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Low molecular thiols, pH and O_2 modulate H_2S-induced S-nitrosoglutathione decomposition – •NO release

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Abstract. We studied the involvement of O_2, pH and low molecular thiols in H_2S-induced decomposition of S-nitrosoglutathione (GSNO). The GSNO decomposition – •NO release was evaluated by UV-VIS spectroscopy and Griess assay. The H_2S donor Na_2S was used. O_2 slightly increased, but was not necessary for the H_2S-induced GSNO decomposition. The rate of GSNO decomposition depended on pH; the maximum rate was observed at pH 7.4–8.0, and this decreased with lowering pH (6.4–4.5) as well as with increasing pH at 9.0–12.0. H_2S-induced GSNO decomposition was slowed by the presence of other thiols, such as L-cysteine (Cys), N-acetyl-L-cysteine (NAC) and L-glutathione (GSH), but not in the presence of L-methionine (Met) or oxidized glutathione (GSSG). In sharp contrast, at pH 6.0, H_2S-induced GSNO decomposition was negligible, yet the presence of Cys, NAC and GSH induced the H_2S-driven GSNO decomposition (whilst Met and GSSG were inactive). In conclusion we postulate an involvement of low molecular thiols and pH in •NO signaling, by modulating the interactions of H_2S with nitroso compounds, and hence in part they also appear to control H_2S-triggered •NO release. The interaction of H_2S and/or its derivatives with the thiol group may be responsible for the observed effects.

Key words: Hydrogen sulfide — S-nitrosoglutathione — Nitric oxide — pH — Thiols

Abbreviations: ABS, absorbance; Cys, L-cysteine; CysNO, S-nitrosocysteine; DTPA, diethylenetriaminepentaacetic acid; GSH, L-glutathione; GSNO, S-nitrosoglutathione; GSSG, oxidized glutathione; Met, L-methionine; NAC, N-acetyl-L-cysteine; NACNO, S-nitrosoacetylcysteine.

Introduction

Nitrogen monoxide (•NO) is a key biological signaling molecule. While it is involved in the production of cyclic GMP by controlling the enzyme-soluble guanyl cyclase (sGC), this also interacts directly or indirectly with many other proteins by post-translational modifications that can alter their function. During the last decade, it has become apparent that intracellular S-nitrosothiols which are formed by the interaction of •NO and thiol (R-SH) groups, act as an important bio-reservoir for NO (Stamler 1994). Like •NO, endogenously synthesized H_2S is recognized as an important gasotransmitter, which together with •NO influences many biological processes (Hosoki et al. 1997; Coletta et al. 2012; Wang 2012). In addition to its direct effect, H_2S can be stored as sulfane sulphur and transported and released in response to a physiological signal (Ishigami et al. 2009).

A number of recent reports have discussed molecular interactions between H_2S, •NO, O_2 or nitrosothiols. The H_2S

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donors NaHS and Na_2S, for instance, released •NO from nitroso-compounds. In addition, chemical reactions between H_2S and •NO have been postulated and in part confirmed (Whiteman et al. 2006; Ondrias et al. 2008; Teng et al. 2008; Tomaskova et al. 2009, 2011; Bertova et al. 2010; Yong et al. 2010; Coletta et al. 2012; Filipovic et al. 2012b), and the modulation of •NO and H_2S actions by O_2 was observed by Abu-Soud and co-workers (Abu-Soud et al. 1996).

A particularly interesting, but puzzling finding, is that S-nitrosylation and certain endogenous or synthetic thiol compounds, such as L-cysteine (Cys), N-acetyl-L-cysteine (NAC) and L-glutathione (GSH) are presumed to be involved in the •NO signaling pathway and these may modulate or interfere with both •NO and H_2S signaling pathways (Stamler 1994; Ellis et al. 2000; Gaston et al. 2006; Duan and Chen 2007; Li et al. 2009; Yong et al. 2010; Yong et al. 2011; Kasparek et al. 2012). For instance, thiols such as Cys, NAC and GSH have been reported to influence the •NO-H_2S effects on myocyte contraction (Yong et al. 2010, 2011). Although these authors suggested that the mutual effect of H_2S and •NO was in fact sensitive to the presence of thiols, the molecular mechanism(s) by which such thiols interfere with the various interactions between H_2S, •NO, pH are still not fully recognized or understood.

We have therefore decided to investigate the various influences of O_2, pH and low molecular thiols on the H_2S-induced GSNO decomposition (•NO release). The aim of this study involves a closer inspection of the underlying molecular interactions of the complex biological effects of pH, H_2S, •NO and thiols, to contribute to our understanding in this difficult field of research.

Material and Methods

Chemicals

Na_2S was obtained from Alfa Aesar (England). All other chemicals were purchased from Sigma-Aldrich. NaHS and Na_2S were used as H_2S donors, which dissociate in solution and react with H^+ to yield HS^-, H_2S and a trace of S^{2-}. We use the term H_2S to encompass the total mixture of H_2S, HS^- and S^{2-} (Staško et al. 2009). S-nitrosocysteine (CysNO) and S-nitrosoacetylcysteine (NACNO) were synthesized by combining 200 mmol/l $NaNO_2$ and 200 mmol/l Cys or NAC in 0.5 mol/l HCl, in darkness at room temperature for 10 min (Shi et al. 2009). The purity of CysNO and NACNO was not determined. The following buffers (in mmol/l) were used: Hepes/Tris bufer 8.0 pH (160 KCl, 1 $MgCl_2$, 0.1 diethylenetriaminepentaacetic acid (DTPA), 50 Tris, pH was adjusted with HEPES to value 8.0). Hepes/Tris buffer 7.4 pH (160 KCl, 1 $MgCl_2$, 0.1 DTPA, 50 HEPES, pH was adjusted with Tris to value 7.4). Pipes/Tris buffer 6.0 pH (160 KCl, 1 $MgCl_2$, 0.1 DTPA, 50 PIPES, pH was adjusted with Tris to value 6.0). Phosphate buffers 4.5; 6.0; 6.4, 7.0, 7.4; 8.0, 9.0 pH (100 sodium phosphate, 0.004 DTPA) and 12.0 pH (100 sodium phosphate, 16 NaOH, 0.004 DTPA). The stock solutions of NaHS, Na_2S and the compounds studied were prepared prior to measurements and used within 2–4 hours. Samples were prepared, incubated and analyzed at room temperature (23 ± 1°C), under sub-light or dark conditions, unless stated otherwise. In order to account for eventual variations in sample quality and preparation, seven samples of NaHS, two of Na_2S and eight of GSNO were used for the studies. Although there may have been minor variations in results depending on the sample used, the overall quality of the results was not affected by these variations. Two methods were used to study the effect of compounds on H_2S-induced GSNO decomposition (•NO release from GSNO): The Griess assay and UV-VIS measurements of GSNO decomposition.

Griess assay

The assay was used to measure the •NO oxidation product, nitrite (NO_2^-), by the Griess reaction and to study the effects of Cys, NAC, GSH, oxidized glutathione (GSSG) and L-methionine (Met) on NO release from GSNO induced by the H_2S donor NaHS (Ondrias et al. 2008). GSNO (100 μmol/l), NaHS (100 μmol/l) and the studied compounds were mixed in the given buffer, at 23±1°C and incubated for 10 min. The Griess reagent was then added to quantify NO_2^-. The samples were incubated for 10 min, and absorption spectra (ABS) in the wavelength range of 290–700 nm (5 nm step) were measured by a μ-Quant Microplate Scanning Spectrophotometer (BIO-TEK Instruments, Inc.) in polystyrene microplates. Data were acquired using Gen5 Data Analysis software (BIO-TEK Instruments, Inc.). NO_2^- formation was calculated from the ABS of the subtracted spectrum as ABS(540 nm)–ABS(700 nm), which was compared to a NO_2^- calibration curve obtained by using samples with known concentrations of $NaNO_2$. The ABS value obtained from the samples containing 100 μmol/l GSNO and 100 μmol/l NaHS was set as the control (100%). The Griess assay was performed at pH 7.4 and at pH 6.0.

UV/VIS spectroscopy

In order to study the various effects of Cys, NAC, GSH, Met and GSSG on H_2S-induced GSNO decomposition at different pH values, NaHS or Na_2S and the compounds under investigation were mixed in the buffer at the given pH, and the kinetics of •NO release were measured by UV-VIS spectrophotometry for 10–30 minutes. The studies related to the influence of O_2 on these effects were performed under air or in deaerated nitrogen solutions. We use the term "aer-

ated samples" for the samples prepared under atmospheric air. The studies in the deaerated solution imply that the sample was prepared in a closed UV-cuvette containing the appropriate buffer (1 ml) with GSNO and was deaerated with nitrogen for 7 min prior to use. The decrease in the oxygen concentration was checked by oxygen electrode (OXELP, SYS-ISO2, WPI, USA). The O_2 concentration in the deaerated samples decreased to 2–3%. The GSNO-H_2S-compound reaction was monitored by concomitant spectrophotometric analysis: the decomposition of GSNO was signified by the decrease in ABS at 334 nm (Singh et al. 1996), the concentration of HS^- was estimated at 232 nm (Guenther et al. 2001) and the formation of an unknown product appeared at 270 and 412 nm. In order to obtain the 334 nm peak undisturbed by the ABS at 240–340 nm during the H_2S-GSNO interaction, the simulated proportion of the shoulder spectrum was subtracted from the experimental spectrum. To obtain the peak at 412 nm undisturbed by the peak at 334 nm, the proportion of the peak at 334 nm of the GSNO was subtracted from the spectra. Data acquired from spectroscopic measurements were analyzed in Sigma Plot 8.0 (SPSS Inc.) and presented as the mean ± standard error of the mean (SEM, $n \geq 3$). Data were analyzed at the significance level of $\alpha = 0.05$, where a probability value of $p < \alpha$ was considered statistically significant.

