear that much further work needs to be conducted in this area, the above series of studies may open a previously unexplored, and potentially clinically translatable aspect of tumour biology.

The mechanism of the up-regulation of CBS in colon cancer cells is not known. Possibilities include a transcriptional up-regulation, a blockade of its degradation, or targeted delivery to the mitochondria via not yet understood mechanisms. A recent study by Teng and colleagues demonstrating in ischaemic liver and in hypoxic hepatocytes that CBS is targeted to the mitochondria via the hypoxia-sensitive regulation of the mitochondrial matrix protease L on protease (Teng *et al.*, 2013) offers a plausible mechanism (especially in light of the well-known phenomenon of tumour hypoxia), which, nevertheless, remains to be directly tested in future studies.

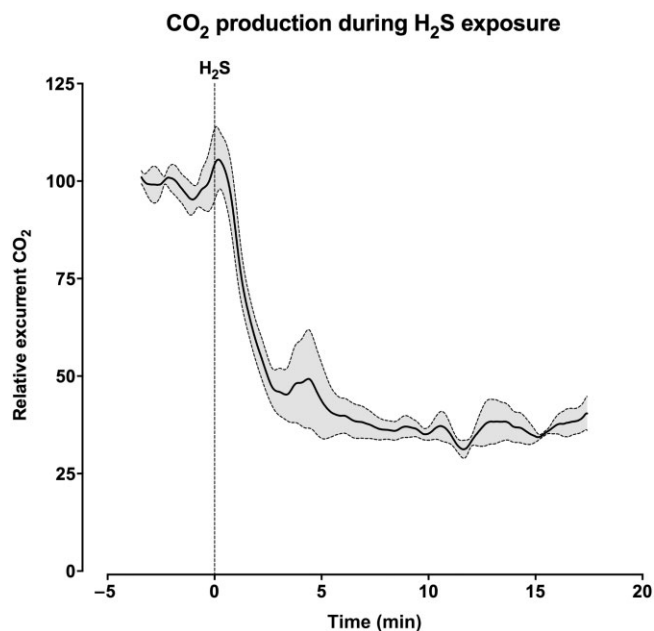
We are well aware of reports in the literature, demonstrating that it is  $\text{H}_2\text{S}$  donation (rather than  $\text{H}_2\text{S}$  biosynthesis inhibi-

tion) that can kill cancer cells (e.g. Shin *et al.*, 2010; Lee *et al.*, 2011; Chattopadhyay *et al.*, 2012a,b; Lim *et al.*, 2012). These studies tend to use relatively high concentrations of various  $\text{H}_2\text{S}$  donors, often linked with molecular scaffolds that have their own sulfide-independent pharmacological actions (e.g. NOSH-aspirin, which has a non-steroidal anti-inflammatory component). These findings are in no way in conflict with the findings related to the bioenergetic and pro-angiogenic effects of endogenously produced  $\text{H}_2\text{S}$  in cancer cells, but, rather, illustrate the complex pharmacology of  $\text{H}_2\text{S}$ , where low concentrations/fluxes of the mediator and higher concentrations/release rates of the same mediator can have radically different biological effects (as discussed in Szabo *et al.*, 2013a). The interpretation of the findings related to the anticancer effect of  $\text{H}_2\text{S}$  donors may therefore be related to the high concentrations of  $\text{H}_2\text{S}$  used, at which concentrations  $\text{H}_2\text{S}$  has cytostatic and cytotoxic properties (possibly, at least in part related to the inhibition of mitochondrial electron transport chain system at complex IV). We predict that one of the technical hurdles of exploiting therapeutic  $\text{H}_2\text{S}$  donation for anticancer therapy will be the delivery: one needs to generate high local levels of  $\text{H}_2\text{S}$ , for reasonably prolonged time, in order to produce anticancer effects. It is hoped that further studies will solve this issue; a similar problem has previously arisen in the context of NO donation and anticancer therapy (NO, too, has bell-shaped dose responses and vital, physiological effects at low concentrations and cytostatic effects at higher concentrations). In this context, photodynamic therapy, with NO-releasing photolabile molecules, has been put forward as one possible technical solution (Bhowmick and Girotti, 2013; Rapozzi *et al.*, 2013).

## Therapeutic induction of 'on-demand suspended animation' with $\text{H}_2\text{S}$

One of the most fascinating biological features of  $\text{H}_2\text{S}$  is the 'suspended animation-like state' that it can induce. The landmark paper on that subject (Blackstone *et al.*, 2005), which utilized gaseous  $\text{H}_2\text{S}$  to induce a hibernation-like state, has sparked interest in the use of sulfide as an inducer of hypometabolism to protect organs and cells against temporary insults. By exposing mice to subtoxic concentrations of  $\text{H}_2\text{S}$  (20–80 p.p.m.), a concentration-dependent state of deeply reduced metabolism was induced, as evidenced by a fast reduction of total-body  $\text{CO}_2$  production and  $\text{O}_2$  consumption (60–70% within 5–10 min), followed by a slow decline of metabolism to around 5% of basal levels. This slower decline parallels the reduction in body temperature that follows the exposure. Core body temperature steadily declines to around  $2^\circ\text{C}$  above ambient temperature over the course of hours. After cessation of  $\text{H}_2\text{S}$  exposure, metabolism recovers to basal levels with no apparent toxic effects (Blackstone *et al.*, 2005). The sulfide-mediated reduction of metabolic demand has been shown to protect mice against the mortality associated with otherwise lethal hypoxia (Blackstone and Roth, 2007).

