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H₂S during circulatory shock: Some unresolved questions

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Abstract

Numerous papers have been published on the role of H₂S during circulatory shock. Consequently, knowledge about vascular sulfide concentrations may assume major importance, in particular in the context of “acute on chronic disease”, i.e., during circulatory shock in animals with pre-existing chronic disease. This review addresses the questions i) of the “real” sulfide levels during circulatory shock, and, ii) to which extent injury and pre-existing co-morbidity may affect the expression of H₂S producing enzymes under these conditions. In the literature there is a huge range on sulfide blood levels during circulatory shock, in part as a result of the different analytical methods used, but also due to the variable of the models and species studied. Clearly, some of the very high levels reported should be questioned in the context of the well-known H₂S toxicity. As long as “real” sulfide levels during circulatory shock are unknown and/or undetectable “on line” due to the lack of appropriate techniques, it appears to be premature to correlate the measured blood levels of hydrogen sulfide with the severity of shock or the H₂S therapy-related biological outcomes. The available data on the tissue expression of the H₂S-releasing enzymes during circulatory shock suggest that a “constitutive” CSE expression may play a crucial role of for the

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maintenance of organ function, at least in the kidney. The data also indicate that increased CBS and CSE expression, in particular in the lung and the liver, represents an adaptive response to stress states.

Keywords

H₂S; NaSH; Na₂S; GYY4137; cystathionine-γ-lyase; cystathione-β-synthase

Introduction

Since the landmark paper by Blackstone *et al.* [1] on the metabolic effects of inhaling gaseous H₂S in awake mice, i.e., the induction of a “*hibernation-like*” status of reduced energy expenditure and consecutive hypothermia, and the subsequent murine study on cardiac stability [2], numerous papers have been published on the role of H₂S during circulatory shock of various etiology. For example: lethal hypoxia [3], ischemia/reperfusion (I/R)-injury [4–30], cardiac arrest [31–36], hemorrhage and resuscitation [37–45], acute lung injury resulting from blunt chest trauma [46,47] and/or injurious mechanical ventilation [48–50], as well as systemic inflammation resulting from endotoxin injection [51–59], acute pancreatitis [60–63], polymicrobial sepsis [64–70], and/or burn injury [71–73]. While the beneficial effect of exogenous H₂S supplementation and maintaining endogenous H₂S production, respectively, are well-established in I/R injury, equivocal results were reported after cardiac arrest, hemorrhage and resuscitation, and, in particular, in sepsis: inhaling gaseous H₂S [33,44,48,50,53,54,65], the injection of the sulfide-containing salts NaSH [31,39,49,51,59,63,64,66–71,120] and Na₂S [32,34–38,41,42,44,47,48,72,73] or the slow-releasing H₂S donor GYY4137 [55] as well as of inhibitors of H₂S production [43,45,58–60,62,63,67–71,120] were associated with attenuation of shock-related organ dysfunction. Consequently, knowledge about vascular sulfide concentrations may assume major importance, in particular in the context of “*acute on chronic disease*”, i.e., during circulatory shock in animals with pre-existing chronic disease, which *per se* may markedly alter endogenous H₂S production, e.g., atherosclerosis [74], arterial hypertension [75,76], chronic kidney disease [77,78], and/or chronic obstructive pulmonary disease (COPD) [79–81]. The present review therefore addresses the questions i) of the “real” sulfide levels during circulatory shock, and, ii) to which extent injury and pre-existing co-morbidity may affect the expression of H₂S producing enzymes under these conditions.

H₂S blood levels during circulatory shock

There is considerable discrepancy in the literature on blood sulfide concentrations. According to the available literature blood sulfide content may vary by three orders of magnitude (Table 1). This is certainly in part due to the different methods that are currently used to measure sulfide blood levels, e.g., the colorimetric methylene blue reaction, sulfide-sensitive fluorescent dye detection, gas chromatography with flame photometry, the monobromobimane assay, and methods using ion-selective or polarographic electrodes, which all differ markedly with respect to the detection limit and yield values representing other molecules rather than dissolved gaseous H₂S and/or free sulfide alone [82–86]. Clearly, blood concentrations of dissolved gaseous H₂S far beyond 1 μM must be questioned

for several reasons: i) Blood-borne dissolved H_2S should equilibrate with the alveolar space during lung passage and appear in the expired gas [87], thus promoting sensation of H_2S odour [88]: the human nose can detect as little as $1 \mu\text{M}$ H_2S [83,89], the odour threshold being 0.01–0.1 ppm [83]. ii) The gas/water coefficient of distribution for H_2S is 0.39, and at physiological pH ~ 20 (at 37°C) – $\sim 40\%$ (at 25°C) of the total free sulfide is present as dissolved gas [83,89] (Figure 1). Assuming that only 20 % of the dissolved gas, i.e., 4 – 10 % of the total free sulfide, disappears from the blood sample due to volatilization [90] during the 2 – 3 seconds of sniffing, a 10 mL blood sample would have a total free sulfide concentration of 20 – 50 μM . This is within the range reported in several studies during circulatory shock, but these blood samples were never reported to smell like rotten eggs. iii) In liver tissue samples *ex vivo*, incubation with Na_2S to achieve concentrations of $\sim 1 \mu\text{M}$ already reduced maximum mitochondrial respiratory activity. Concentrations $> 16 \mu\text{M}$ were associated with a near-complete inhibition of mitochondrial respiration [91,92].