Results

Effect of O_2 on the H_2S-induced GSNO decomposition

Since Hepes is a commonly used biological buffer known to react with peroxynitrite (Lomonosova et al. 1998), we used phosphate and Hepes/Tris buffers in our studies. The UV-VIS spectrum of 200 µmol/l NaHS or Na_2S at 7.4 pH showed a single peak at 232 nm assigned to the hydrogen sulfide anion HS^- (Guenther et al. 2001) (insert in Fig. 1). No ABS of H_2S oxidation products close to 420 nm were seen in these spectra (Hughes et al. 2009). The spectrum of 200 µmol/l GSNO exhibited two peaks; the first peak at 220 nm and the

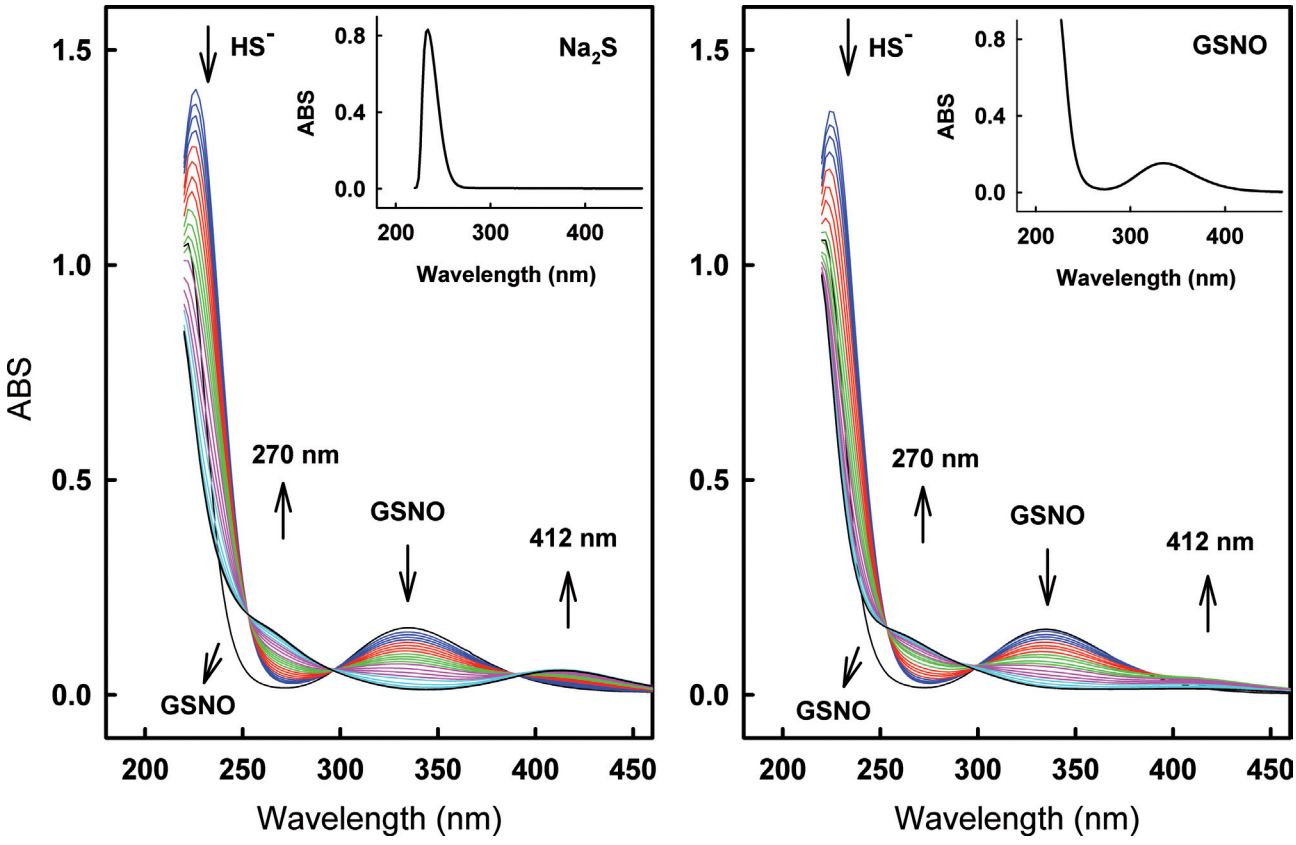

Figure 1. Time dependence of H_2S-induced GSNO decomposition on O_2. Representative spectra of the comparison of the time dependent UV-VIS spectra of the GSNO-Na_2S mixture in the nitrogen de-aerated (left) and aerated (right) samples. Absorbance spectra of 200 µmol/l GSNO before and after addition of 200 µmol/l Na_2S. The arrows indicate changes in spectral intensities at HS^--232, 270, 334 and GSNO-412 nm during 10 min incubation. Insert of UV-VIS spectrum of 200 µmol/l Na_2S and 200 µmol/l GSNO. The phosphate buffer pH 7.4 was used.

second peak at 334 nm at 7.4–9.0 pH (at pH 12.0 the last peak shifted to 328 nm). The latter peak was assigned to the sulfur-nitrogen bond linking the •NO group to the sulfur atom of a thiol (Hogg 2000) (insert in Fig. 1).

We then studied the time-dependent changes of the UV-VIS spectra after mixing 200 μmol/l GSNO with 200 μmol/l Na_2S. At 100 mmol/l sodium phosphate buffer (7.4 pH), the time dependence of the spectra in the aerated and deaerated samples was similar. In both cases, during 10 min of incubation, the peaks characteristic of HS^- at 232 nm and GSNO at 334 nm gradually diminished, whilst the ABS at 270 nm increased and a new peak at 412 nm appeared (Fig. 1). While overall patterns of change in the aerated and deaerated samples were similar, some more specific differences were observed (see the Supplementary Material, Fig. S1). When compared to deaerated samples, the rate of change was higher in the aerated samples; with ABS at 334, 270 and 232 nm changing more rapidly in the aerated ones (Fig. 2). The main difference was observed at 412 nm, where the ABS increase was higher and relatively stable for the deaerated samples, whereas it exhibited a biphasic shape for the aerated ones (Fig. 2). Isosbestic points of the deaerated sample were at 252.5, 296 and 390 nm, and of the aerated sample at 253 and 298 nm, while the point in the 390–410 nm region was not clearly resolved (Fig. S1).

The time dependence of the UV-VIS spectra of the Na_2S-GSNO in the Hepes/Tris buffer in the aerated and deaerated samples was qualitatively similar to the phosphate buffer. The time dependence of the spectra obtained at pH 6.0 (Fig. S2) was different to that at 7.4 obtained with the Hepes/Tris and phosphate buffers (Figs. 1, S1). When Na_2S was added to GSNO at pH 6.0, the peak at 230 nm assigned to HS^- and the ABS at 250–340 nm increased. At pH 6.0, the peak at 412 nm was notably absent, while the peak at 334 decreased only slightly compared to that at pH 7.4.

As expected, Na_2S increased the rate of GSNO decomposition in a concentration-dependent manner in the aerated samples (Figs. S3, S4). The peaks at around ~232 and 334 nm decreased as product(s) of GSNO decomposition at 270 and 412 nm increased.

