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Hypothesis

The hypothesis of the main role of H_2S in coupled sulphide-nitroso signalling pathway

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Abstract. As a part of the nitroso signalling pathway, nitroso-compounds serve as stores and carriers of NO; as part of the sulphide signalling pathway, bound sulfane-sulphur compounds serve as stores and carriers of H_2S. Here we hypothesise a coupled sulphide-nitroso signalling pathway, in which H_2S plays a main role. H_2S releases NO from the endogenous S-nitroso-compounds nitroso-cysteine, nitroso-acetylcysteine and nitroso-albumin. Relaxation of noradrenaline-precontracted aortic rings by H_2S is also enhanced in the presence of nitroso-albumin, which may implicate the involvement of the nitroso signalling pathway. Pretreatment of albumin, cysteine, N-acetylcysteine and lipids with H_2S results in binding of sulphur to these compounds creating thus new-modified sulphur compounds that release NO from nitroso-compounds directly and/or through released H_2S, which suggests sulphide-nitroso signalling pathway participation. This hypothesis is supported by the observation that the pretreatment of noradrenaline-precontracted aortic rings with H_2S significantly enhanced relaxation induced by nitroso-glutathione in the absence of H_2S. We assume that the NO release from nitroso-compounds directly by H_2S or indirectly by the H_2S-induced sulphur-bound compounds represents coupled sulphide-nitroso signalling, which may explain some of the numerous biological effects of H_2S that are shared with NO.

Abbreviations: NA, noradrenaline; Cys, cysteine; NAC, N-acetylcysteine; GSH, glutathione; BSA, bovine serum albumin; Cys-NO, nitroso-cysteine; NAC-NO, nitroso-N-acetylcysteine; GS-NO, nitroso-glutathione; BSA-NO, nitroso-bovine serum albumin; Cys-S, H_2S bound to Cys; NAC-S, H_2S bound to NAC; GS-S, H_2S bound to GSH; BSA-S, H_2S bound to BSA.

Key words: H_2S — Nitroso signalling — Sulphide signalling — NO release — Nitroso-glutathione

Introduction

Endogenously produced H_2S is recognised as a gasotransmitter, which together with NO influences numerous biological processes, including muscle relaxation, hypertension, proliferation, gene expression, cardioprotection, neuroprotection, intestinal secretion, visceral pain, penile erection, diabetes, apoptosis, atherosclerosis and inflammation (Zhao et al. 2001; Wang 2002; Chen et al. 2007; Lowicka and Beltowski 2007; Pacher et al. 2007; Szabo 2007; Yang et al. 2008; Wang et al. 2009; Kimura 2010). Nevertheless, the molecular mechanism of H_2S signalling is not fully understood. Many bacteria, most fungi and plants, and all higher organisms endogenously produce H_2S and NO, which are involved in many biological functions. In mammals, H_2S is produced

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from cysteine (Cys) by at least three enzymes, which are widely expressed in many tissues (Stipanuk and Beck 1982; Shibuya et al. 2009; Kimura 2010). The synthesised H_2S can be released from cells to affect tissues in a paracrine fashion, or H_2S can initially be stored, transported and then released in response to a physiological signal (Ishigami et al. 2009; Kimura 2010). Newly-synthesised H_2S is stored as sulfane sulphur, which is a divalent sulphur molecule bound to another sulphur molecule, such as outer sulphur atoms of the persulphides and inner chain atoms of polysulphides (Nagahara and Nishino 1996; Furne et al. 2008; Whitfield et al. 2008; Ishigami et al. 2009; Shibuya et al. 2009; Kimura 2010). The steady state concentration of free H_2S molecules in mammalian blood and brain tissue is in the nanomolar range (<20 nmol/l) (Furne et al. 2008; Ishigami et al. 2009). Thus, free H_2S does not circulate in plasma in measurable concentrations, but rather circulates bound in sulphur stores (Furne et al. 2008; Ishigami et al. 2009; Kimura 2010). Two types of cellular sulphur stores have been identified; under acidic conditions, H_2S is released from acid-labile sulphur, whereas under reducing conditions, H_2S is released from sulfane stores (Stipanuk and Beck 1982; Savage and Gould 1990; Ogasawara et al. 1994; Ishigami et al. 2009; Kimura 2010). The process of H_2S synthesis and its effects on cellular targets are part of the sulphide signalling pathway.