Exogenous sulfide is rapidly bound and/or metabolized so that dissolved gaseous H_2S rapidly disappears after bolus administration of the sulfide salts Na_2S or NaSH [56]. At least *ex vivo*, slow-releasing H_2S -donors (e.g. GYY4137) allow overcoming this effect [93,94] (figure 2A). Sulfide binding is even more pronounced in blood than in plasma and buffer or culture media (Figure 2B): using a polarographic sensor with a detection limit for H_2S gas corresponding to 100 nM total sulfide in blood at pH = 7.4, a $10 \mu\text{M}$ Na_2S spike only transiently increased sulfide from undetectable levels to about $0.5 \mu\text{M}$ [95]. In rats ($4 \text{ mg}\cdot\text{kg}^{-1}$ bolus or $20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ infusion [96]) and swine ($0.5 \text{ mg}\cdot\text{kg}^{-1}$ bolus followed by $1 - 4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ infusion [12,15,18]) a primed-continuous intravenous (*i.v.*) Na_2S infusion increased sulfide levels from $0.4 - 0.9$ to a maximum values of $4 - 9 \mu\text{M}$ as measured with the monobromobimane assay [96] (Figure 3). After repetitive (at 12 and 18 hours after inoculation of autologous feces to induce fecal peritonitis) *i.v.* injection of a GYY4137 bolus ($10 \text{ mg}\cdot\text{kg}^{-1}$) in swine with long-term septic shock treated with fluid resuscitation and continuous *i.v.* noradrenaline to maintain adequate systemic hemodynamics, we found H_2S levels of $0.5 - 2.5 \mu\text{M}$ (Figure 4). For comparison: in wild type and $\text{CSE}^{-/-}$ -mice after blunt chest trauma, mean sulfide levels were respectively (Figure 5). In the two latter studies, the sulfide blood concentrations were assessed with a modified routine gas chromatography/mass spectrometry technique for sulfide quantification *via* a bis-pentafluorobenzyl derivative [97] using 1,3,5-tribromo-benzene as internal standard (see Appendix for explicit methodology).

In addition to the above-mentioned methodological issues, the effect of circulatory shock *per se* on H_2S blood levels remains a matter of debate, depending on the species studied and the type of acute illness investigated. For example, in murine pancreatitis [60], polymicrobial sepsis induced by cecal-ligation- and puncture [67–70], and burn injury [71], blood sulfide concentrations were reported to increase by 50 – 100 %. In contrast, in endotoxic and hemorrhagic shock, or I/R injury sulfide levels either *increased* [20,45,56], *decreased* [21,53] or *did not change* [10]. In anesthetized and mechanically ventilated $\text{CSE}^{-/-}$ -mice undergoing blunt chest trauma, the acute challenge *per se* did not affect sulfide blood concentrations (figure 5), and similar results were found in swine after cardiopulmonary bypass [15,18] and kidney I/R injury [12]. Hemorrhage and resuscitation

per se, nor therapeutic hypothermia and/or hyperoxia in swine also had no significant effect on blood sulfide levels (Figure 6). Human studies are also somewhat ambiguous, and from clinical studies are even more conflicting. On the one hand, a marked reduction of blood sulfide levels was reported during pre-eclampsia [75], acute exacerbation of COPD [81,98], severe acute asthma [99], and bacterial pneumonia [100]. On the other hand, in another study non-survivors of circulatory shock showed twice as high serum sulfide levels as survivors (32 vs. 13 μM [101]). Median values as high as 150 [59] and 300 μM [102] were found in patients with septic shock and severe asthma, respectively!

In summary, in the literature there is a huge range on sulfide blood levels during circulatory shock, in part as a result of the different analytical methods used, but also due to the variable of the models and species studied. Clearly, some of the very high levels reported should be questioned in the context of the well-known H_2S toxicity. In addition, as long as “real” sulfide levels during circulatory shock are unknown and/or undetectable “on line” due to the lack of appropriate techniques, it appears to be premature to correlate the measured blood levels of hydrogen sulfide with the severity of shock or the H_2S therapy-related biological outcomes.

H_2S producing enzymes during acute injury and “acute on chronic disease”

There are three known enzymatic sources of endogenous H_2S production: cystathionine- β -synthase, (CBS), cystathionine- γ -lyase (CSE) and 3-mercaptopyruvate-sulphurtransferase (MST). As mentioned above, equivocal data on the effects of H_2S during acute illness are available, inasmuch both ameliorated or exacerbated injury were reported. Impaired endogenous H_2S production as a result of *down-regulation* of CSE and CBS is associated with *chronic* cardiovascular pathology, e.g., arterial hypertension, atherosclerosis, and chronic kidney disease [77,78,103], and homocystinurea resulting from CBS deficiency being a prominent example. In contrast, chronic exposure to cigarette smoke lead to elevated CSE expression in the lungs [98]. CSE and CBS *up-regulation* was also found during acute hyper-inflammatory states resulting from polymicrobial sepsis [69,70], burn injury [71], and blunt chest trauma [47], whereas acute liver failure induced by D-galactosamine in combination with endotoxin lead to reduced expression of both CSE and CBS [52]. Ambiguous data are available on the regulation of H_2S producing enzymes after endotoxin injection: either increased [56,58] or decreased [53] enzyme expression was reported. Comparably controversial results were obtained after I/R-injury: unchanged [5], increased [11,17,20,23] or decreased [4,6,104] enzyme expression was found. Moreover, Xu *et al* even reported a different response of CSE and CBS, respectively, after kidney I/R-injury: while CSE activity remained unchanged, CBS activity was markedly reduced [21]. Finally, Bos *et al* reported recently that in rats CSE mRNA was *up-regulated* after kidney I/R-injury, whereas the CSE protein expression was *down-regulated*, which persisted at low levels up to 21 days post I/R [4]. On the one hand, this observation may suggest that the CSE mRNA was not translated into protein. However, when looking for CSE protein expression using various antibodies, Fu *et al* also reported that CSE protein was undetectable in mouse and rat cardiac tissue despite the presence of CSE mRNA and convincing evidence of CSE-dependent H_2S formation [105]. Rapid turnover of the CSE protein and/or the existence of different CSE isoforms may explain these findings. CBS mRNA was increased 30 minutes