Although the above findings of metabolic suppression have been repeatedly confirmed in mice (Blackstone and Roth, 2007; Volpato *et al.*, 2008; Bos *et al.*, 2009) (Figure 10), it has been challenging to translate these findings to larger



**Figure 10**

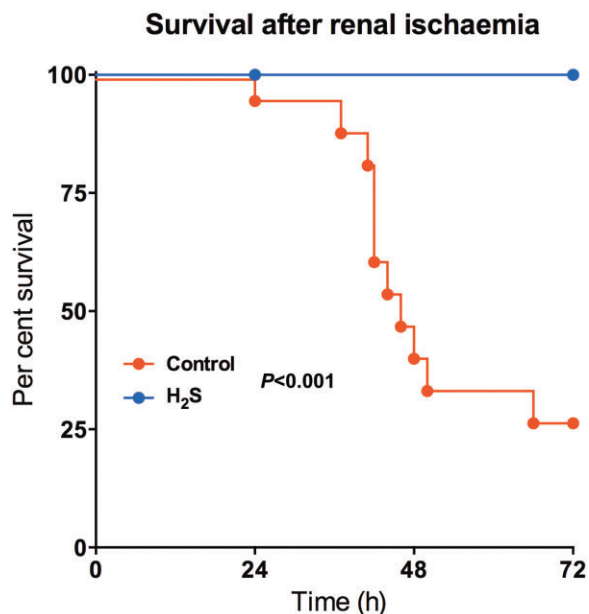
'On-demand suspended animation' induced by H<sub>2</sub>S: reduced CO<sub>2</sub> production of mice exposed to H<sub>2</sub>S. Mean CO<sub>2</sub> production (solid line) and standard error of the mean (dashed lines) of three mice, shown before and after exposure to 100 p.p.m. H<sub>2</sub>S. Reproduced with permission from Bos *et al.* (2012).

mammals. Different studies have been performed with varying results. Some studies showed no effect on metabolic parameters in rats (Haouzi *et al.*, 2009), sheep (Haouzi *et al.*, 2008) or swine (Li *et al.*, 2008; Drabek *et al.*, 2011), while others showed metabolic suppression in rats (Florian *et al.*, 2008; Aslami *et al.*, 2010; 2013a; Seitz *et al.*, 2012) and swine (Simon *et al.*, 2008). One human toxicity study shows that inhalation of 10 p.p.m. H<sub>2</sub>S during exercise reduces V·O<sub>2</sub>, possibly by reducing aerobic capacity (Bhambhani *et al.*, 1997). A comparison of the effects of sulfide to the effects of anaesthetics shows that H<sub>2</sub>S does not have the hypnotic or immobilizing effects of anaesthetic compounds (Li *et al.*, 2012). The last word has not been written on the interspecies variability, but it seems that the translation to larger mammals is more difficult than initially presumed. A possible explanation lies in the different types of metabolism between smaller and larger animals. Mice have a relatively high metabolic rate in relation to their body weight, and a larger proportion of their metabolism is geared towards maintaining core body temperature. It is well established that in response to hypoxia, small rodents can reduce their energy expenditure by decreasing 'non-shivering thermogenesis', irrespective of any pharmacological intervention. Hypoxia-induced hypometabolism is present in numerous mammals (Mortola, 2004) and humans (van Marken Lichtenbelt and Schrauwen, 2011), but due to the high area/volume ratio and, consequently, the higher heat dissipation, it is inversely related to body size (Mortola, 2004), that is, to the ratio of O<sub>2</sub> consumption and body weight (VO<sub>2</sub>/kg). This is substantiated by the fact that (i) no matter the species, newborns present with

more pronounced hypoxia-induced hypometabolism than adults, and (ii) in adults with low VO<sub>2</sub>/kg, hypoxia-induced hypometabolism becomes manifest when normoxic VO<sub>2</sub> is increased, for example, during exercise (Mortola, 2004). Because non-shivering thermogenesis does not result in ATP production due to the activity of uncoupling proteins (van Marken Lichtenbelt and Schrauwen, 2011), reduced VO<sub>2</sub> and the consecutive hypothermia represent a unique adaptation to hypoxia only present in small animals but not in larger species or humans. Consequently, with respect to the putative induction of a 'suspended animation'-like hypometabolism, it has been questioned whether any therapeutic potential of H<sub>2</sub>S observed in mice and rats can be transferred to the clinical setting (Leslie, 2008, Derwall *et al.*, 2010, Drabek *et al.*, 2011, Haouzi, 2011; 2012). Clearly, data in sheep and swine subjected to shock, ischaemia/reperfusion (Sodha *et al.*, 2008; Osipov *et al.*, 2009; Sodha *et al.*, 2009; Osipov *et al.*, 2010; Simon *et al.*, 2011; Hunter *et al.*, 2012), haemorrhage (Bracht *et al.*, 2012b) or burn injury (Esechie *et al.*, 2009) suggest that the beneficial effects of infusing Na<sub>2</sub>S are independent of core temperature: any induction of moderate hypothermia is due to an attenuation of systemic inflammation rather than an effect on energy expenditure (Simon *et al.*, 2011; Bracht *et al.*, 2012b). Further studies need to be conducted to study dose responses and to compare different methods of administration (i.e. gaseous, i.v./i.a. soluble salt injection, slow release donor injection).