NO, which is synthesised by three isoforms of NO synthase, posttranslationally modifies proteins, e.g., the formation of S-nitrosothiol (RSNO), to alter protein function (Miersch and Mutus 2005). Thus, as a part of the nitroso signalling pathway, nitroso-compounds serve as stores and carriers of NO (Miersch and Mutus 2005); as part of the sulphide signalling pathway, bound sulfane-sulphur compounds serve as stores and carriers of H_2S (Ishigami et al. 2009). Many of the effects of H_2S are shared with NO, suggesting a possible connection between the sulphide and nitroso signalling pathways. Therefore, the aim of the present study was to explore the connection between the sulphide and nitroso signalling pathways.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich including nitroso-glutathione (GS-NO) and asolectin from soybean (highly purified phospholipid product containing lecithin, cephalin, inositol phosphatides and soybean oil). In all experiments NaHS was used as the H_2S donor, which dissociates in solution to $HS^- + H^+$ and trace amounts of S^{2-}. The proportion of H_2S and HS^- at 37°C and pH 7.4 is 30% and 70%, respectively. In the present work, though, the term H_2S will refer to the total mixture of H_2S, HS^- and S^{2-} (Ondrias et al. 2008). Nitroso-cysteine (Cys-NO) and nitroso-N-acetylcysteine (NAC-NO) were synthesised by combining 200 mmol/l $NaNO_2$ and 200 mmol/l L-cysteine or N-acetyl-L-cysteine in 0.5 mol/l HCl in the dark at room temperature for 10 min. In case of BSA-NO, 2 mmol/l $NaNO_2$ and 2 mmol/l bovine serum albumin (BSA) were combined. The solution was neutralized to pH 7.2 with 1 mol/l NaOH, and its concentration estimated by absorbance at 334 nm using GS-NO as standard (Shi et al. 2008). Yield of Cys-NO, NAC-NO and BSA-NO was 31 mmol/l, 37 mmol/l and 50 μmol/l, respectively. The stocks of nitroso compound solutions were stored at −70°C. Stock solutions of NaHS and studied compounds in buffer A (in mmol/l: 160 KCl, 1 $MgCl_2$, 0.1 DTPA, 10 HEPES, pH was adjusted with Tris to value 7.4) were prepared at the beginning of measurement and were used within a few hours.

Measurement of rat aorta reactivity

All procedures were approved by the State VET and Nutritive Administration of Slovak Republic. Experiments were carried out as previously described (Ondrias et al. 2008). Briefly, the rings of thoracic aorta were prepared from male Wistar rats (250–300 g, 12 weeks old) and were mounted for recording of isometric tension changes in pneumoxid-oxygenated (95% O_2: CO_2; 37°C) Krebs-bicarbonate solution. The effects of H_2S, GS-NO, BSA and BSA-NO were given relative to the difference of the tension induced by 1 μmol/l noradrenaline and the original tension. This difference was set at 100%. The rings were washed out three times with 20 ml of solution. Paired *t*-test was used to determine significance of an effect of the compounds. Data represent means ± SD, $n \geq 3$. The procedure was used to obtain results showed in Figs. 1 and 3.

Preparation of BSA-S

All experiments were performed at 22 ± 1°C, in buffer A. The stock solutions of H_2S bound to BSA (BSA-S) were produced by mixing 1 mmol/l BSA with 1 mmol/l NaHS, 1 mmol/l BSA with 5 mmol/l NaHS and 0.5 mmol/l BSA with 50 mmol/l NaHS. All stock solutions were incubated for 30 minutes and dialysed against H_2O for 24 hour at 22°C; the H_2O was exchanged 6 times during dialysis. The ratio of BSA to BSA-S (sulphur bound to BSA) was not known. The procedure was used to obtain results showed in Fig. 2A.