post ischemia, reduced below sham levels at 6 hours, and normalized only on day 9 post ischemia. Interestingly however, no matter whether enzyme *expression* was increased or reduced, at least after ischemia/reperfusion, inhibiting the enzyme *activity* aggravated organ damage, whereas exogenous H₂S administration attenuated the severity of organ injury.

Table 2 summarizes the above-mentioned studies on the expression of H₂S generating enzymes in states of acute stress. Clearly and in particular in the context of I/R-injury, their regulation remains controversial. Moreover, all these data on enzyme expression in kidney I/R injury originate from un-resuscitated rodent models, the relevance of which has recently been challenged, at least with respect to experiments on mice [106]. As far as acute kidney injury is concerned, the underlying anatomical differences between rodents on the one hand, and large animal species or humans on the other hand may particularly hinder clinical translations: Mice, rat and rabbits share a unilobular, unipapillary kidney in contrast to multilobular, multipapillary kidneys shared by pigs, monkeys, and humans [107]. In addition, in rodents the urine empties directly into the renal pelvis, whereas in pigs and humans urine empties into a branched caliceal network that distributes to the renal pelvis. Finally, an intricate system of interlobar and segmented arteries provides blood flow to numerous kidney lobes in humans and pigs, which is not present in rodents and dogs because of the lack of multiple medullary pyramids [107]. Consequently, while kidney I/R injury in rodents leads to extensive necrosis of proximal tubules, the severity of which depends on the length of the ischemic insult. In contrast, in humans “*frank tubular necrosis*” is infrequent, less pronounced, and only patchy if present at all [108]. Given these species-specific considerations as well as the above-mentioned variable expression of the H₂S producing enzymes after I/R-injury, we assessed the expression of CSE and CBS after porcine aortic balloon occlusion-induced kidney I/R-injury. Animals were resuscitated according to standard intensive care protocols that allowed for maintaining target hemodynamics, and thereby exclude any influence of compromised organ blood flow to kidney dysfunction [12]. Despite only moderate overall histopathological damage, a five-fold fall in creatinine clearance demonstrated a degree of organ dysfunction consistent with the development of acute kidney failure. CSE was abundantly expressed in the pre-ischemic biopsies (Figure 7), whereas there was only minimal detection of CBS; I/R injury reduced tissue CSE expression by approximately one third (Figure 7), and CBS co-localized with infarct regions. These findings well agree with what is reported for the human kidney: CBS mRNA levels were three to fivefold lower than that of CSE mRNA, and CSE expression was related to glomerular filtration rate at two weeks after organ transplantation [4].

Scarce data are only available on the effects of other therapeutic interventions rather than inhibition of endogenous H₂S production and/or its exogenous supplementation on the enzyme expression during acute circulatory distress. However, H₂S is referred to play a crucial role in “O₂ sensing” [109], inasmuch the cellular H₂S concentration is a result of the balance between cytoplasmatic production and mitochondrial oxidation of H₂S: hypoxia will decrease the oxidation of H₂S and thereby increase its concentration. Besides *decreased* H₂S oxidation hypoxia can also increase cellular H₂S concentrations due to *increased* intra-mitochondrial formation: on the one hand, *in vitro* near-anoxia (O₂ concentration 1 %) caused translocation of the CSE protein from the cytosol into the mitochondria [110]. In