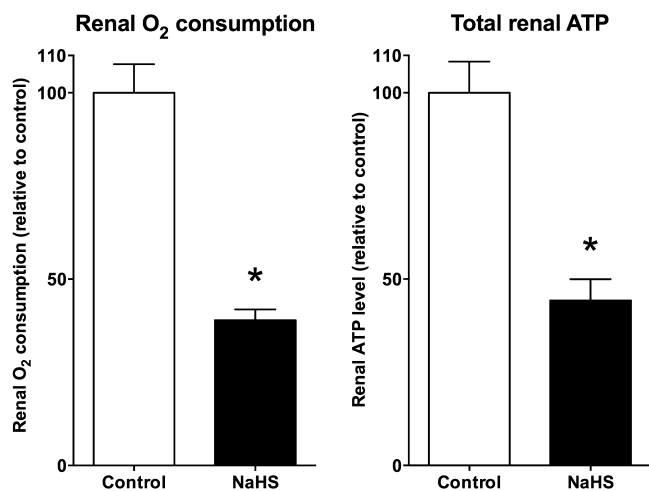
Sulfide-induced hypometabolism is highly protective in different models of hypoxia or ischaemia. During H<sub>2</sub>S-hypometabolism, mice can survive hypoxia (5% O<sub>2</sub>) for up to 6 h, while all the animals in the control group died within 17 min (Blackstone and Roth, 2007). Similarly, hydrogen sulfide-induced hypometabolism has been shown to improve survival in lethal haemorrhagic shock in the rat; the mechanism has been attributed to suppression of tissue oxygen demand by sulfide (Morrison *et al.*, 2008). Tokuda and colleagues have reported that inhaled H<sub>2</sub>S gas suppresses inflammatory response and prolongs survival in endotoxin shock, effects that have been attributed to suppression of metabolism (Tokuda *et al.*, 2012). Inducing a state of hypometabolism using 100 p.p.m. H<sub>2</sub>S before clamping the renal artery can prevent the onset of kidney damage in mice (Figure 11 and Figure 12) (Bos *et al.*, 2009). Induction of hypometabolism during the reperfusion phase has some protective potency, but much less than pretreatment does (Bos *et al.*, 2009). Similar results were obtained for hepatic ischaemia, in which hypometabolic pretreatment protected livers from ischaemic damage (Bos *et al.*, 2012). In both of these studies, hypometabolism was induced while core body temperature was kept at 37°C (thus preventing hypothermia). This shows that hypothermia itself is not needed for hypometabolic protection (but, in theory, it could offer additional protection). H<sub>2</sub>S-induced hypometabolism, when established before the onset of ischaemia, was also protective in cardiac ischaemia (Snijder *et al.*, 2013). Sub-hypometabolic concentrations of H<sub>2</sub>S are mainly associated with anti-inflammatory and anti-fibrotic action, while higher concentrations of H<sub>2</sub>S (those that induce hypometabolism) offer additional protection against early myocardial necrosis.

It is likely that the protective effects of H<sub>2</sub>S can be attributed to several factors, hypometabolism being just one of



**Figure 11**

H<sub>2</sub>S improves survival after bilateral renal ischaemia. Control mice or mice that were in a hypometabolic state (caused by exposure of 100 p.p.m. H<sub>2</sub>S for 30 min) were subjected to bilateral renal ischaemia at 37°C. While in the control group 73% mortality was observed, the H<sub>2</sub>S-pretreated mice all survived the insult. Adapted from Bos *et al.* (2009).



**Figure 12**

H<sub>2</sub>S induces organ hypometabolism *ex vivo*. Isolated perfused rat kidneys treated with NaHS show reduced oxygen consumption and reduced total renal ATP levels. Reproduced with permission from Bos *et al.* (2009).

them. As noted in the previous sections, H<sub>2</sub>S has many pharmacological effects in addition to metabolic suppression, as it can act as an anti-oxidant, anti-fibrotic, anti-inflammatory and anti-apoptotic substance (Zanardo *et al.*, 2006; Yao *et al.*, 2010; Bos *et al.*, 2013; Snijder *et al.*, 2013). In addition, treat-

ment with H<sub>2</sub>S during hypoxia protects the integrity and function of mitochondria (Elrod *et al.*, 2007; Bos *et al.*, 2009).

Although it is of a mixed pharmacological action, sulfide remains to be an intriguing molecule as an on-demand inducer of hypometabolism, with the capability to protect organs from ischaemia or hypoxia. Several sets of studies show the ability of sulfide to hibernate *isolated organs* which could be promising in the setting of organ transplantation, where warm and cold ischaemia times affect transplant survival and function (Table 1, Figure 12) (Hu *et al.*, 2007; Henderson *et al.*, 2010; Hosgood and Nicholson, 2010; Balaban *et al.*, 2011; Bos *et al.*, 2012; George *et al.*, 2012; Hunter *et al.*, 2012; Lobb *et al.*, 2012; Siriussawakul *et al.*, 2012; Xie *et al.*, 2012; Zhu *et al.*, 2012; Wu *et al.*, 2013). In addition, hypometabolic effects (if they prove to be translatable to large animals and humans) could be of potential use in other clinical situations as well, such as vascular surgery, infarction and stroke.