Measurement of NO release from NO donors

NO-donors, H_2S and studied compounds were mixed in the buffer A, incubated at 22 ± 1°C for 10 min, and to measure NO oxidation product, nitrite (NO_2^-), the Griess reagent was added. The samples were incubated for 15 min, and

absorption spectra at 540 nm were measured (Ondrias et al. 2008). Concentration of NO_2^- was calibrated by $NaNO_2$. Data represent means ± SD of a triplicate experiment. The procedure was used to obtain results showed in Fig. 2A, B and D.

Time-dependent release of NO from GS-NO

The stock solutions of 5 mmol/l NaHS alone and the mixtures of 2.5 mmol/l N-acetylcysteine (NAC), Cys or glutathione (GSH) with 5 mmol/l NaHS, and 1 mmol/l BSA with 5 mmol/l NaHS in the buffer A were incubated at 22 ± 1°C under air. Since the H_2S evaporated from the solution under air, the surface : volume ratio of 4.52 cm^2 : 1.47 ml was constant for all the incubated samples. The Griess reagent was used to measure NO release from GS-NO. The procedure was used to obtain results showed in Fig. 2B.

Preparation of H_2S-pretreated asolectin and measurement GS-NO concentration

The stock solutions of NaHS alone (5 mmol/l) or asolectin (1.25 mmol/l) alone or with NaHS (5 mmol/l) were incubated at 25°C for 5 hours at the surface : volume ratio of 4.52 cm^2 : 1.47 ml. The samples were then diluted to give 320 μmol/l NaHS and 80 μmol/l asolectin, and GS-NO (200 μmol/l) was added. The samples were further incubated at 25°C or 37°C, and the concentration of GS-NO (200 μmol/l = 100%) was measured by absorbance at 334 nm (Gordge et al. 1998). The procedure was used to obtain results showed in Fig. 2C.

Results

To investigate the involvement of H_2S in sulphide and nitroso signalling pathways, we studied the effects of endogenous NO-donor GS-NO on noradrenaline (NA)-precontracted aortic rings that were pretreated with H_2S (Fig. 1). As shown in Fig. 1A, the repeated application and removal (wash out) of NA and GS-NO to aortic rings caused a relaxation effect that slightly increased after each application. This effect was not studied in more details. However, when aortic rings were pretreated with H_2S and then washed prior to the application of GS-NO, the observed relaxation effect significantly increased (Fig. 1B and C). From these results, we hypothesise that the increased relaxation of aortic rings was due to the enhanced release of NO from GS-NO, and that sulphur from