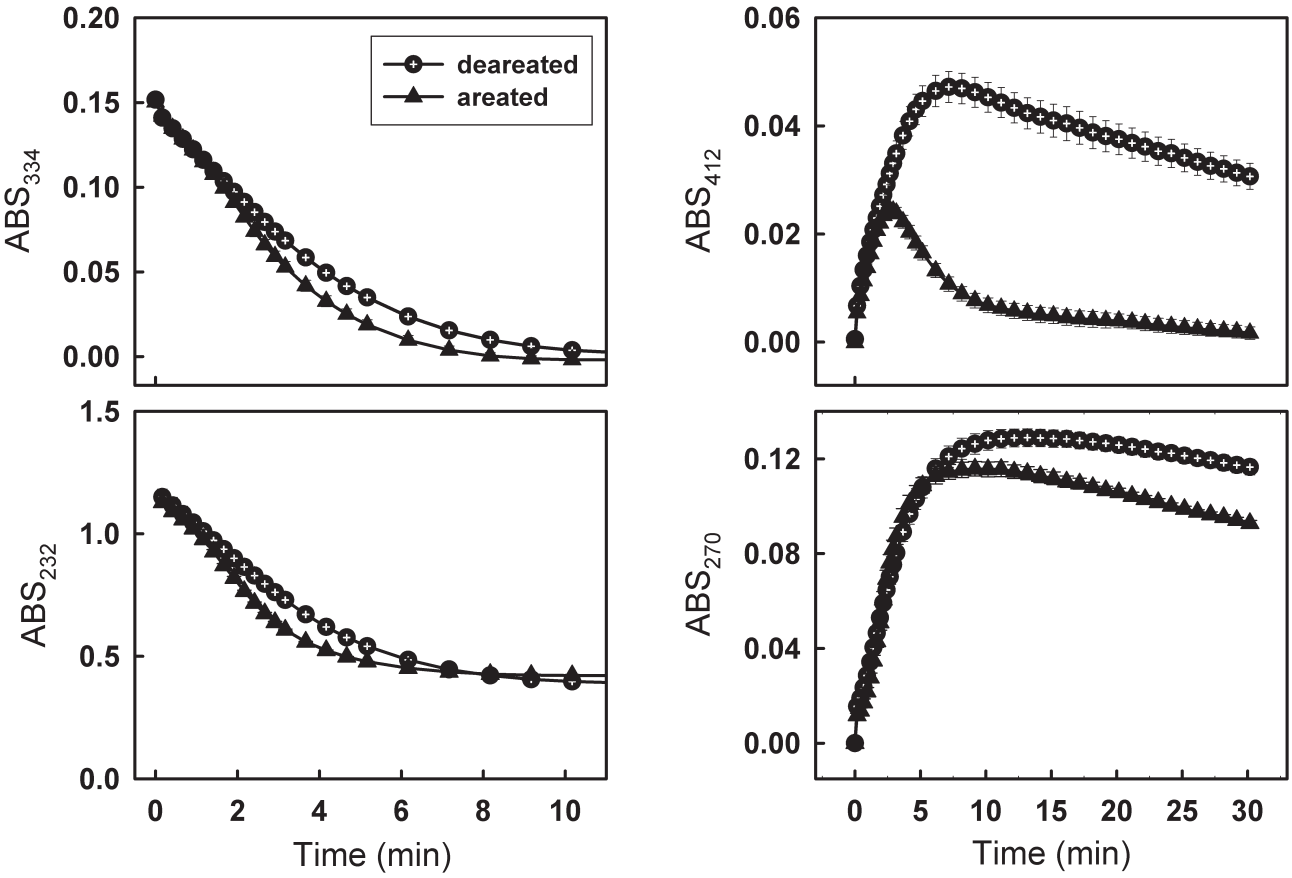

Figure 2. Time dependence of UV-VIS spectra parameters during H_2S-induced GSNO decomposition on O_2. The time dependence of ABS at 334, 412, 232 and 270 nm after mixing 200 μmol/l GSNO with 200 μmol/l Na_2S in deaerated (circles) and aerated (triangles) samples. The phosphate buffer pH 7.4 was used.

Effect of pH on the H_2S-induced GSNO decomposition

In order to better understand the observed pH effects, more detailed pH studies were performed in aerated samples. Overall, the time dependence of ABS spectra of the Na_2S-induced GSNO decomposition was complex and significantly depended on pH (Figs. 3, S5). Na_2S had a negligible effect on the GSNO decomposition at pH 4.5 and only a minor effect at pH 6.0, but it also exhibited effect at pH 12.0. The decomposition rate evaluated as the time required for 50% of the GSNO to decompose was highest at pH 7.4–8.0 and decreased at lower or higher pH values (Fig. 3). Similarly, formation of the decomposition product(s) with ABS at 270 and 412 nm significantly depended on pH (Fig. S5). The ABS at 412 nm was absent at pH 4.5, 6.0 and 6.4, but transiently increased at pH 7.0, 7.4 and 8.0 and significantly increased at pH 9.0 and 12.0 (Fig. S5). The ABS increase at 270 nm was minor at pH 4.5, and it increased and was stable at pH 6.0–8.0, and gradually, yet significantly increased at pH 9.0 and 12.0 (Fig. S5). The ABS at 232 nm did not increase at pH 4.5, 6.0 and increased only slightly at pH 6.4 after Na_2S (200 μmol/l) was added to 200 μmol/l GSNO (Fig. S5). Although the ABS at 232 nm increased at pH 7.0, 7.4, 8.0, 9.0 and 12.0 after Na_2S addition, decreases were later noted at all these pH values except for 12.0 pH, where corresponding species appeared stable (Figs. S5–S8). Therefore, after 30 min of Na_2S-GSNO incubation at pH 12.0 in phosphate buffer, HCl was added to the sample, thus decreasing the pH from 12.0 to 5.4 and resulting in the disappearance of the 412 nm peak and the increase in a broad peak around 300 nm (Figs. S7, S8). Similarly, the peak at 412 nm disappeared when Hepes/Tris buffer at pH 8.0 was used, and HCl was added to decrease the pH to 5.2. When this study of pH dependence of the GSNO decomposition was extended to other S-nitrosothiols, we found that decomposition of CysNO and NACNO followed the similar pH dependent pattern observed for the decomposition of GSNO at pH 7.4 and 6.0. The decomposition was rapid at pH 7.4 and slower at pH 6.0 (data not shown).

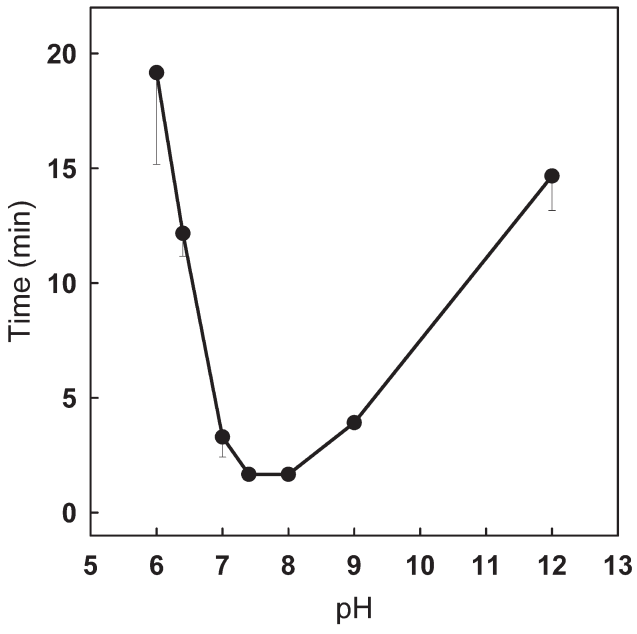

Figure 3. Time dependence of H_2S-induced GSNO decomposition on pH. The pH dependence on the time period during which ABS at 334 decreased to 50%. The phosphate buffers were used.

Effect of compounds on H_2S-induced $^{\bullet}NO$ release detected by Griess assay

NaHS (0–200 μmol/l) did not interfere *per se* with Griess assay when $NaNO_2$ was used to obtain the data for the calibration curve. Furthermore, the spontaneous release of $^{\bullet}NO$ from GSNO (100 μmol/l) was negligible in buffer at pH 7.4 (~0.6% of control). NaHS released $^{\bullet}NO$ from GSNO, and the ABS of the mixture of 100 μmol/l NaHS and 100 μmol/l GSNO in buffer at pH 7.4 was set as 100% (40 μmol/l, control). Addition of the low molecular thiols (NAC, Cys and GSH) to the NaHS/GSNO mixture in the buffer at pH 7.4 diminished the measured ABS, highlighting decreased $^{\bullet}NO$ release in a concentration-dependent manner (Fig. 4A). On the other hand, GSSG and Met did not show such an effect on the $^{\bullet}NO$ release, indicating an effect closely associated with reduced thiol groups. At the concentration of 320 μmol/l and pH 7.4, the compounds produced a significantly different effect on the release of $^{\bullet}NO$. The potency order of the compounds in inhibiting $^{\bullet}NO$ release was NAC > GHS > Cys > control = GSSG = Met (Fig. 4A). Further increase in NAC concentration (up to 3.2 mmol/l), did not have any additional inhibitory effect. Cys applied at a concentration of 3.2 mmol/l reduced $^{\bullet}NO$ release to 36.2 ± 1.2% of the control (data not shown). In comparison to the effects observed at pH 7.4, the ability of NaHS to release $^{\bullet}NO$ from GSNO (100 μmol/l) at pH 6.0 was negligible (~2% of the $^{\bullet}NO$ release at pH 7.4). While addition of Cys here promoted $^{\bullet}NO$ release in the presence of NaHS (Fig. 4B), the addition of NAC, GSH, GSSG and Met (0–320 μmol/l) had no effect.

In order to establish whether NAC was interacting directly with either GSNO or NaHS, the following procedure was used. The NO_2^- ABS of GSNO (100 μmol/l) was measured at increased concentrations of NaHS in the absence and the presence of the constant concentration of NAC (80 μmol/l). Then the ABS in the presence and the absence of NAC was divided. The ABS ratio is depicted on x-axis (Fig. 4C). The ratio increased with NaHS concentration (Fig. 4C). The NO_2^- ABS at increased concentrations of GSNO was measured at the constant concentration of NaHS (100 μmol/l) in the absence and the presence of NAC (80 μmol/l). Then the