## Therapeutic sulfide administration: pharmacological and biochemical considerations

The unique characteristics of the bioenergetic consequences of sulfide exposure must be carefully considered when designing experimental therapeutic approaches related to its administration. The same characteristics of sulfide must also be considered in formulating a critical analysis of the published findings showing the biological effects of sulfide administration *in vitro* or *in vivo*. With regard to *in vitro* models versus *in vivo* models, first of all it must be recalled that – while mammalian cells *in vivo* cannot survive for more than a few minutes when oxidative bioenergetics (i.e. mitochondrial function) is shut off (e.g. complete tissue ischaemia or severe cyanide poisoning) – the vast majority of mammalian cells in culture can survive to a prolonged eclipse or even a permanent inactivation of mitochondrial bioenergetics, if glucose concentrations are sufficient to sustain an intense anaerobic glycolysis. [Part of this may be explained by the fact that most cells grown in culture are transformed cells, with an up-regulation of glycolysis (Warburg effect).] It must therefore be kept in mind that cultured cells can, in fact, tolerate a severe transient or even permanent shutdown of mitochondrial function by high doses/steady rates of sulfide exposure, without impairing cell viability. (In fact, some cultured cells can even tolerate combined oxygen/glucose deprivation fairly well by mobilizing emergency pathways of ATP generation, e.g. by metabolizing purine nucleotides via adenosine kinase; Módis *et al.*, 2009; Szoleczky *et al.*, 2012). At the same time, such gross alterations in mitochondrial function may, in fact, initiate mitochondrion-mediated stress signalling. It has been suggested (Whitfield *et al.*, 2008; Olson and Whitfield, 2010; Olson, 2012) that transient inhibitory effects of sulfide on mitochondrial electron transport may, in fact, be at least in part responsible for some of the observed cytoprotective effects of sulfide *in vitro* (and possibly in some of the *in vivo* studies as well). In other words, experiments aiming to promote or explain a sulfide-based pharmacological treatment have thus to be considered with regard to the

Table 1

Protective effects of hydrogen sulfide during organ storage and transplantation

Organ	Experimental conditions	Findings	Reference
Heart	Isolated rat hearts; Langendorff perfusion system; Krebs–Henseleit solution. Cold heart storage for 6 h, followed by warm reperfusion for 30 min with or without sulfide (1 μM).	Sulfide accelerated the recovery of contractility during reperfusion, improved myocardial contractility, increased ATP content and decreased myocardial apoptosis index.	Hu <i>et al.</i> , 2007.
Lung	Rabbit heart/lung blocs stored in cold low-potassium dextrane sulfate solution for 18 h, followed by a 2 h reperfusion with donor blood in an <i>in vitro</i> perfusion system. Lungs from donor animals ventilated with room were compared with lungs of donor animals ventilated for 1 h with 150 p.p.m. H <sub>2</sub> S prior to the start of the excision/storage of the heart/lung blocs.	Perfusion pressures were improved in the sulfide group. In addition, H <sub>2</sub> S-treated lungs had better oxygenation and ventilation indices. Finally, sulfide treatment reduced ROS formation during reperfusion and resulted in a better maintenance of mitochondrial cytochrome c content.	George <i>et al.</i> , 2012.
	Rabbit heart/lung blocs stored in cold Perfadex solution for 18 h, followed by a 2 h reperfusion with donor blood in an <i>in vitro</i> perfusion system in the presence or absence of a bolus dose of NaHS (100 μg·kg <sup>-1</sup> bolus + 1 mg·kg <sup>-1</sup> ·h <sup>-1</sup> infusion) starting at reperfusion.	Sulfide treatment reduced reactive oxygen species formation during reperfusion. Perfusion pressures were similar in all groups.	George <i>et al.</i> , 2012.
	Single left lung transplantation in rats, with 3 h of cold storage/ischaemia. NaHS was administered to the recipient animal at 14 μmol·kg <sup>-1</sup> 15 min before the start of the transplantation.	Sulfide improved pulmonary function (e.g. PaO <sub>2</sub> /FiO <sub>2</sub> ratio) and pulmonary histology, reduced lung oedema formation and reduced the accumulation of neutrophils in the lung. Sulfide resulted in a reduction in IL-1β and an increase in IL-10 levels.	Wu <i>et al.</i> , 2013.
Kidney	Porcine kidneys subjected to 25 min of warm ischaemia followed by 18 h of cold storage on ice after perfusion with a hyperosmolar citrate solution. Sulfide (or vehicle treatment) was applied as 0.5 mM NaHS infused 10 min prior and during reperfusion in an <i>in vitro</i> perfusion system.	Renal blood flow and renal function (measured as creatinine clearance, fractional excretion of sodium and urine output) was improved in the sulfide group. Sulfide treatment also resulted in reduced isoprostane and NO levels. No significant differences were observed in histological parameters between groups.	Hosgood and Nicholson, 2010.
Liver	Cold storage of rat livers in Wisconsin solution for 48 h, in the presence or absence of the sulfide donor diallyl disulfide (3.4 mM), followed by perfusion in an isolated constant-pressure perfusion system.	Hepatic clearance (bromosulphthalein depuration) was enhanced by sulfide treatment; most other parameters (vascular resistance, oxygen consumption, LDH release as an index of cell injury) were unaffected by sulfide.	Balaban <i>et al.</i> , 2011.
Cutaneous tissue	<i>In vitro</i> model of cutaneous tissue transplantation: endothelial cells and fibroblasts exposed to hypoxia or anoxia for 24 h, followed by 6 h of normoxia, in the absence or presence of NaHS (10 μM–1 mM).	Sulfide reduced apoptotic index (percentage of TUNEL-positive cells).	Henderson <i>et al.</i> , 2010.
Stem cells	<i>In vitro</i> model of stem cell transplantation: rat mesenchymal stem cells exposed to hypoxia or anoxia for 6 h, in the absence or presence of NaHS (200 μM), followed by intramyocardial injection of stem cells and measurement of cardiac function in an ischaemia/reperfusion model in the rat.	Sulfide reduced hypoxia-induced stem cell apoptosis and enhanced donor cell survival in the heart after intramyocardial injection, resulting in improved cardiac contractility. Sulfide treatment was associated with increased Akt, Erk and GSK-3β activation in the stem cells, which has been suggested to contribute to the functional changes observed <i>in vivo</i> .	Xie <i>et al.</i> , 2012.