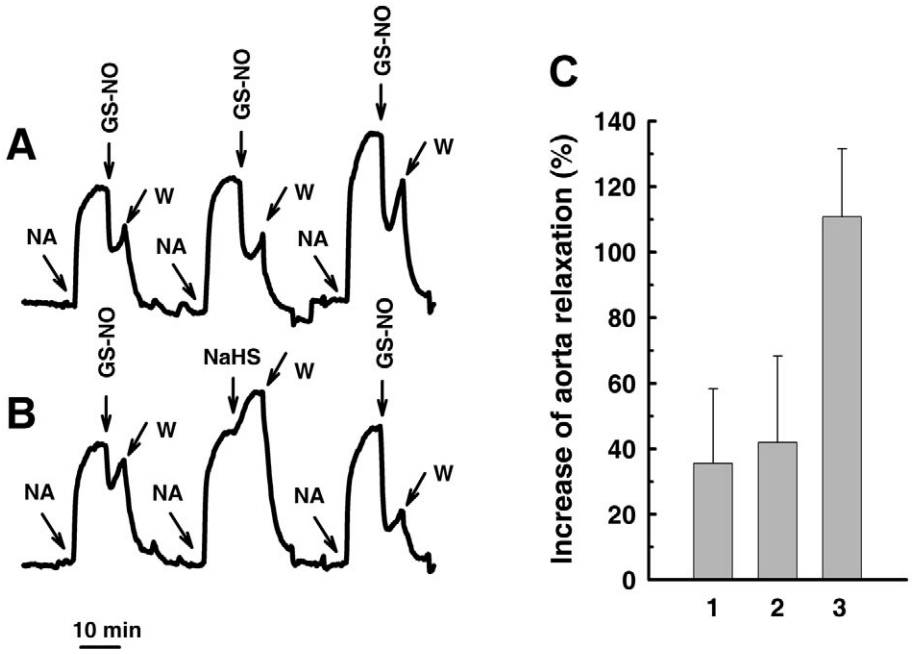

Figure 1. Representative traces showing the effect of GS-NO (0.5 μmol/l) and NaHS (100 μmol/l) on noradrenaline (NA, 1 μmol/l)-precontracted aortic rings. **A.** The effect of thrice-repeated GS-NO application and wash out (W) in arbitrary units. **B.** The sequential effect of GS-NO, NaHS and GS-NO in arbitrary units. **C.** The increase in NA-precontracted aortic ring relaxation by GS-NO: 1, the second addition of GS-NO *versus* the first addition from Fig. 1A; 2, the third addition of GS-NO *versus* the first addition from Fig. 1A; 3, the third addition of GS-NO after pretreatment with H_2S *versus* the first addition from Fig. 1B (one-tailed *t*-test; column 3 *versus* column 2: $p = 0.0017$, $n = 6$; column 3 *versus* column 1: $p = 0.0003$, $n = 6$).

the H_2S pretreatment bound to component(s) of the aortic rings to induce NO release.

To study our assumption, we investigated whether endogenous compounds having free thiol groups could release NO from the NO-donor, GS-NO, after H_2S pretreatment. BSA was used since it represents the largest fraction of free thiols in circulation. As shown in Fig. 2A, the BSA pretreated with H_2S followed by the subsequent dialysing out of the H_2S for 24 hours, released NO from GS-NO. To confirm this result by a different approach, we measured the time-dependent release of NO from GS-NO by H_2S alone or after mixing H_2S with Cys, NAC, GSH or BSA (Fig. 2B). During these experiments, free H_2S concentration in solution decreased significantly, since the ability of H_2S to release NO from GS-NO in solution decreased over time. The H_2S solution alone did not cause the release of NO from GS-NO after 90 minutes, indicating that at this time the solution was apparently free of H_2S. However, the inclusion of Cys or BSA significantly prolonged the time-dependent release of NO from GS-NO. These results may suggest that H_2S bound to BSA, Cys, NAC and GSH to form "S-compounds" (we named them as BSA-S, Cys-S, NAC-S and GS-S), which released NO from GS-NO. The ability of the S-compounds to release NO from GS-NO differed (Cys-S >> BSA-S ≥ NAC-S ≥ GS-S), depending on the chemical structure, whereas GS-S had no significant effect. The most potent combination was Cys and BSA-S, which released NO four hours after mixing with H_2S (Fig. 2B) and for longer than 24 hours when the H_2S was dialysed out (Fig. 2A). Based on the data showing the time-dependent decrease in the ability of S-compounds to release NO from GS-NO, we may assume that sulphur was gradually released