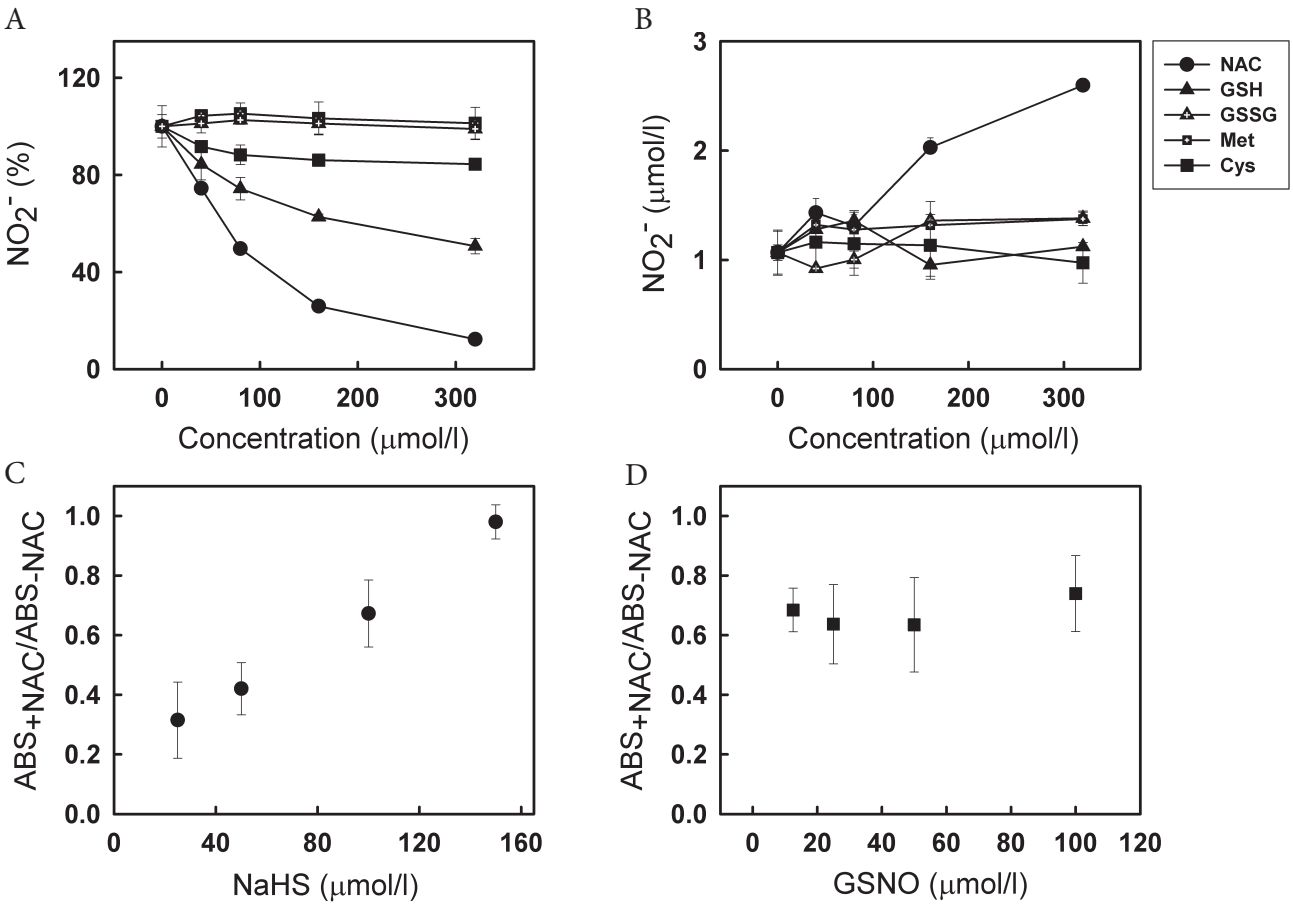

Figure 4. Concentration-dependent effect of NAC, GSH, Cys, Met and GSSG on the release of •NO from 100 μmol/l GSNO at 7.4 pH (**A**) and at 6.0 pH (**B**), induced by 100 μmol/l NaHS as indicated by the formation of NO_2^- and monitored by the Griess assay. The ABS of the mixture NAC/NaHS/GSNO (ABS_{+NAC}) expressed relative to the ABS of corresponding mixture of NaHS/GSNO without NAC (ABS_{-NAC}). The mixture was measured at constant concentrations of NAC (80 μmol/l) and GSNO (100 μmol/l) and different concentrations of NaHS (**C**), and at fixed concentrations of NaHS (100 μmol/l) and NAC (80 μmol/l) and different concentrations of GSNO (**D**).

ABS in the presence and the absence of NAC was divided. The ABS ratio is depicted on x-axis (Fig. 4D). The ratio was constant within the GSNO concentrations (Fig. 4D).

Effect of Cys on H_2S-induced GSNO decomposition

We studied the time, pH and concentration dependence of the effect of Cys, Met, NAC, GSH and GSSG on the H_2S-induced GSNO decomposition by UV-VIS spectroscopy. The compounds were studied at a concentration of 200 μmol/l, and they showed no ABS at 270, 334 or 412 nm, apart from GSSG (ABS 0.039 at 270 nm) and GSNO (peak at 334 nm). The H_2S donors NaHS or Na_2S were used. Cys, Met, NAC, GSH and GSSG alone did not trigger GSNO decomposition at pH 8.0, 7.4 and 6.0, as detected by changes at 334 and 412 nm (data not shown). Cys affected the kinetics of H_2S-induced GSNO decomposition, measured as ABS changes at both the 334 and 412 nm peaks. At pH 8.0 and 7.4, Cys decreased the rate of GSNO decomposition (Figs. 5, 6). Increasing the Cys concentration from 200 to 400 μmol/l did not further enhance the effect of Cys. The potency of Cys to decrease the rate of GSNO decomposition was higher at pH 8.0 compared to pH 7.4 (Fig. 5). On the other hand, at pH 6.0, Cys significantly increased the GSNO decomposition (Fig. 5). Although the high concentration of Cys at 5 mmol/l and 7.4 pH decreased the rate of Na_2S-induced GSNO decomposition, there was minor increase of ABS at 270 nm, but no increase was noted at 412 nm (Figs. S9, S10).

At pH 8.0, Cys delayed and significantly decreased the ABS maximum peak at 412 nm in comparison to the control value, at pH 7.4 (Fig. 6). In addition, the control ABS at 412 nm at pH 6.0 was negligible (Fig. 3), and Cys at 200

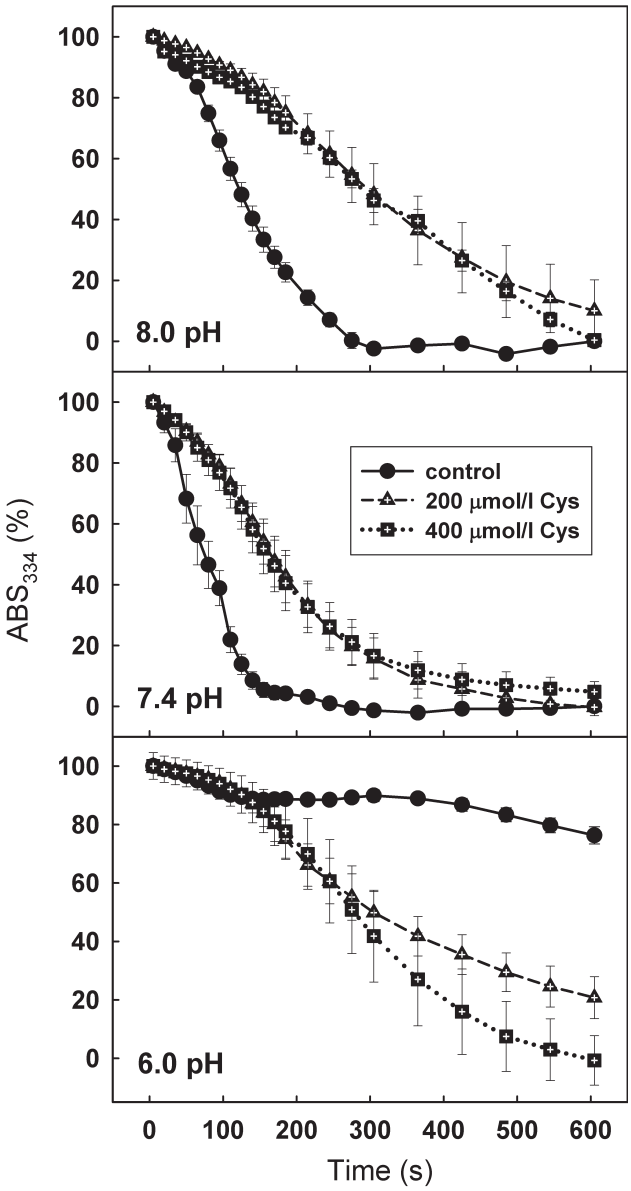

Figure 5. Time dependent effect of Cys on NaHS-induced GSNO decomposition at pH 8.0, 7.4 and 6.0 as detected by ABS at 334 nm. Control: 200 μmol/l GSNO with 200 μmol/l NaHS (full line, circles); effect of 200 μmol/l Cys (dashed line, triangles) and 400 μmol/l Cys (dotted line, squares).

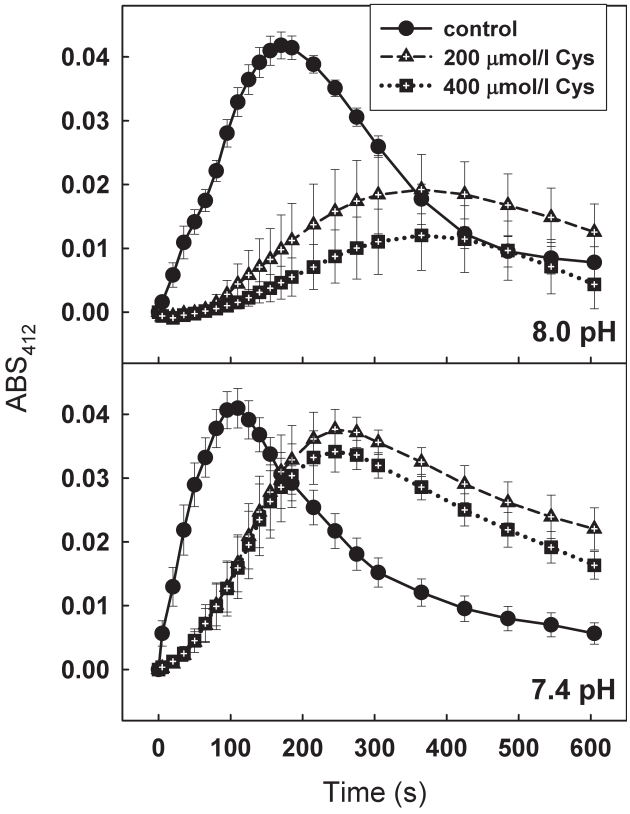

Figure 6. Time dependent effect of Cys on NaHS-induced GSNO decomposition at pH 8.0 and 7.4 as detected by ABS at 412 nm. Control: 200 μmol/l GSNO with 200 μmol/l NaHS (full line, circles); effect of 200 μmol/l Cys (dashed line, triangles) and 400 μmol/l Cys (dotted line, squares).

or 400 μmol/l did not increase the ABS at 412 nm within experimental error (data not shown).