addition, both *in vitro* and *in vivo* hypoxia or ischemia lead to intra-mitochondrial CBS accumulation resulting from reduced protein degradation and thereby increased total cellular H₂S formation [111]. In contrast to the effects of *hypoxia* or *anoxia*, there is no data on the effect of *hyperoxia* on the expression of H₂S producing enzymes. Hyperoxia may, however, counteract the systemic inflammatory response that is triggered by tissue hypoxia resulting from either alveolar hypoxia, hypoxemia and/or impaired tissue perfusion [112]. Therefore, we investigated the effect of a short-term exposure to 100% O₂ on the lung tissue CSE and CBS expression in mice after combined femoral fracture [113] and blunt chest trauma [47]. Immediately and nine hours after trauma, animals were exposed to 100% O₂ over three hours each, enzyme expression was evaluated until day 21. Both CBS and CSE were up-regulated after injury, the compound injury causing a higher CSE expression than femoral fracture alone. Despite a gradual decrease over time, the enzyme expression had not returned to native levels even at day 21. Hyperoxia, however, markedly attenuated both CBS and CSE expression, the latter being normalized back to normal levels already after the first three hours of 100 % O₂ exposure (Figure 8). The reduced post-traumatic enzyme expression coincided with attenuated pulmonary histological damage and reduced tissue concentrations of pro-inflammatory cytokines. In mice that underwent mechanical ventilation immediately after chest trauma, four hours of ventilation with 100 % O₂ were also associated with lower CSE and CBS expression than in controls ventilated with air (Figure 9). Interestingly, the reduced *pulmonary* CBS and CSE expression in these animals coincided with higher enzyme expression in the *kidney* (Figure 9). These data fit well with recent findings on the effects of pure O₂ ventilation on renal tissue CSE expression in swine undergoing hemorrhage and resuscitation (withdrawal of 30 % of calculated blood volume and subsequent titration of mean blood $\approx$ 35 mmHg over four hours [38,114]): ventilation with 100 % O₂ increased renal blood flow and urine output, ultimately resulting in attenuation of kidney dysfunction. This protective effect of hyperoxic ventilation went along with a two-fold higher CSE expression at the end of the experiment (Figure 10).

In their seminal paper Blackstone *et al* [1] had shown that in awake mice inhaling gaseous H₂S decreased energy expenditure and lowered core body temperature close to ambient levels, and this H₂S-induced hypometabolism was shown to be organ-protective in various shock states (see above). Data from larger species such as sheep or swine, however, suggest that beneficial effects of H₂S were due to an attenuation of systemic inflammation rather than an effect on energy expenditure [12,38]. Hence, any coincidence of induction of moderate hypothermia and attenuation of the inflammatory response related to H₂S administration raises a “*the chicken or the egg*” problem. Therefore, we sought to separate the effects of exogenous H₂S (using the sulfide salt Na₂S) administration and induced hypothermia on the expression of H₂S releasing enzymes. In mice after blunt chest trauma, continuous *i.v.* Na₂S had no effect during normothermia. In contrast, hypothermia alone reduced both CBS and CSE expression, and Na₂S administration during hypothermia even further reduced CBS expression, which was ultimately associated with the least activation of apoptosis [47]. In swine undergoing hemorrhage and resuscitation, moderate pre-treatment hypothermia (32 and 35 °C, respectively, vs. 38 °C) not only caused a switch from necrotic to apoptotic cell death, most likely as a result of less energy deprivation during the shock phase [114], but also had a clear effect on the H₂S producing enzymes in the kidney: i) the

otherwise marked loss of CSE expression after hemorrhagic shock was attenuated (Figure 11); ii) any expression of CBS was abolished, which was hardly detectable in native kidney samples and expressed in very low quantities only, limited to focal regions of necrosis (Figure 11B).

Taken together, the above-mentioned findings on the effects of hyperoxia and hypothermia confirm our previous results in mechanically ventilated mice undergoing blunt chest trauma that increased pulmonary CBS and CSE expression after trauma most likely is an adaptive stress response [47]. In addition, the results discussed above also confirm the important role of a “*constitutive*” CSE expression for the maintenance of kidney function during circulatory shock, in particular after I/R injury, reported by other authors [4,20,21].

As already mentioned, impaired endogenous H₂S production as a result of *down-regulation* of CSE and CBS is associated with *chronic* cardiovascular pathology, e.g., hypertension, atherosclerosis, and chronic kidney disease. Close to nothing is known on the effect of circulatory shock in animals with pre-existing co-morbidity, i.e., “*acute on chronic disease*”. Nevertheless, Gao *et al* suggested a “self-protective mechanism of endogenous H₂S” against myocardial infarction in diabetic rats [11]. Since CSE and CBS expression were also shown to be affected by age and modulated by diet [115], we compared CSE expression before and after porcine kidney I/R-injury in otherwise healthy animals and swine with ubiquitous atherosclerosis resulting from a mutation of the LDL receptor together with a cholesterol-enriched diet [116]. In this swine strain, the underlying atherosclerosis is associated with reduced tissue expression of the erythropoietin [117] and the PPAR- β/δ [118] receptors. We could show that atherosclerosis was associated with a much stronger drop of the CSE expression after I/R-injury than observed in the healthy controls (Figure 7). This effect was paralleled with a higher increase of blood isoprostane levels and lower nitrate concentrations during reperfusion when compared to I/R-injury effects in healthy control animals [117,118], indicating that more severe oxidative stress and impaired adaptive NO production is associated with more severe CSE down-regulation.

Conclusions and future perspectives

The huge range of sulfide blood levels reported in the literature is not only due to the variable models and species studied, but also due to different analytical methods used. Currently, to the best of our knowledge, there is no universally accepted “gold standard” for the measurement of blood or tissue sulfide concentrations. Therefore, measured blood sulfide levels so far cannot be correlated with the severity of shock or the effects of H₂S-related therapy, and some of the very high levels must be questioned completely. A GC-MS based method such as we have used has the advantage of giving unequivocal structural identity to products measured. Nevertheless, as highlighted in a recent issue of this journal there is “...*need for rigorous and reliable measurement techniques to monitor the biological levels of H₂S...*” [85], and without the possibility to “...*accurately determine and control for the levels of H₂S in experimental settings...*” [85], any comparison of H₂S effects between various shock models will remain flawed.