mitochondrial bioenergetics. The first issue is whether or not a partially toxic concentration (tenth of micromolar) has been reached. If so, the cells concerned would experience something similar to an ischaemic period (sulfide toxicity period) followed by a reperfusion-like episode in which sulfide concentration would be brought back to non-toxic levels. In this case, complex cellular responses such as those induced by a drop in the ATP/ADP ratio would be triggered and while mobilization of such defences might be of interest (as they induce subsequent protection via preconditioning) the physiological responses would not necessarily represent specific 'sulfide-signalling' events. An indirect support of this hypothesis is that, so far, all of the pathways identified as downstream effectors of sulfide-mediated preconditioning (e.g. Nrf2,  $K_{ATP}$  channels, etc.) are, in fact, largely identical to the ones involved in the protective effect of all other forms of preconditioning (e.g. ischaemic, free radical/oxidant mediated, etc.) (e.g. Broadhead *et al.*, 2004; Halestrap *et al.*, 2007; Sadat, 2009; Yang *et al.*, 2010; Bell *et al.*, 2011). An alternative interpretation of the above-listed mechanisms may be, however, that sulfide (and possibly other gaseous transmitters) maintain a 'controlled, adverse bioenergetic effect' that may control cell signalling, cell viability and/or cardiovascular function.

With respect to the implications of the above mechanisms for *in vivo* experiments, similar considerations can be made. With a bolus administration of sulfide (or a short-acting sulfide donor), the fact that the animal tolerated the sulfide exposure means that a critical toxic concentration of sulfide was not reached/maintained for a significant amount of time in the vicinity of cells that control vital biological functions (such as a set of neurons controlling basic physiological functions). After entering the circulation, due to its diffusive properties, free sulfide will quickly distribute into the entire organism, where the cells equipped with the sulfide-oxidizing unit (SOU) will tend to metabolize it to low levels. [Part of the circulating sulfide may also exit in the gas phase via the lungs (Insko *et al.*, 2009; Toombs *et al.*, 2010).] We estimate that with fast-acting sulfide donors (sulfide salts), the rise in sulfide concentration compatible with survival probably does not exceed a few tenths of a micromolar for a few minutes. The resulting transient inhibition of mitochondrial function, and the subsequent preconditioning-type responses would then (similar to the *in vitro* considerations detailed in the previous paragraph) be expected to be at least in part responsible for subsequent protective/beneficial biological effects. When administering long-acting sulfide donors, or when applying a continuous infusion of short-acting hydrogen sulfide donors (e.g. infusion of H<sub>2</sub>S gas dissolved in physiological solutions), the above-mentioned 'peak effects' would not occur. In the latter case, steady-state levels of circulating sulfide would be maintained when equilibrium exists between sulfide administration (or the release rate of the sulfide donor) and the rate of sulfide elimination (i.e. overall sulfide-oxidizing capacities of the animal plus sulfide exhalation). An extreme example illustrates the biological consequences of impaired sulfide elimination: genetic deletion of ETHE1 (encoding for a  $\beta$ -lactamase-like, iron-coordinating metalloprotein, with sulfur dioxygenase activity) in mice results in a severe, toxic accumulation of sulfide, producing a severe

inhibition of cytochrome c oxidase activity as well as a severe form of encephalopathy (Tiranti *et al.*, 2009; Tiranti and Zeviani, 2013).