Effect of NAC on H_2S-induced GSNO decomposition

In a similar manner to Cys, 200 and 400 μmol/l NAC affected the kinetics of H_2S-induced GSNO decomposition, as monitored by ABS at 334 and 412 nm. NAC slowed the rate of GSNO decomposition in a concentration dependent manner at both pH 7.4 and 8.0. The potency of NAC to delay GSNO decomposition was higher at pH 8.0 compared to pH 7.4, and the rate of GNSO decomposition was slower when higher NAC concentration was applied (Fig. 7). For both pH values, there was a statistically significant difference between the effect of 200 μmol/l and 400 μmol/l NAC concentration ($p < 0.0001$). In addition, at pH 6.0, NAC significantly increased •NO release relative to the control, with the statistically significant difference of $p < 0.0001$ between the 200 and 400 μmol/l concentrations. At pH 7.4 and 8.0, NAC delayed and significantly decreased the peak at 412 nm in a concentration dependent manner compared to the effect exerted by the control. Its effect was more pronounced at pH 8.0 than at pH 7.4; raising the NAC concentration further diminished the ABS at 412 nm (Fig. 8). The control ABS at 412 nm at pH 6.0 was negligible (Fig. S2), and NAC at concentration of 200 and 400 μmol/l did not increase ABS at 412 nm, within experimental error (data not shown).

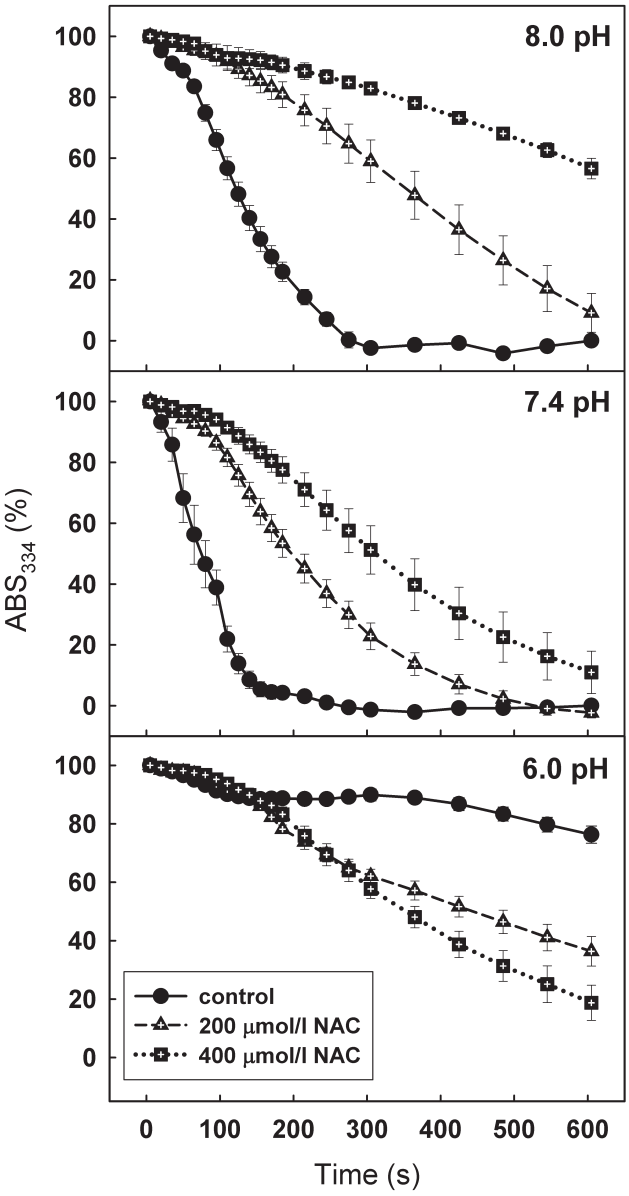

Figure 7. Time dependent effect of NAC on NaHS-induced GSNO decomposition at pH 8.0, 7.4 and 6.0 as detected by ABS at 334 nm. Control: 200 μmol/l GSNO with 200 μmol/l NaHS (full line, circles); effect of 200 μmol/l NAC (dashed line, triangles) and 400 μmol/l NAC (dotted line, squares).

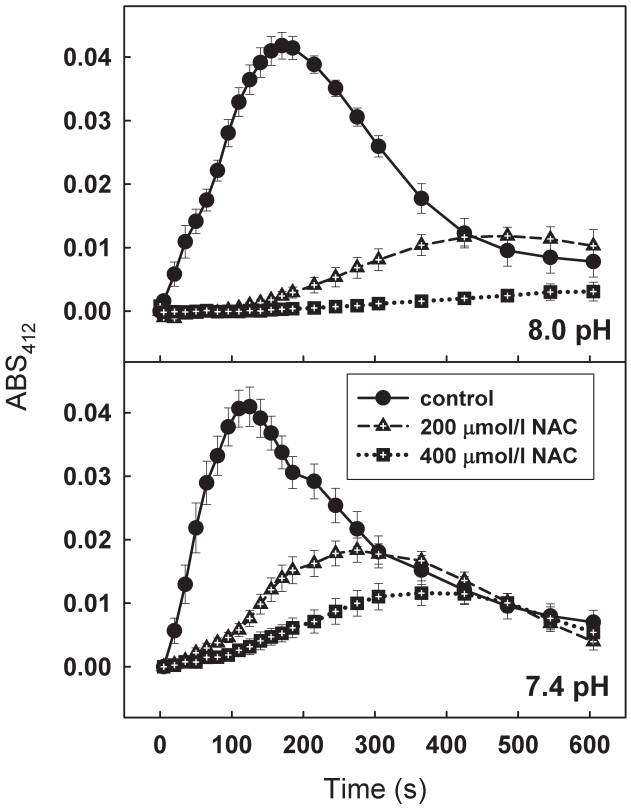

Figure 8. Time dependent effect of NAC on NaHS-induced GSNO decomposition at pH 8.0 and 7.4 as detected by ABS at 412 nm. Control: 200 μmol/l GSNO with 200 μmol/l NaHS (full line, circles); effect of 200 μmol/l NAC (dashed line, triangles) and μmol/l NAC (dotted line, squares).

Effect of GSH, GSSG and Met on H_2S-induced GSNO decomposition

In order to evaluate the particular role of the thiol group in the effects of the studied compounds on GSNO decomposition, we compared the effect of reduced and oxidized glutathione (GSH and GSSG) and Met on GSNO decomposition. The effect of GSH on GSNO decomposition, monitored as changes in ABS at 334 nm at pH 7.4 and 6.0, was similar to the effect of Cys and NAC (Fig. 9). At pH 7.4, GSH (400 μmol/l) inhibited the GSNO decomposition induced by Na_2S (GSH *vs.* control: $p < 0.0001$), and while Met had no effect, the GSSG at concentration of 400 μmol/l increased the rate of GSNO decomposition when compared to the control ($p < 0.0001$). Meanwhile, at pH 6.0, GSH enhanced GSNO decomposition compared to the control, while Met and GSSG exhibited no effect. GSH at 5 mmol/l decreased the rate of Na_2S-induced GSNO decomposition, exactly as observed with Cys at this high concentration, with concomitant minor increase of ABS at 270 nm, but no increase at 412 nm (Figs. S9, S10).

Discussion

O_2 and pH dependence of H_2S-induced GSNO decomposition

After H_2S-induced GSNO decomposition, •NO has been detected by the electron paramagnetic resonance spectroscopy

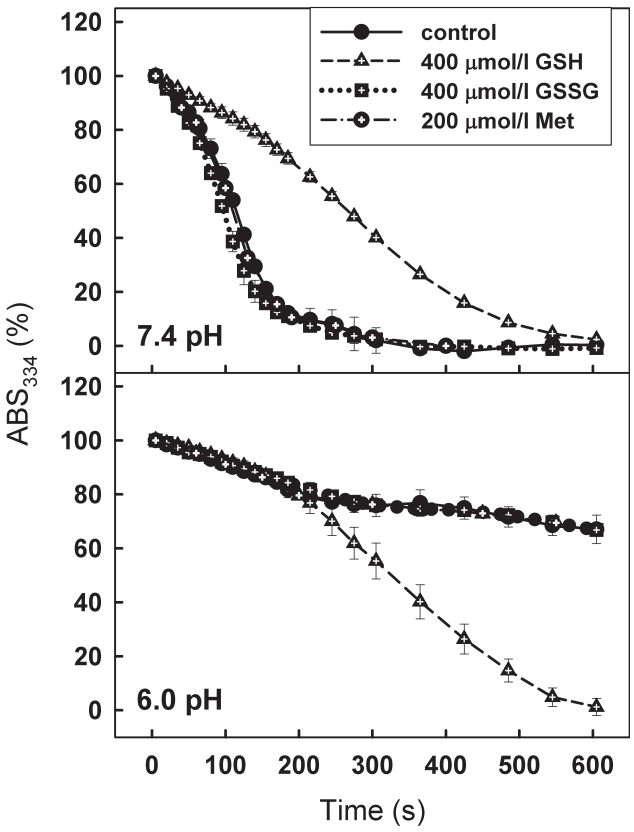

Figure 9. Time dependent effect of GSH, GSSG and Met on Na_2S-induced GSNO decomposition at pH 7.4 and 6.0 as detected by ABS at 334 nm. Control: 200 μmol/l GSNO with 200 μmol/l Na_2S (full line, circles); effect of 400 μmol/l GSH (dashed line, full triangles), 400 μmol/l GSSG (dotted line, squares) and 200 μmol/l Met (dash-dotted line, open triangles).

of spin trap, Griess assay and chemiluminescence methods (Ondrias et al. 2008; Teng et al. 2008). We suppose that Na_2S-induced GSNO decomposition observed in our study by UV-VIS spectroscopy produced •NO or its derivatives.