The available data on the tissue expression of the H₂S-releasing enzymes during circulatory shock suggest that a “*constitutive*” CSE expression may play a crucial role of for the

maintenance of organ function, at least in the kidney. The data also indicate that increased CBS and CSE expression, in particular in the lung and the liver, represents an adaptive response to stress states. In contrast, it remains to be elucidated whether exogenous H₂S supplementation is beneficial during circulatory shock. Inhaling gaseous H₂S or injection of the soluble sulfide salts NaSH or Na₂S most likely will not become clinical practice due to damage of airway mucosa and possibly toxic peak sulfide concentrations, but slow H₂S-releasing molecules may allow avoiding these problems. Despite the promising findings in rodent models [1–3,16,49,50], the role of a possible H₂S-induced hypometabolism during shock states is unclear as well [123]: most studies so far suggest that any beneficial effect of H₂S is at least in part independent of metabolic depression, but other data suggest that H₂S-related hypometabolism may enhance its organ-protective properties. In addition, while the feasibility *per se* of on demand, H₂S-induced *protective reduction* rather than *toxic inhibition* of cellular respiration is still matter of debate, “hibernating” isolated organs remains an option.

How can these issues be reconciled? In addition to the above-mentioned need for a commonly accepted method to assess sulfide concentrations, consensus on the design of appropriate models of circulatory shock will certainly help. Some of these issues have been highlighted in a recent review on sepsis and septic shock, i.e. to study “...*animals that are more genetically diverse, are older, or have preexisting disease. Longer experiments with more advanced supportive care would allow better mimicry ... in a more realistic setting ...*” [124].

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Appendix: Gas chromatography/mass spectrometry method for sulfide determination

Sulfide concentrations were measured with a modified gas chromatography/mass spectrometry (GC/MS) method of sulfid quantification in blood and plasma using a bis-pentafluorobenzyl derivative [97]. 100 and 25 μL blood samples were used in swine and mice, respectively.

Derivatization

100 μL of blood were added to the derivatization mixture consisting of 400 μL of 5 $\mu\text{g}\cdot\text{mL}^{-1}$ 1,3,5-tribromobenzene in isooctane as internal standard, 400 μL of 2 $\text{mg}\cdot\text{mL}^{-1}$ tetradecyldimethylbenzylammoniumchloride in sodiumtetraborate saturated water, and 200 μL of 10 $\mu\text{L}\cdot\text{mL}^{-1}$ pentafluorobenzylbromide in isooctane. The closed vial was vortexed for 1 minute. Thereafter, 400 μL of water saturated with KH_2PO_4 were added. The sample was vortexed again for another 10 seconds. For phase separation, the vial was centrifuged at 10000 rpm for 5 minutes. An aliquot of the organic layer was analyzed by GC/MS.

GC/MS determination

Analysis was performed with an Agilent 5890/5970 GC/MS system (Waldbronn, Germany), housing an MN 5-MS capillary column (12m $\times$ 0.2mm, 0.33 μm film thickness; Macherey-Nagel, Düren, Germany). Helium was used as carrier gas at a column head pressure of 5 psi. The injector temperature was 250°C. A 2 μL sample was injected at an oven temperature of 80°C. After a 1 minute hold, the temperature was raised to 200°C at a rate of 25 $^{\circ}\cdot\text{min}^{-1}$ and, after a subsequent 2.6 minutes hold, to 280°C at 50 $^{\circ}\cdot\text{min}^{-1}$. The mass spectrometer run in electron impact mode at an ionisation energy of 70eV. In the selected ion monitoring mode, ions m/z 313.7, 394.0 and 396.0 were recorded for internal standard and the derivatized $^{32}\text{S}^{2-}$ and $^{34}\text{S}^{2-}$, respectively. Retention times were 4.7 and 5.8 minutes for the internal standard and the sulfide derivatives, respectively. Mass spectra of derivatized sulfide species are shown in figure 1.

Figure 1.
Mass spectra of bis-pentafluorobenzyl derivatives of $^{32}\text{S}_2^-$ and $^{34}\text{S}_2^-$.

Calibration

Sodium sulfide (Na_2S , Alfa Aesar, Karlsruhe, Germany) was dissolved in 0.1 M phosphate buffer, pH 7.4 to prepare calibration samples. Response ratios (analyte/internal standard) were plotted versus amount ratios to obtain calibration curves. Calibration ranged from 0.2 – 4 μM for sulfide baseline determination in blood and plasma, and from 0.2 – 100 μM for spiking experiments.

The calibration graph for baseline determinations is depicted in figure 2.

Figure 2.

Detection limit

The signal to noise ratio was at least 5 for the lowest calibration concentration 0.2 μM . Therefore this concentration was considered as detection limit.

Stable isotope approach

In blood and plasma samples, sulfide may be released from sulfur containing compounds during storage or sample work up. Therefore it is not possible to monitor sulfide levels of exogenously added sulfide. To overcome this problem, we used the stable, non-radioactive sulfur isotope ^{34}S for all spiking experiments. In combination with our GC/MS method, this approach allowed to distinguish between genuine and externally added sulfide. See mass spectra of compounds in figure 1.