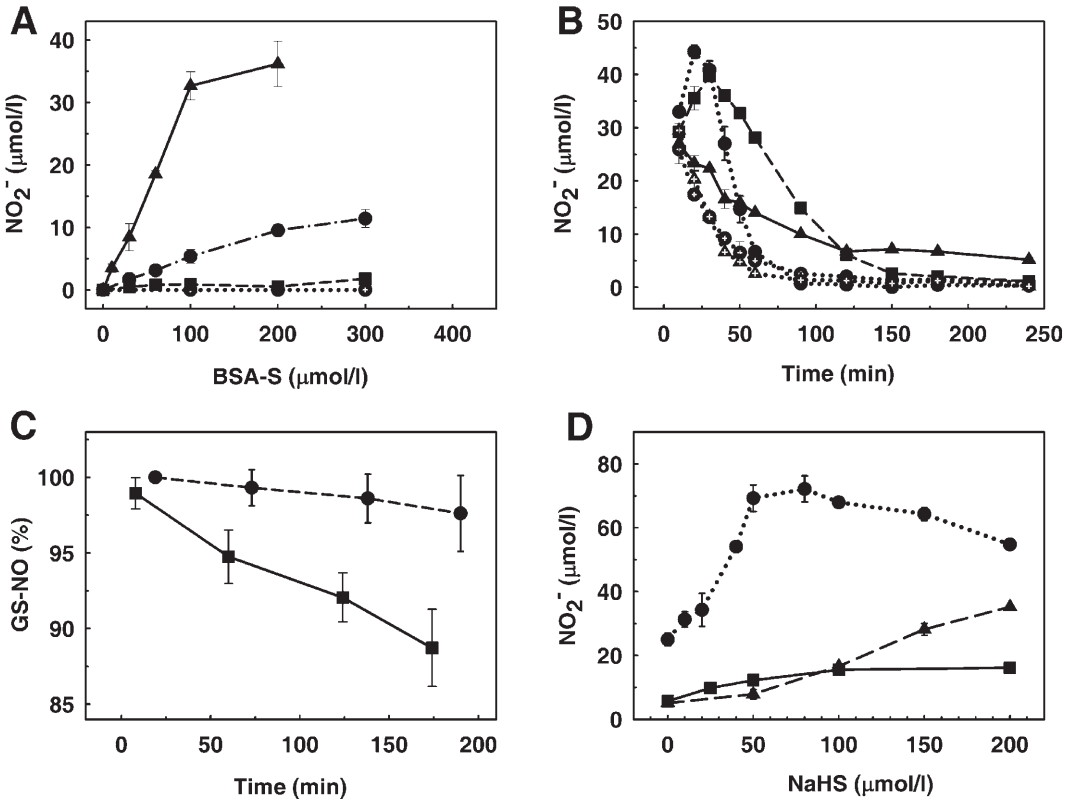

Figure 2. NO release from GS-NO at different experimental conditions. **A.** Concentration-dependent release of NO from 100 μmol/l GS-NO, induced by BSA alone (open circles, dotted line) and BSA pretreated with different concentrations of NaHS (BSA-S, n = 3, ±SD): By mixing 1 mmol/l BSA with 1 mmol/l NaHS (squares, dash line), 1 mmol/l BSA with 5 mmol/l NaHS (circles, dash-dot line) and 0.5 mmol/l BSA with 50 mmol/l NaHS (triangles, full line). **B.** Time-dependent release of NO from 100 μmol/l GS-NO, induced by 400 μmol/l NaHS alone (full circles, dotted line) or mixed with 200 μmol/l NAC (open circles, dotted line), 200 μmol/l Cys (squares, dash line), 200 μmol/l GSH (open triangles, dotted line) or 80 μmol/l BSA (full triangles, full line). **C.** Time- and temperature-dependent comparison of NO release from GS-NO (200 μmol/l) by NaHS-pretreated asolectin (molecular weight ≈770; 80 μmol/l; n = 3, ±SD) at 25°C (circles, dash line) and 37°C (squares, full line) measured as decrease of GS-NO concentration at 334 nm absorbance. **D.** Concentration dependence of NaHS induced NO release (NO_2^-) from Cys-NO (100 μmol/l, circles, dotted line), NAC-NO (100 μmol/l, triangles, dash line) and BSA-NO (25 μmol/l, squares, full line).

from the S-compounds to form H_2S, which then may have partially evaporated from the solution, or its concentration decreased after unknown chemical interactions (Fig. 2B). Although the exact mechanism of the S-compound-mediated release of NO from the NO-donor is unknown, we may assume that the formation of H_2S using the sulphur released from S-compounds is responsible for the NO release; however, any possible direct effect of S-compounds on NO release cannot be ignored.