Even if the physiological sulfide degradation pathways are functioning normally, a steady-state plasma level of sulfide will produce heterogeneous cell/tissue sulfide 'exposure levels' for several reasons. First of all, a 'Krogh cylinder' type phenomenon (similar to that of oxygen) is likely to exist for sulfide, in which sulfide concentrations diminish as one moves away from the blood vessel and as one moves from the arterial to the venous side of the circulation. In addition, different cells and tissues have different levels of 'anti-sulfide defences' (e.g. sulfide binding and SOU in tissues). Moreover, within each cell, there may be subcellular organelles in which the degradation of sulfide is higher than in other areas of the cell. As discussed elsewhere (Bouillaud and Blachier, 2011), mitochondria are expected to produce steep decreases in the intracellular or transcellular sulfide gradients. Sulfide gradients and 'anti-sulfide defences' are also important in the context of the microflora resident in the colonic lumen and the colonic wall (Bouillaud and Blachier, 2011).

Notwithstanding the complex theoretical issues related to therapeutic sulfide administration, many pathophysiological conditions are associated with a relative deficiency of H<sub>2</sub>S (due to impaired production and/or enhanced degradation), and, in these circumstances, therapeutic H<sub>2</sub>S donation is warranted, either to serve as a pharmacological replacement (as in diabetes, see above) or to induce transient metabolic suppression (as in the context of suspended animation, see above). It is hoped that future therapeutic H<sub>2</sub>S donation approaches will benefit from recent studies enabling the direct comparison of the doses and associated pharmacological actions of gaseous (inhalational) sulfide versus circulating, blood-borne sulfide. These studies yielded an approximate biological equivalency between 200 p.p.m. inhaled H<sub>2</sub>S and 10 mg·kg<sup>-1</sup>·h<sup>-1</sup> i.v. infusion of a H<sub>2</sub>S solution (Wintner *et al.*, 2010), and concluded that the therapeutic doses of sulfide *in vivo* are below those inhalational exposure levels that are associated with systemic toxicity.

## Pharmacological exploitation of the mitochondrial effects of H<sub>2</sub>S: future directions

The above sections only represent selected examples of the pathophysiological and therapeutic aspects related to the complex mitochondrial modulatory effects of H<sub>2</sub>S. Based on several lines of experimental data (Hu *et al.*, 2009; 2010; Pun *et al.*, 2010; Caro *et al.*, 2012; Guo *et al.*, 2012b; Tang *et al.*, 2012; Aslami *et al.*, 2013b; Lu *et al.*, 2013; Wen *et al.*, 2013), it is likely that H<sub>2</sub>S as a mitochondrial protectant – similar to the example of diabetic endothelial dysfunction discussed above – also protects the mitochondria of neurons, stem cells, cardiac myocytes and various other cell types. It is also likely that bioenergetic effects play important roles in H<sub>2</sub>S-mediated angiogenesis (Cai *et al.*, 2007; Papapetropoulos *et al.*, 2009; Coletta *et al.*, 2012), where it may serve to support the increased energy demand of the growing, migrating and dividing endothelial cells. At the opposite end of the spec-

trum is cancer therapy, in which high concentrations of H<sub>2</sub>S donors can cause inhibition of cell division and promotion of cell death (Lee *et al.*, 2011; Tsubura *et al.*, 2011; Wu *et al.*, 2012; Kashfi and Olson, 2013), effects that are, at least in part, likely to be related to the inhibition of mitochondrial electron transport by high local concentrations of H<sub>2</sub>S. This latter concept does not conflict with the role of endogenously produced H<sub>2</sub>S in cancer, as the exogenously administered H<sub>2</sub>S donors produce much higher concentrations than endogenous sulfide levels, yielding a different biological response.

The interaction between the effects of H<sub>2</sub>S on mitochondrial function and the effects of H<sub>2</sub>S on cell signalling needs to be characterized in future studies as well. There are convincing data in the field of carbon monoxide (CO, another gaseous transmitter) showing that partial inhibition of mitochondrial electron transport, followed by a 'leakage' of ROS from the mitochondrial electron transport chain, plays a key role in the initiation of CO-dependent cellular signalling (Zuckerbraun *et al.*, 2007). Whether similar processes are also at play in the case of H<sub>2</sub>S remains to be elucidated. In fact, the complex relationship between the three gasotransmitters (NO, CO and H<sub>2</sub>S) in the regulation of cellular signalling and cellular bioenergetics (Lane, 2006; Pun *et al.*, 2010; Szabo, 2010) needs to be studied in further detail, in light of the effect of each of these transmitters on mitochondrial electron transport and glycolysis (Cooper and Brown, 2008; Pun *et al.*, 2010). Furthermore, their emerging interdependence and mutual regulation in the context of many biological processes (e.g. Whiteman *et al.*, 2006; Minamishima *et al.*, 2009; Tomaskova *et al.*, 2009; Yusof *et al.*, 2009; Coletta *et al.*, 2012; Carballal *et al.*, 2013; Kondo *et al.*, 2013; Polhemus *et al.*, 2013) need to be further studied as well. We must also point out that in the current review, very little discussion was devoted to the 'third' H<sub>2</sub>S-producing enzyme, 3-MST (while its physiological roles have been detailed in the complementary article; Szabo *et al.*, 2013a). At this point, there is essentially no information on the potential pathophysiological role of the 3-MST system; future work may identify pathophysiological conditions where the 3-MST system plays a role. The recent generation of the 3-MST deficient mice (Nagahara *et al.*, 2013) may catalyse further work in this direction. Finally, the potential modulation of the H<sub>2</sub>S-dependent direct mitochondrial effects by other downstream effector pathways of H<sub>2</sub>S [such as cysteine modification (sulfhydration)] (Gadalla and Snyder, 2010), modulation of potassium channels (Zhao *et al.*, 2001; Cheng *et al.*, 2004; Cheang *et al.*, 2010) and effects on calcium signalling (Bauer *et al.*, 2010) remain to be explored in future studies.