The absorption maximum at 334 nm, characteristic of GSNO, decreased over time in the presence of NaHS and Na_2S H_2S donors. This agrees with the recent study (Filipovic et al. 2012b), and it is attributed to the decomposition of GSNO (Munro and Williams 2000; Manoj et al. 2006; Okado-Matsumoto and Fridovich 2007). Results obtained were relatively similar for both Hepes/Tris and phosphate buffers. From the similarities of the time dependent ABS spectra of the H_2S-GSNO reaction in both aerated and deaerated samples, we conclude that O_2 is not necessary for GSNO decomposition (Figs. 1, 2), although the decomposition rate decreased in the deaerated samples. Similarly, Singh et al. reported that GSH excess at 0.5–10 mmol/l caused decomposition of 1 mM GSNO during 5–90 min of incubation at pH 7.4 (Singh et al. 1996). In the absence of O_2, the rate of GSNO decomposition in the presence of GSH decreased by a factor of two. The increased rate of GSNO decomposition and the pronounced effect on the product appearing with an ABS at 412 nm in the aerated sample indicates that O_2 modulates the GSNO-H_2S reaction(s), but ultimately, it does not result in different end-products. This conclusion is supported by comparison of the isosbestic points. The deaerated samples had three isosbestic points in comparison to the aerated samples, where the points at ~400 nm were "broadened" and not clearly resolved (Figs. 1, S1). The three isosbestic points observed during the time dependent reaction of H_2S with GSNO in the deaerated samples indicate that there are only two species in varying concentrations which contribute to the ABS around this isosbestic point. NaHS or Na_2S at 200 μmol/l do not have ABS > 260 nm, and although 200 μmol/l GSNO has a peak at 334 nm, there is no other peak at ABS > 270 nm. Therefore, we may conclude that deaerated samples have one reaction product with ABS at 230–330 and 412 nm, or alternatively there are two products, one at each of these peaks. In our study, the absolute values and kinetics of ABS at 412 nm depended on O_2, and this could indicate connection of the second product with O_2. Among these possible chemical products, for instance, one may postulate the transient presence of a highly reactive inorganic di- or polysulfide (S_x^{2-}, $x \geq 2$), formed by the oxygen-driven oxidation of S^{2-}, which then reacts with the GSNO. Alternatively, an oxidized sulfur- and/or nitrogen species may be involved in initial release from GSNO, which is then further transformed to result in •NO.

The ABS at 232 nm reflects the concentration of the hydrogen sulfide anion HS^- (Guenther et al. 2001). This decreased to zero during the GSNO decomposition, indicating that HS^- was consumed during the GSNO decomposition. The decrease in HS^- qualitatively correlated with the decomposition of GSNO and the product(s) formation at ~230–330 and 412 nm. This is partly supported by our pH studies. H_2S is a weak acid in water and it dissociates to $HS^- + H^+$ and traces of S^{2-}. The pK_{a1} and pK_{a2} values at 20°C are 7.04 and 11.96, respectively (Staško et al. 2009). This implies that at pH 4.5 and 6.0, the HS^- concentration is low, leading to minor GSNO decomposition. At approximately pH 7, the H_2S-driven GSNO decomposition is highest, in line with the notion of H_2S de-protonation and HS^- formation. However, at pH > 7.0, although the concentration of HS^- increases, the rate of GSNO decomposition does not increase and at pH > 8.0 this release even decreases (Fig. 3). This indicates that other or additional chemical mechanism(s) besides the simple de-protonation of H_2S may be involved in GSNO decomposition at pH > 7.0. It is possible, for instance, that the initial release involves NO^-, and that this transient species is no longer removed rapidly from the equilibrium, for example by protonation to HNO or by oxidation to •NO, at

higher pH. Since a significant amount of S^{2-} is present at pH 12.0, we suggest that S^{2-} may play a role in the slow •NO rate release at this pH (Fig. 3).

The decrease in ABS at 232 nm was paralleled by the appearance of an ABS at 230–330 and 412 nm (Figs. 1–3, S1, S4). Since the ABS at 412 nm was not observed during the decomposition of GSNO by one-electron reduction (Manoj et al. 2006), we assume that the ABS at 412 nm did not result from the decomposition of GSNO itself, but from the interaction of GSNO, or its decomposition products, with H_2S/HS^-. Filipovic and co-workers reported $HSNO_2$ (thionitrate), predominantly present as sulfinyl nitrite, HS(O)NO, as the reaction product of H_2S with peroxynitrite, which had two absorption maxima at 289 and 408 nm (Filipovic et al. 2012a). However, our product from the H_2S-GSNO reaction had no significant ABS peak at 289 nm (Figs. 1, S1). An ABS peak at 410 nm was reported after the interaction of •NO or GSNO with Na_2S, NaSH or Na_2S_2, and this was thought to be from the nitrosodisulfide (or perthionitrite) ion $ONSS^-$ (Seel and Wagner 1988; Munro and Williams 2000). The recent study of Filipovic et al. (Filipovic et al. 2012b), however, observed that the ABS at 412 nm resulted predominantly from the mixture of polysulfides, and they confirmed the presence of $HSNO/SNO^-$ (ABS at 340 nm) in aqueous solution at pH 7.4. In contrast, the ABS < 340 nm may be due to the formation of GSSH or polysulfides (Linkous et al. 2004; Francoleon et al. 2011). Mixing GSSG and Na_2S at pH 7.4 resulted in absorbance with maximum around 280 nm, which was presumed to be for GSSH (Francoleon et al. 2011). When NaOH was added, the 280 nm peak disappeared and a new absorbance with a maximum at 340 nm was detected, which suggested a persulfide anion absorbing at this wavelength. Similarly, a broad ABS of S_2^{2-} at ~< 340 nm after photochemical oxidation of aqueous Na_2S was observed (Linkous et al. 2004). From these reports and our findings, we speculate that increased ABS in the ~230–330 nm range resulted from GSSH, S_2^{2-} or polysulfides formed during the GSNO decomposition.

The unknown product with ABS at 412 nm seems to be absent at pH 4.5, 6.0 and 6.4, yet its presence is apparent at pH ≥7.0, and pronouncedly increased at pH 12.0 (Fig. 3, S5). The ABS at 412 nm diminished instantaneously when solutions of pH 8.0 and 12.0 were acidified to pH < 6.0 (Fig. S8). It is therefore most likely that this product with ABS at 412 nm constitutes either an anion, or an extremely acid sensitive compound. It is possible that this species is either NO^- or a transient, reactive polysulfide intermediate (RS_x^- or HS_x^-, x ≥ 2). Since pH dependence of H_2S-induced decomposition of CysNO and NACNO was qualitatively similar to that for GSNO, this indicates that the pH dependence observed for GSNO may be generally valid for nitrosocompounds.

Our working hypothesis postulates the following reaction scheme which appears to explain most of our observations, including the pH dependence of the reactions and the disappearance of one of the products upon acidification.

a) strictly anaerobic pathway

$$RSNO + HS^- \rightarrow RSSH + NO^- \quad (1)$$

$$NO^- + Ox \rightarrow {}^\bullet NO + Red \quad (2)$$

$$2\ RSSH \rightarrow RSH, RSSR, H_2S_x, S_x \ldots. \quad (3)$$

b) additional or alternative pathways in the presence of O_2:

$$4\ HS^- + O_2 \rightarrow 2\ HSS^- + 2\ HO^- \quad (4)$$

$$RSNO + HSS^- \rightarrow RSSSH + NO^- \quad (5)$$

$$NO^- + Ox \rightarrow {}^\bullet NO + Red \quad (2)$$

$$2\ RSSSH \rightarrow RSH, RSSR, H_2S_x, S_x \ldots. \quad (6)$$

Effect of Cys, NAC, GSH, GSSH and Met on H_2S-induced GSNO decomposition

Based on our initial working hypothesis, we then studied the influence of thiols, sulfides and disulfides on H_2S-induced GSNO decomposition. These studies were conducted with two goals in mind. Firstly, we aimed to better understand the possible control of •NO release by these thiols, and secondly, we wanted to test our proposed reaction scheme in Eq. 1 to Eq. 6, in an additional scenario.

Using both the Griess assay and UV-VIS spectroscopy, we observed qualitatively similar results of the effects of compounds on H_2S-induced GSNO decomposition or •NO release from GSNO. Cys, NAC and GSH decreased the rate of the H_2S-induced GSNO decomposition at pH 7.4 and 8.0 and activated the rate of GSNO decomposition at pH 6.0. The potencies of the compounds depended on the particular thiol used. •NO release increased with the concentration of H_2S donors at constant GSNO/NAC ratio, but the release was constant when the GSNO concentration increased at constant GSNO/NaHS ratio (Fig. 4C and D). This result could indicate that NAC decreased the •NO release because less H_2S was available to interact with GSNO, and thus less •NO was released. We can assume that the NAC chemically interacts with H_2S, most likely with the free thiol group, and hence decreases effective HS^- concentration available for the interaction with GSNO, as we suggested for the inhibition effect of asolectin (Tomaskova et al. 2009). We can assume that a similar mechanism is also valid for the inhibitory effects of CYS and GSH. This assumption is supported by our reported results that pre-treatment of serum albumin, Cys,

NAC and lipids with H_2S results in sulfur binding to these molecules, and these modified sulfur compounds release •NO from nitroso-compounds directly and/or through reliberated H_2S (Bertova et al. 2010). Our observation that 400 μmol/l Cys was not more efficient in decreasing GSNO decomposition than 200 μmol/l Cys (Fig. 5) may indicate nitrosylation of Cys, whereby the resulting product of CysNO shows an ABS at 334 nm. Cys, NAC and GSH decreased the ABS at 412 nm (Figs. 6, 8). There are two alternative explanations for this finding. On the one hand, it is possible that thiols prevent the formation of this particular product, or on the other hand, there may be a direct interaction of the thiols or their derivatives with the products(s) absorbing at 412 nm. This latter explanation could apply to species such as polysulfides which are almost certainly present in this sequence of reactions (see Eq. 1, 3, 4, 5, 6) (Filipovic et al. 2012b). Nonetheless, the chemical nature of the transient and final products and also the exact sequence of transformations, remain unknown.