As shown in Fig. 2B, combining BSA, Cys, NAC or GSH with H_2S significantly reduced the rate of NO release over H_2S alone during the first 10–30 minutes of the incubation period. These data supported our assumption that some amount of H_2S bound to BSA, Cys, NAC and GSH, and therefore the concentration of free H_2S decreased and less H_2S was available to release NO from GS-NO.

We previously reported that asolectin, a mixture of lipids, inhibited the H_2S-induced release of NO from GS-NO (Tomaskova et al. 2009) due to a possible reaction of H_2S with unsaturated fatty acid or lipid bonds (Lykakis et al. 2007; Tomaskova et al. 2009). Fig. 2C suggests that lipids are involved in the coupled sulphide-nitroso signalling, as H_2S-pretreated asolectin released NO from GS-NO at 37°C, but the effect was not statistically significant at 25°C (Fig. 2C). Parallel samples of H_2S and asolectin alone had no effect on NO release after 5 hours of incubation time (data not shown).

Endogenous Cys-NO, NAC-NO, GS-NO and nitroso-bovine serum albumin (BSA-NO) may act as an intermediate in the storage and/or transport of nitric oxide as a part of nitroso-signalling (Miersch and Mutus 2005). We therefore tested if H_2S could release NO from these nitroso-compounds, and we found that NaHS (100 μmol/l) released NO from all of these nitroso-compounds in a concentration-dependent manner (Fig. 2D), similarly as we previously have found for GS-NO (Ondrias et al. 2008).

To support the involvement of nitroso-proteins in the biological effects of H_2S nitroso signalling, the NaHS-induced release of NO from BSA-NO was studied in NA-precontracted aortic rings (Fig. 3). Although the application of 1 μmol/l BSA, followed by 100 μmol/l NaHS, to the rings did not have a significant effect on relaxation (Fig. 3A), 1 μmol/l BSA-NO caused small relaxation effects (33 ± 16%, n = 3, means ± SD) (Fig. 3B) and the subsequent addition of 100 μmol/l NaHS markedly increased relaxation (82 ± 6%, n = 5). A similar effect was observed when 100 μmol/l NaHS was applied to the rings first, causing a slight contraction, but the subsequent addition of 1 μmol/l BSA-NO caused significant relaxation (78 ± 15%, n = 3) (Fig. 3C). These results showed that 100 μmol/l NaHS significantly increased relaxation in the presence of BSA-NO, whereas NaHS (100 μmol/l) alone caused contraction rather than relaxation (Fig. 3D), indicating that NaHS may release NO from BSA-NO to cause aortic