General protocol for sulfide spiking experiments

1.95 mL of heparinized blood, plasma or 0.1 M phosphate buffer at pH 7.4 were spiked with 50 μL of 4 mM Na_2^{34}S (Campro Scientific, Berlin, Germany) to prepare an initial concentration of 100 μM $^{34}\text{S}^{2-}$. 10 μM concentrations were generated by adding 25 μL of 1 mM sulfide solution to 2.475 mL of blood, plasma or buffer. After vortexing three aliquots each were obtained at 1, 10, 30, 60, 120, and 180 minutes. Baseline levels were measured in the sample pools before spiking.

Figure 1.

A schematic representation of various forms of H₂S in solid, aqueous and gaseous phase. The crystalline compounds sodium sulfide (Na₂S) and sodium hydrogen sulfide (NaHS) can be dissolved in aqueous solutions, to yield an equilibrium between dissolved hydrogen sulfide gas (H₂S) and dissolved hydrosulfide anion (HS⁻). The gaseous form of hydrogen sulfide is hydrogen sulfide gas (H₂S), which can escape into the headspace. Reprinted with permission from [47].

Figure 2A

Figure 2B

Figure 2.

2A) Sulfide concentrations determined in swine blood *ex vivo* without addition of sulfide (open circles, broken line) and spiked with 1 mM Na₂S to a target concentration of 10 μM (closed squares, solid line) or 1 mM GYY4137 (open triangles, dotted line) (n = 4 in each group). All data are mean ± SD. **2B)** Sulfide concentrations after spiking with 4 mM Na₂³⁴S in a phosphate buffer (pH = 7.4) to a target concentration of 100 μM (squares, dotted line; n = 4), plasma (rhombus, solid line; n = 6), and blood (triangles, broken line; n = 6). All data are mean ± SD. All measurements were performed using a modified gas chromatography/mass spectrometry assay according to Kage *et al* [97]. For details see appendix.

Figure 3.

Sulfide concentrations after an initial Na₂S bolus (0.2 mg·kg⁻¹) followed by a continuous i.v. infusion (2.4 (open rhombus, n = 4) and 4.7 (open triangles, n = 7) mg·kg⁻¹·h⁻¹, respectively; vehicle: solid squares, n = 5) in swine. All data are mean ± SD, measurements were performed using the monobromobimane assay as described by Wintner *et al* [96].

Figure 4.

Sulfide concentrations (solid squares) in swine ($n = 5$) *before* (time points -1 and 0 hours, respectively) and *during* long-term, resuscitated, fecal peritonitis-induced septic shock. Samples were taken hourly, at 0 hours autologous feces were inoculated into the abdominal cavity to induce fecal peritonitis. At 12 and 18 hours of sepsis (see arrows), a bolus of GYY4137 ($10 \text{ mg}\cdot\text{kg}^{-1}$) was injected. After GYY injection additional measurements were obtained at 20 and 40 minutes post-injection, respectively (open circles). All data are mean $\pm$ SD. All measurements were performed using a modified gas chromatography/mass spectrometry assay according to Kage *et al* [97]. For details see appendix.

Figure 5.

Sulfide concentrations in CSE-ko ($\text{CSE}^{-/-}$) undergoing sham procedure (open squares, $n = 11$) or blunt chest trauma (TxT) (solid rhombus, $n = 8$) and wild type (solid triangles, $n = 5$) mice. All data are mean $\pm$ SD. All measurements were performed using a modified gas chromatography/mass spectrometry assay according to Kage *et al* [97]. For details see appendix.

Figure 6.

Sulfide concentrations in swine before, immediately after hemorrhagic shock, as well as over 24 hours of resuscitation using standard treatment (normothermia, inspired O₂ fraction titrated to arterial oxygenation, n = 5, open circles), therapeutic hypothermia (core temperature 34 °C, n = 6, squares), hyperoxia (mechanical ventilation with 100 % O₂, n = 7, triangles) and the combination of the two latter interventions (n = 6, rhombus). All data are mean ± SD. All measurements were performed using a modified gas chromatography/mass spectrometry assay according to Kage *et al* [97]. For details see appendix.

Figure 7.

Examples (upper panel) and quantitative analysis (lower panel) of the tissue CSE expression in kidneys from young and healthy German Landrace (“Landrace”, open boxplots) as well as adult swine with ubiquitous atherosclerosis resulting from homozygous modification of the LDL receptor and an atherogenic diet (“FBM”, grey boxplots) after Sham procedure (dotted boxplots, $n = 8$ each) or kidney I/R injury induced by intra-aortic balloon occlusion (hatched boxplots, $n = 8$, and $n = 7$, respectively). All data are median (quartiles, range), § designates $p < 0.05$ sham versus I/R injury, # designates $p < 0.05$ FBM versus German Landrace swine.

Figure 8A

Figure 8B

Figure 8.

Time course of lung tissue CSE (**8A**) and CBS (**8B**) expression in mice undergoing femoral fracture alone (“Fx”, dotted boxplots), combined femoral fracture and blunt chest trauma (“Fx + TxT”, hatched boxplots), combined femoral fracture and blunt chest trauma with exposure to 100 % O₂ over three hours each, immediately and nine hours after the trauma (“Fx + TxT + O₂”, vertically lined boxplots) (n = 7–9 each), as well as in animals without surgery (“Native”, open boxplots, n = 5). All data are median (quartiles, range), § designates p < 0.05 versus combined femoral fracture and blunt chest trauma.

Figure 9.