There are a number of contentious, intensively debated issues in the field of H<sub>2</sub>S (for further reading, please see: Whiteman *et al.*, 2011; Olson, 2013b,c), one of them being the circulating/plasma levels of H<sub>2</sub>S, and the methods used to measure it (especially the most commonly used method, the 'methylene blue method', which yields low- to mid-micromolar plasma concentrations). As discussed elsewhere (Whitfield *et al.*, 2008; Wintner *et al.*, 2010; Whiteman *et al.*, 2011; Olson, 2013b; Szabo *et al.*, 2013a), there are various 'pools' of sulfide (or reactive sulfur species) in the circulation (free H<sub>2</sub>S gas being only a minor component of it, if any), and the methylene blue method is almost certainly measuring a net sum of these various pools, and it does not represent 'free'

H<sub>2</sub>S levels. There may also be methodological issues that can result in a significant overestimation of the absorbance values detected by the methylene blue method (e.g. Van de Louw and Hauzi, 2012). Due to the fact that there are various biological 'sinks' of sulfide (e.g. in red blood cells), it is also difficult to estimate 'steady-state' levels of sulfide, or to be certain that such 'steady-state' levels even exist in biological systems (Olson, 2013c). Clearly, additional work is needed to clarify these issues and to improve the methods to detect H<sub>2</sub>S in biological systems. While this refinement is ongoing, we do not have a better option than to keep these limitations in mind, and to interpret the reported differences in the sulfide levels between two experimental groups (when reported in the same publication) as probably *directionally* correct, while remaining critical about the absolute values reported. Another issue that represents a severe limitation of the field is the lack of selective pharmacological tools. As discussed elsewhere (Whiteman *et al.*, 2011; Asimakopoulou *et al.*, 2013), the available compounds (PAG, AOAA, etc.) are neither particularly potent nor are they sufficiently selective or specific; they show cross-inhibition between the two pyridoxal 5'-phosphate (PLP)-dependent enzymes CSE and CBS, and they also inhibit a variety of other enzymes. One way to minimize this limitation is by using genetically modified animals or transient or permanent silencing of each enzyme (with the caveat that these tools, too, have certain limitations). While these issues remain to be refined, we must remain careful with the interpretation of most of the published studies utilizing these inhibitors (especially when they are not accompanied by corroborating studies using genetic tools).

Finally, we would like to emphasize that a substantial portion of the data reviewed in the current article, as well as its companion paper (Szabo *et al.*, 2013a), stems from *in vitro* studies, and the number of studies investigating the stimulatory or inhibitory effect of exogenously administered or endogenously produced sulfide *in vivo* is limited (Table 2). As discussed elsewhere (Budde and Roth, 2011; Vozdek *et al.*, 2012; Módis *et al.*, 2013d), the model organism *Caenorhabditis elegans* provides a convenient system to study H<sub>2</sub>S-mediated biological regulatory mechanisms. In this organism, a CBS homologue enzyme is primarily responsible for endogenous H<sub>2</sub>S production (Vozdek *et al.*, 2012). siRNA-mediated silencing of this enzyme attenuates the bioenergetic function of *C. elegans*, consistent with the hypothesis that endogenous H<sub>2</sub>S production plays a stimulatory bioenergetic role (Szabo *et al.*, 2013a). One known example of *endogenously produced* H<sub>2</sub>S suppressing mitochondrial function is the phenomenon of physiological hypoxia sensing (e.g. in the arterial chemoreceptors or the kidney) where, at biological sites associated with low O<sub>2</sub> tension (e.g. the carotid body or the kidney medulla), the inhibition of complex IV by endogenous H<sub>2</sub>S (produced by CSE) plays a physiological regulatory role (Beltowski, 2010; Olson and Whitfield, 2010; Peng *et al.*, 2010; Makarenko *et al.*, 2012; Olson, 2013a). (Although it must be noted that the role of H<sub>2</sub>S in physiological hypoxia sensing remains a field of intensive research and debate, and other lines of data suggest that it may be a decreased oxidation of H<sub>2</sub>S during hypoxia, and the subsequent accumulation of H<sub>2</sub>S, and a subsequent signalling process that may be involved; see Olson, 2013c).