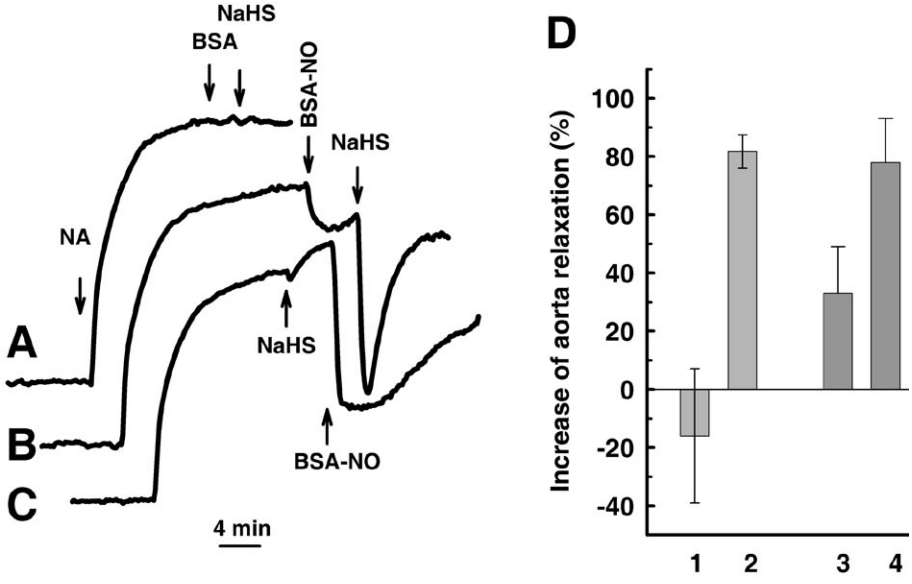

Figure 3. Representative traces showing the effects of BSA-NO (1 μmol/l) and NaHS (100 μmol/l) on noradrenaline (NA, 1 μmol/l) – precontracted aortic rings. **A.** Control addition of BSA (1 μmol/l) and NaHS (100 μmol/l) in arbitrary units. **B.** The relaxation effect of BSA-NO followed by an enhanced relaxation effect of NaHS in arbitrary units. **C.** The contraction effect of NaHS followed by the relaxation effect of BSA-NO in arbitrary units. **D.** The effects of NaHS and BSA-NO on aortic relaxation: 1, the first addition of NaHS (–16 ± 23%, n = 11); 2, the addition of NaHS in the presence of BSA-NO (82 ± 6%, n = 5); 3, the first addition of BSA-NO (33 ± 16%, n = 3); 4, the addition of BSA-NO in the presence of NaHS (78 ± 15%, n = 3).

rings relaxation. The contraction effect of NaHS and its nonsignificant effect after BSA application were not studied in more details.

Discussion

Based on this *in vitro* study, we hypothesise that sulphide-nitroso signalling pathway *in vivo* does exists (Fig. 4) involving at least Cys, albumin and lipids, and that H_2S plays a main role in this pathway. However, it is not known whether S-compounds can directly release NO from nitroso compounds, or whether the H_2S released from the S-compounds releases NO.

Several reports indicate connection between H_2S and NO. A "cross-talk" between H_2S and NO was suggested. Mixing of H_2S donor NaHS with NO donors (SNP or SNAP) diminished vasorelaxant effect of each NO donor on rat aorta and low concentrations of H_2S contracted human internal mammary artery (Ali et al. 2006; Webb et al. 2008). It was suggested that H_2S was able to inactivate NO by chemical reaction with NO to produce an unidentified nitrosothiol (Ali et al. 2006; Whiteman et al. 2006; Webb et al. 2008). Munro and Williams (2000) supposed nitrosodisulfide ion $ONSS^-$ as the result of RSNOs reaction with sulfur-centred nucleophiles. H_2S gas and H_2S donors NaHS were found to reduce RSNOs and release NO from RSNO (Ondrias et al. 2008; Teng et al. 2008). Using EPR spectroscopy of spin probe, NO release from GSNO, biological membranes and L1210 cells was detected upon addition of H_2S donor NaHS. From the pH-dependence of the effects, it was supposed that HS^- rather than H_2S was responsible for the NO-releasing effect (Ondrias et al. 2008). Recently we observed that lipids, depending on their structure, influenced H_2S/HS^- induced NO release from S-nitrosoglutathione (Tomaskova et al. 2009).

The role of H_2S in sulphide-nitroso signalling is also supported by several published findings, which described the biological absorption and release of H_2S and showed that H_2S and NO pretreatment caused similar biological effects in several model systems. The existence of H_2S stores was reported based on the observation that exogenously applied and endogenously produced H_2S were both absorbed as bound sulfane sulphur (Ogasawara et al. 1994; Ishigami et al. 2009; Shibuya et al. 2009; Kimura 2010). H_2S was released from bound sulphur, an intracellular store of sulphur, in neurons and astrocytes of mice and rats in the presence of physiologic concentrations of the endogenous reducing substances, GSH and Cys, at pH 8.4 (Ogasawara et al. 1994). Considering that H_2S is absorbed by biological systems (Ishigami et al. 2009) and because a gas evaporates during longer experimental procedures, the results from the published biological experiments using exogenous H_2S may actually be an experimental artifact of H_2S pretreatment. Direct H_2S pretreatment has been reported to have biological effects in multiple model systems; the survival