Examples (upper panel) and quantitative analysis (lower panel) of the tissue CSE and CBS expression in lungs (**9A**) and kidneys (**9B**) of mice undergoing mechanical ventilation with air (open boxplots) or 100 % O₂ (grey dotted boxplots) after blunt chest trauma (n = 6–7 each). All data are median (quartiles, range), § designates p < 0.05 versus air ventilation.

Figure 10.

Kidney CSE expression in swine after hemorrhagic shock and 22 hours of resuscitation using either standard treatment (normothermia, inspired O₂ fraction titrated to arterial oxygenation, n = 7, dotted grey boxplots) or therapeutic hyperoxia (mechanical ventilation with 100 % O₂, n = 5, open boxplots). All data are median (quartiles, range), § designates p < 0.05 versus standard treatment.

Figure 11.

Examples (upper panel) and quantitative analysis (lower panel) of the kidney CSE (**11A**) and CBS (**11B**) expression in swine after hemorrhagic shock and 22 hours of resuscitation either maintained at normothermia (core temperature 38 °C, hatched grey boxplots, n = 6) or hypothermia at 35 (dotted grey boxplots, n = 7) or 32 °C (grey boxplots, n = 7), respectively, as well as animals without surgery (“Native”, open boxplots, n = 5). Data are the results of post hoc analysis of tissue material obtained in [114]. All data are median (quartiles, range), # designates $p < 0.05$ versus normothermia.

Literature data on H₂S blood levels in experimental shock models as well as during acute illness. The concentration range presented refers to the minimum/maximum values in the vehicle- or drug-treated and/or control and shock groups, respectively.

Table 1

Author	Type of shock	Species	Intervention to modulate H ₂ S level	H ₂ S [μM]	Ref.
Wang 2013	Pre-eclampsia	Mouse	PAG 25/50 mg/kg	7 – 14	75
Zhao 2013	Myocardial I/R injury	Mouse	Penicillamine-based perthiol derivatives	0.1 – 0.5	119
Sidhapuriwala 2012	Acute pancreatitis	Mouse	PAG 10 mg/kg	6 – 20	60
Tokuda 2012	LPS	Mouse	H ₂ S 80 ppm	1.0 – 4.5	54
Wagner 2011	CLP sepsis	Mouse	H ₂ S 100 ppm	0.7 – 0.9	65
Ang 2010	CLP sepsis	Mouse	PAG 50 mg/kg; NaSH 10 mg/kg	11 – 24	120
Zhang 2010	Burn injury	Mouse	PAG 50 mg/kg; NaSH 10 mg/kg	50 – 80	71
Tripatara 2009	Kidney I/R injury	Mouse	NaSH 100 μmol/kg	11 – 25	20
Florian 2008	Stroke	Mouse	H ₂ S 80 ppm	14 – 30	22
Zhang 2008	CLP sepsis	Mouse	PAG 50 mg/kg; NaSH 10 mg/kg	9 – 19	67
Zhang 2007	CLP sepsis	Mouse	PAG 50 mg/kg; NaSH 10 mg/kg	8 – 23	68
Zhang 2007	CLP sepsis	Mouse	PAG 50 mg/kg; NaSH 10 mg/kg	6 – 27	69
Zhang 2006	CLP sepsis	Mouse	PAG 50 mg/kg; NaSH 10 mg/kg	9 – 23	70
Li 2005	LPS	Mouse	PAG 50mg/kg; NaSH 14μmol/kg	20 – 67	59
Aminzadeh 2012	% nephrectomy	Rat	Ø	30 – 48	78
Chai 2012	Hemorrhage	Rat	NaSH 28 μmol/kg	19 – 42	39
Van de Louw 2012	Hemorrhage	Rat	Hydroxocobalamin 140 mg/kg	1.5	40
Chen 2011	COPD	Rat	PAG 37.5 mg/kg; NaSH 14 μmol/kg	7 – 28	98
Li 2009	LPS	Rat	Dexamethasone 1 mg/kg	25 – 37	56
Xu 2009	Kidney I/R injury	Rat	NaSH 100 μg/kg	20 – 35	21
Geng 2004	Heart failure	Rat	Isoproterenol 3 – 20 mg/kg	18 – 63	121
Mok 2004	Hemorrhage	Rat	PAG 50 mg/kg	24 – 43	45
Simon 2011	Kidney I/R injury	Swine	Na ₂ S (bolus/infusion) 0.2 mg/kg/2 mg/kgxh	0.5 – 2.5	12
Osipov 2010	Cardiopulmonary bypass	Swine	Na ₂ S (bolus/infusion) 0.2 mg/kg/2 mg/kgxh	0.3 – 4.0	15

Author	Type of shock	Species	Intervention to modulate H ₂ S level	H ₂ S [μ M]	Ref.
Osipov 2009	Cardiopulmonary bypass	Swine	Na ₂ S (bolus/infusion) 0.2 mg/kg/2 mg/kgxh	0.5 – 9.0	18
Saito 2013	Asthma	Human	Ø	30 – 580	102
Wang 2013	Pre-eclampsia	Human	Ø	3 – 35	75
Goslar 2011	Circulatory shock	Human	Ø	2 – 110	101
Chen 2009	Community-acquired pneumonia/COPD exacerbation	Human	Ø	8 – 53	100
Wu 2008	Exacerbated asthma	Human	Ø	28 – 88	99
Chen 2005	Exacerbated COPD	Human	Ø	21 – 62	81
Li 2005	Septic shock	Human	Ø	32 – 249	59

CLP cecal ligation and puncture; COPD chronic obstructive pulmonary disease; I/R ischemia/reperfusion injury; LPS lipopolysaccharide, PAG propargylglycine.