The influence of Cys, NAC and GSH on H_2S-induced GSNO decomposition was pH dependent. It differed at pH 6.0 from the effects observed at pH 7.4. At pH 6.0 the compounds increased the rate of GSNO decomposition in the presence of NaHS or Na_2S (Figs. 5, 7, 9). The molecular mechanism of this effect is not known. Since Met and GSSG have no RSH group and they also did not decrease GSNO decomposition at 7.4 or increase it at 6.0 pH (Fig. 9), we assume that the thiol group is essential to trigger these effects. In fact, it is possible that such thiol groups interfere with the interaction of HS^- and GSNO, or prevent or reverse the formation of per- and polysulfanes and sulfides (RS_x^- or HS_x^-, $x \geq 2$). These latter may be formed as transient reactive intermediates, thus explaining the role of O_2 in this process, and they could also be attacked, for example, by reduction by thiols. Since such intermediates are not affected by Met and GSSG, their involvement would explain a number of key observations.

Biological significance

H_2S, •NO, O_2, Cys, NAC, Met, GSH and GSSG constitute common endogenous compounds involved in many biological functions, in which their mutual chemical and biochemical interactions play a significant role. Our *in vitro* study presents evidence of the mutual interactions of these compounds resulting in the modulation of H_2S-induced GSNO decomposition and •NO release from nitroso compounds. These results support a hypothesis of a "coupled sulphide-nitroso signalling pathway" (Bertova et al. 2010). In spite of the fact that the steady-state free H_2S concetrations *in vivo* are less than 20 nmol/l (Furne et al. 2008), its storage forms, such as bound sulfane sulfur and acid-labile sulfide, may be critical in regulating H_2S availability under different intracellular conditions (Ishigami et al. 2009). It was shown that the free H_2S and bound sulfane sulfur pools in plasma from healthy human volunteers was in a nanomolar range but that the acid-labile pool was in the low-micromolar range. The results supported the theory of a reversible sulfide sink into and from which H_2S can be stored or released to affect biological functions (Ogasawara et al. 1994; Ishigami et al. 2009; Shen et al. 2012). Plasma nitrosothiol levels are in the range of 0.5–2 μmol/l (Massy et al. 2003). We used H_2S and GSNO concentrations several times higher than they exist *in vivo.* Since local and transient concentrations of H_2S and S-nitrosothiols *in situ* are unknown, however, the biological relevance of our *in vitro* results is still a challenge.

There are several observations which may indicate that the mutual interactions observed in our study may play a role *in vivo*. H_2S shares many biological effects with •NO. These include vasorelaxant (Hosoki et al. 1997), antiinflammatory (Zanardo et al. 2006), cardioprotective (Sivarajah et al. 2006), antiproliferation (Du et al. 2004) and erectile (Srilatha et al. 2007) functions, which may result from the ability of H_2S to release •NO *in vivo*. H_2S was found to influence several dysfunctions (e.g. ischemia/reperfusion, smooth muscle tonus, mitochondria function) in which low pH, oxygen conditions, and/or thiol status play a role (Endoh 2001; Singh et al. 2011; Wang 2012). From biological experiments, chemical interaction between H_2S and •NO or •NO-donors was suggested (Whiteman et al. 2006; Ondrias et al. 2008; Teng et al. 2008; Bertova et al. 2010; Yong et al. 2010; Coletta et al. 2012; Filipovic et al. 2012b). Cys, NAC and GSH diminished the effects of a mixture of •NO or L-arginine and H_2S donors on myocyte contraction (Yong et al. 2010, 2011). Endogenous and exogenous thiols are often used in studies involving •NO and/or H_2S signaling pathways (Stamler 1994; Ellis et al. 2000; Gaston et al. 2006; Duan and Chen 2007; Li et al. 2009; Yong et al. 2010, 2011; Kasparek et al. 2012). Our results may suggest that in these studies, a possible modulation of H_2S-induced •NO release from nitroso-compounds by thiols might be considered. Since S-nitrosothiols act as bio-reservoirs for •NO *in vivo* (Stamler et al. 1992), we suggest that H_2S may act to release •NO from such S-nitrosothiols *in vivo*.

In conclusion, we present evidence of the involvement of O_2, pH and low molecular thiols in a mechanism of H_2S-induced •NO release from nitroso compounds, where the interaction of H_2S and/or its derivatives with thiol groups may be responsible for the release effect. We hypothesize that an involvement of low molecular thiols and pH in •NO signaling may be due in part to interactions with H_2S or with some of its transient reaction products (such as polysulfides). Ultimately, the results obtained as part of this study may contribute to a better understanding of the mutual biological effects of H_2S, nitrosocompounds, •NO, O_2, pH and thiols.

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Supplementary Material

Low molecular thiols, pH and O_2 modulate H_2S-induced S-nitrosoglutathione decomposition - •NO release

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Supplementary Figures

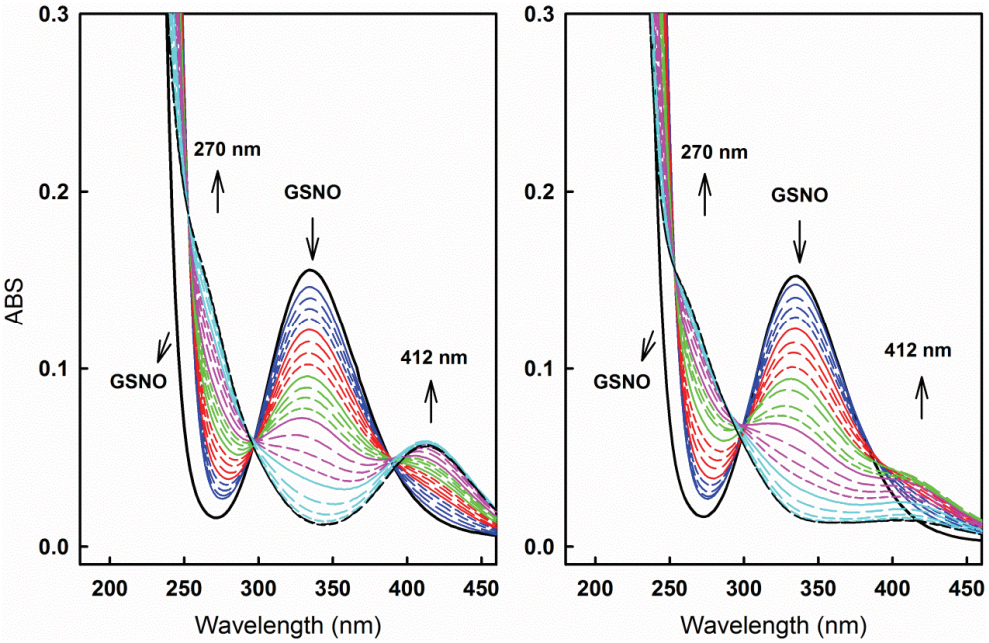

Figure S1. Representative spectra of the comparison of the time dependent UV-VIS spectra of the S-nitrosoglutathione (GSNO)-Na_2S mixture in the nitrogen deaerated (left) and aerated (right) samples. Absorbance spectra of 200 μmol/l GSNO before (black line marked by GSNO) and after addition of 200 μmol/l Na_2S. The arrows indicate changes of the spectral intensities at 270, GSNO-334 and 412 nm during 10 min incubation. The 100 mmol/l sodium phosphate, pH 7.4 buffer was used.

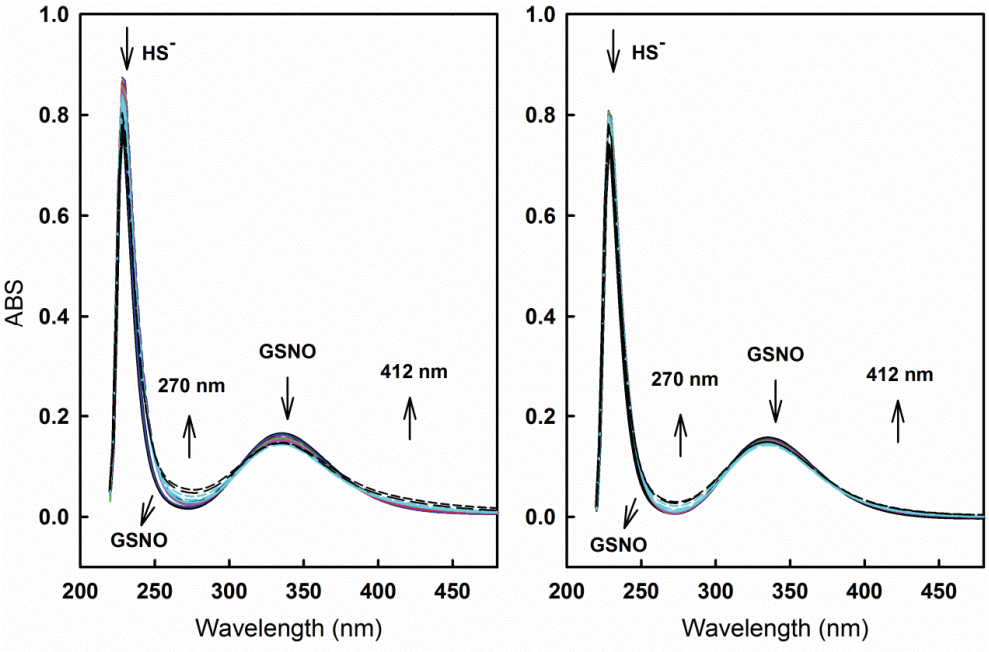

Figure S2. Representative spectra of the comparison of the time dependent UV-VIS spectra of the S-nitrosoglutathione (GSNO)-Na_2S mixture in the nitrogen deaerated (left) and aerated (right) samples. Absorbance spectra of 200 µmol/l GSNO before (black line marked by GSNO) and after addition of 200 µmol/l Na_2S. The arrows indicate changes of the spectral intensities at HS^--232, 270 and GSNO-334 nm during 10 min incubation. The 50/25 mmol/l Pipes/Tris buffer pH 6.0 was used.