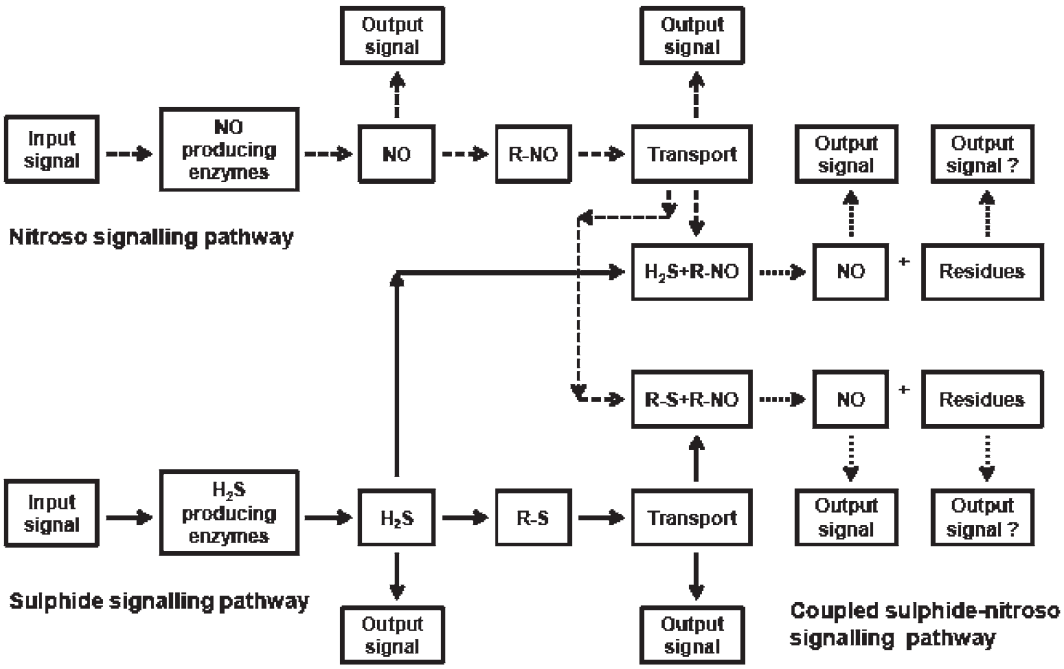

Figure 4. Block scheme of the suggested sulphide (full lines), nitroso (dash lines) and coupled sulphide-nitroso (dotted lines) signalling pathways. R-NO, nitroso-compounds; R-S, sulphur-bound-compounds.

of mice with sepsis was increased by a pretreatment with H_2S, which improved leukocyte rolling/adhesion in the mesenteric microcirculation as well as neutrophil migration, reduced bacteremia levels, and prevented hypotension and lung lesions (Spiller et al. 2010); pretreatment of oxidised low density lipoprotein with H_2S destroyed lipid hydroperoxides (Muellner et al. 2009); H_2S pretreatment of isolated cardiomyocytes and an *in vivo* rat model of myocardial infarction produced potent cardioprotection against lethal ischemia (Pan et al. 2006, 2009); pretreatment of brain endothelial cells with H_2S protected the cells from oxidative damage (Tyagi et al. 2009) and pretreatment of mice with H_2S gas inhalation protected the animals from lethal hypoxia (Blackstone and Roth 2007).

An involvement of H_2S in the coupled sulphide-nitroso signalling may also be supported by the observations that H_2S shares many biological effects with NO, including vasorelaxant (Ignarro et al. 1987; Zhao et al. 2001), anti-inflammatory (Guzik et al. 2003; Zanardo et al. 2006), cardioprotective (Cohen et al. 2006; Sivarajah et al. 2006), neuroprotective (Contestabile and Ciani 2004; Kimura and Kimura 2004), anti-proliferation (van der Veen et al. 1999; Du et al. 2004), antiatherosclerothic (Kawashima and Yokoyama 2004; Wang et al. 2009) and erectile (Burnett et al. 1992; Srilatha et al. 2007) properties. We suppose that H_2S-coupled sulphide-nitroso signalling may underline some of the numerous biological effects of H_2S.

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