Table 2

Metabolic/bioenergetic effects of exogenously administered or endogenously produced hydrogen sulfide in cultured cells *in vitro* and in various species *in vivo*

	Stimulatory effect of H <sub>2</sub> S		Inhibitory effect of H <sub>2</sub> S	
Exogenously administered H <sub>2</sub> S	Cultured epithelial cells, hepatocytes and macrophages: Low concentrations of sulfide are consumed by the cells and stimulate mitochondrial electron transport and cellular bioenergetics.	Leschelle <i>et al.</i> , 2005; Goubern <i>et al.</i> , 2007; Lagoutte <i>et al.</i> , 2010; Módis <i>et al.</i> , 2013a,b,c	Cultured epithelial cells, hepatocytes, and macrophages: High concentrations of sulfide inhibit mitochondrial electron transport (complex IV/cytochrome c oxidase) resulting in a suppression of cellular bioenergetics.	Khan <i>et al.</i> , 1991; Thompson <i>et al.</i> , 2003; Leschelle <i>et al.</i> , 2005; Truong <i>et al.</i> , 2006; Goubern <i>et al.</i> , 2007; Lagoutte <i>et al.</i> , 2010; Buckler, 2012; Groeger <i>et al.</i> , 2012; Sun <i>et al.</i> , 2012; Módis <i>et al.</i> , 2013a,b,c
	Sulfur-oxidizing species of bacteria, clams, tubeworms living in sulfide-rich environments: Multiple present-day lower species (e.g. in deep-sea hydrothermal vents, sulfur springs, sewage-treatment plants) take up, consume and utilize sulfide to drive electron transport and ATP generation ('sulfur chemolithotrophism').	Powell and Somero, 1986; Gaill, 1993; Volkel and Grieshaber, 1996; Lloyd, 2006	Inhalational toxicology: H <sub>2</sub> S gas inhalation reduces cytochrome c oxidase activity in lung tissue <i>ex vivo</i> .  'On-demand suspended animation': H <sub>2</sub> S gas inhalation reduces oxygen consumption and CO <sub>2</sub> production in conscious mice.  Protection from reperfusion injury: Systemic sulfide administration to mice suppresses mitochondrial activity in the heart.  Organ transplantation: Treatment of the transplanted organ with H <sub>2</sub> S induces organ protection, attributed at least in part to a reversible metabolic suppression.	Khan <i>et al.</i> , 1990  Blackstone <i>et al.</i> , 2005; Volpato <i>et al.</i> , 2008; Bos <i>et al.</i> , 2009  Elrod <i>et al.</i> , 2007; Szabo <i>et al.</i> , 2011; Snijder <i>et al.</i> , 2013  Table 1
Endogenously produced H <sub>2</sub> S	<i>Caenorhabditis elegans</i> : Silencing of CBS suppresses basal and uncoupler-induced oxygen consumption.	Szabo <i>et al.</i> , 2013a	Carotid body hypoxia sensing: Hypoxia-induced increase in H <sub>2</sub> S may exert metabolic inhibitory effects. H <sub>2</sub> S production and hypoxic carotid body responses are attenuated by the CSE inhibitor PAG or in CSE <sup>-/-</sup> mice.	Peng <i>et al.</i> , 2010; Makarenko <i>et al.</i> , 2012
	Colorectal cancer cells: Silencing of CBS suppresses oxygen consumption in colon carcinoma cells, indicating that endogenously produced sulfide exerts stimulatory bioenergetic effects.	Szabo <i>et al.</i> , 2013b	Whole-body hypoxia: The CSE inhibitor PAG suppresses hypoxia-induced decrease in VO <sub>2</sub> .  ETHE1 deficiency: Impaired degradation of H <sub>2</sub> S results in accumulation of toxic levels of sulfide, resulting in metabolic suppression, mitochondrial toxicity and neurotoxicity.	Del Rio <i>et al.</i> , 2013  Tiranti <i>et al.</i> , 2009; Tiranti and Zeviani, 2013
			Down syndrome: CBS (which is localized on chromosome 21) is overexpressed in patients with Down syndrome, resulting in H <sub>2</sub> S overproduction. This has been suggested to contribute to some of the observed toxicities.	Kamoun <i>et al.</i> , 2003; Robert <i>et al.</i> , 2003



Pharmacological inhibition of H<sub>2</sub>S production in the carotid body may be a potential future therapeutic approach for the therapy of chemoreceptor hypersensitivity and the associated cardiovascular imbalance, such as the one associated with chronic heart failure (Del Rio *et al.*, 2013). It is also interesting to note that in a recent study, the CSE inhibitor PAG has been shown to block the hypoxia-induced decrease in oxygen uptake (VO<sub>2</sub>) in rats (Del Rio *et al.*, 2013), perhaps indicative of a role endogenous H<sub>2</sub>S production in hypoxia-induced hypometabolism. One must keep in mind, nevertheless, that the selectivity of PAG is limited. Thus, it is hoped that the results of the above study will be confirmed and extended in future studies using CSE<sup>-/-</sup> mice. Additional studies in mammalian systems are necessary to clarify the complex bioenergetic roles of sulfide in various diseases *in vivo* and the interrelationship between bioenergetic regulation and the various other facets of sulfide biology (such as its diverse roles in the cardiovascular, central nervous and immune systems), in order to allow the design and testing of future therapeutic approaches.

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## Conflict of interest

C.S and M.H. are stockholders and corporate officers and A.P. is a stockholder and consultant to CBS Therapeutics Inc., a for-profit organization involved in the therapeutic exploitation of sulfide biosynthesis inhibitors for cancer therapy.

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