Table 2

Literature data on the expression of H₂S generating enzymes in experimental shock models as well as during acute illness. The concentration range presented refers to the minimum/maximum values in the vehicle- or drug-treated and/or control and shock groups, respectively.

Author	Type of shock	Species	Intervention	Endogenous Enzyme	Ref.
Kondo 2013	Heart failure	Mouse	TAC SG1002	TAC: CSE ↑, CBS no change SG1002: CSE no change, CBS ↓	122
Shirozu 2013	Acute liver Failure	Mouse	Galactosamine/LPS	CBS mRNA/protein no change	52
Wang 2013	Pre-eclampsia	Mouse	PAG 25/50 mg/kg GY4137 250 µg/kg	Intrauterine growth restriction: CSE mRNA ↓	75
Yamamoto 2013	Diabetic nephropathy	Mouse	Ø	CSE ↓; CBS no change	77
Tokuda 2012	LPS	Mouse	H ₂ S 80 ppm	Liver, lung: CSE mRNA ↓, CSE protein no change Liver, lung + H ₂ S: CSE mRNA/protein no change	54
Wagner 2011	Blunt chest trauma	Mouse	Na ₂ S ≈ 7.5 nmol/g 32° vs. 37°C	Lung: CSE ↑↑, CBS ↑↑ + Na ₂ S: CSE ↑↑, CBS ↑↑ 32°C: CSE ↑, CBS ↑ 32°C + Na ₂ S: CSE, CBS no change	47
Zhang 2010	Burn injury	Mouse	PAG 50 mg/kg NaSH 10 mg/kg	Liver, lung: CSE mRNA ↑ Liver, lung + PAG: CSE mRNA no change	71
Tripata 2009	Kidney I/R injury	Mouse	NaSH 100 µmol/kg	CSE ↑	20
Zhang 2008	CLP sepsis	Mouse	PAG 50 mg/kg NaSH 10 mg/kg	Liver: CSE activity ↑ CLP + PAG: Liver CSE activity ↓	69
Zhang 2006	CLP sepsis	Mouse	PAG 50 mg/kg NaSH 10 mg/kg	Liver: CSE activity ↑ CLP + PAG: Liver CSE activity ↓	70
Li 2005	LPS	Mouse	PAG 50mg/kg NaSH 14µmol/kg	Liver, kidney: CSE mRNA ↑ Liver, kidney + PAG: CSE activity ↓	59
Bos 2013	Kidney I/R Injury	Rat	Ø	CSE, CSE mRNA ↓ CBS mRNA ↓	4
Cui 2013	Gastric I/R injury	Rat	PAG 50 mg/kg L-cysteine 50 mg/kg	Mucosa: CSE no change + PAG: CSE ↓ + L-cysteine: CSE no change	5
Wu 2013	Lung I/R injury	Rat	Transplant PAG 37.5 mg/kg NaSH 14 µmol/kg	CSE ↓	6
Aminzadeh 2012	CKD	Rat	Nephrectomy	Kidney, liver: CBS ↓, CSE ↓ Brain: no difference	78
Chen 2011	COPD	Rat	Cigarette smoke PAG 37.5 mg/kg NaSH 14 µmol/kg	Lung: CSE ↑	98

Author	Type of shock	Species	Intervention	Endogenous Enzyme	Ref.
Gao 2011	Diabetes Heart I/R injury	Rat	PAG 37.5 mg/kg NaSH 14 µmol/kg	Heart: CSE mRNA ↑ + PAG: CSE activity ↓ + NaSH: CSE activity no change	11
Wu 2010	Kidney I/R injury	Rat	∅	CBS mRNA ↓, CBS ↓	104
Kang 2009	Liver I/R injury	Rat	PAG 50 mg/kg NaSH 14 µmol/kg	CSE mRNA ↑	17
Li 2009	LPS	Rat	Dexamethasone Mifepristone	Liver: CSE ↑ Dex (pre/post): CSE ↓	56
Fu 2008	Lung I/R	Rat	Isolated organ PAG 2 mmol/L NaSH 50/100 µmol/L	CSE protein no change CSE activity ↑	23
Mok 2008	Hemorrhage	Rat	PAG 50 mg/kg	Liver, kidney: CSE activity ↓	43
Mok 2004	Hemorrhage	Rat	PAG 50 mg/kg β-cyanoalanine 50 mg/kg glibenclamide 40 mg/kg	Liver: CSE mRNA ↑	45
Bos 2013	Kidney I/R injury	Human	Transplant	CSE mRNA ↓; CSE protein, CBS mRNA no change	4
Wang 2013	Pre-eclampsia	Human	∅	Placenta: CSE mRNA ↓	75

CKD chronic kidney disease; CLP cecal ligation and puncture; COPD chronic obstructive pulmonary disease; I/R ischemia/reperfusion injury; LPS lipopolysaccharide, PAG propargylglycine, TAC transverse aortic constriction. Data refer to enzyme protein expression unless otherwise states. Relative changes are in comparison to sham control animals.