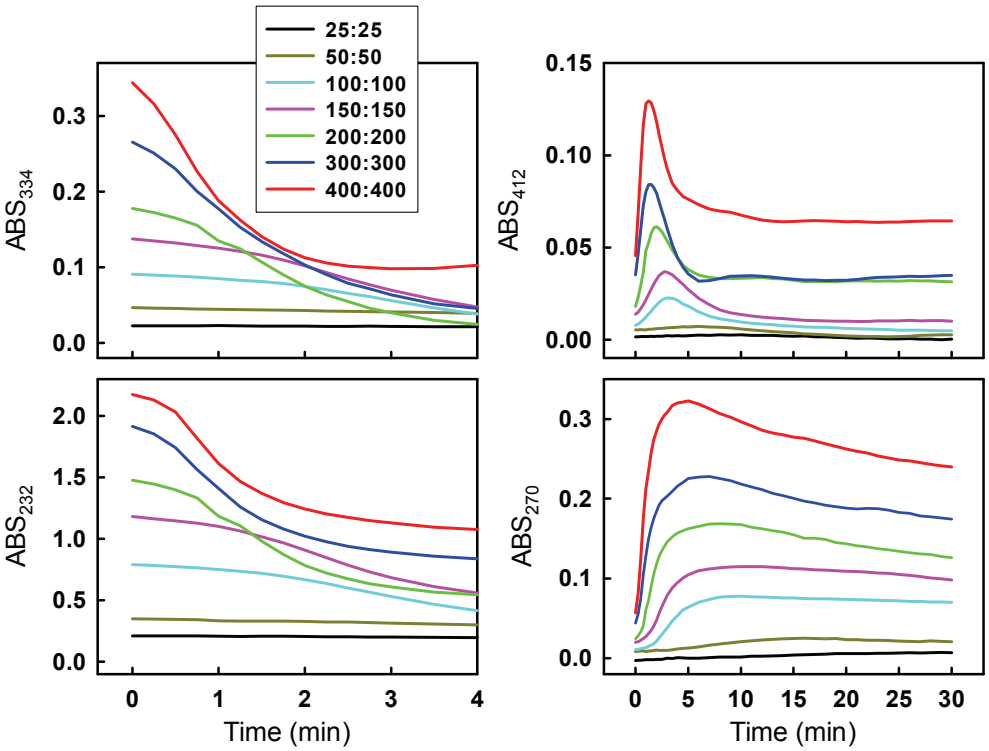

Figure S3. The time and concentration dependence of absorbance (ABS) at 334, 412, 232 and 270 nm after mixing of S-nitrosoglutathione (GSNO):Na_2S at the equal molar concentrations of 25 (black), 50 (dark yellow), 100 (cyan), 150 (pink), 200 (green), 300 (blue) and 400 µmol/l (red). The 100 mmol/l phosphate buffer pH 7.4 was used.

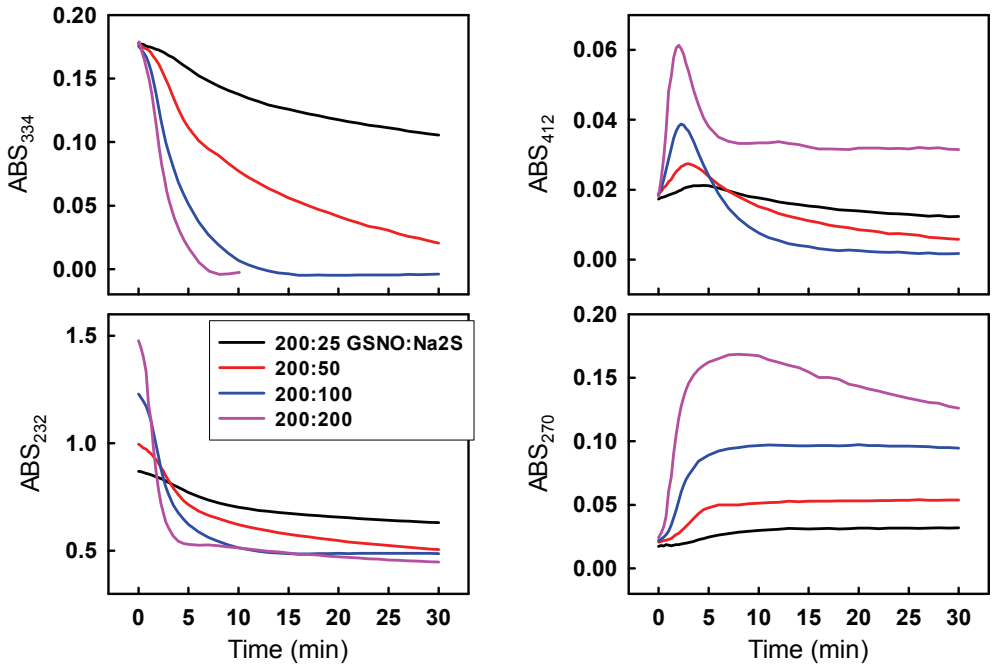

Figure S4. The time and concentration dependence of absorbance (ABS) at 334, 412, 232 and 270 nm after mixing of 200 μmol/l S-nitrosoglutathione (GSNO) with 25 (black), 50 (red), 100 (blue) and 200 μmol/l (pink) Na_2S. The 100 mmol/l phosphate buffer pH 7.4 was used.

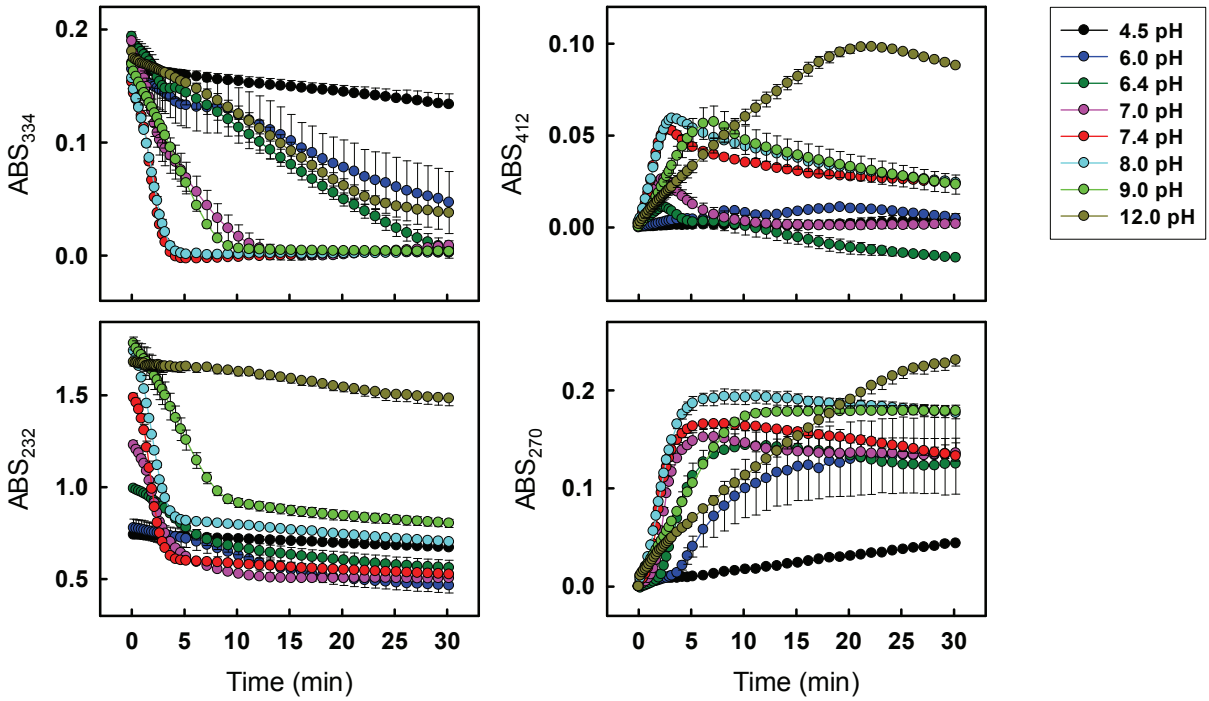

Figure S5. The time dependence of Na_2S induced S-nitrosoglutathione (GSNO) decomposition on pH. The time and pH dependence of absorbance (ABS) at 334, 412, 232 and 270 nm after mixing of GSNO:Na_2S at the equal molar concentrations (200 μmol/l) at 4.5 (black), 6.0 (blue), 6.4 (dark green), 7.0 (pink), 7.4 (red), 8.0 (cyan), 9.0 (green) and 12.0 (dark yellow) pH. The 100 mmol/l phosphate buffers were used.

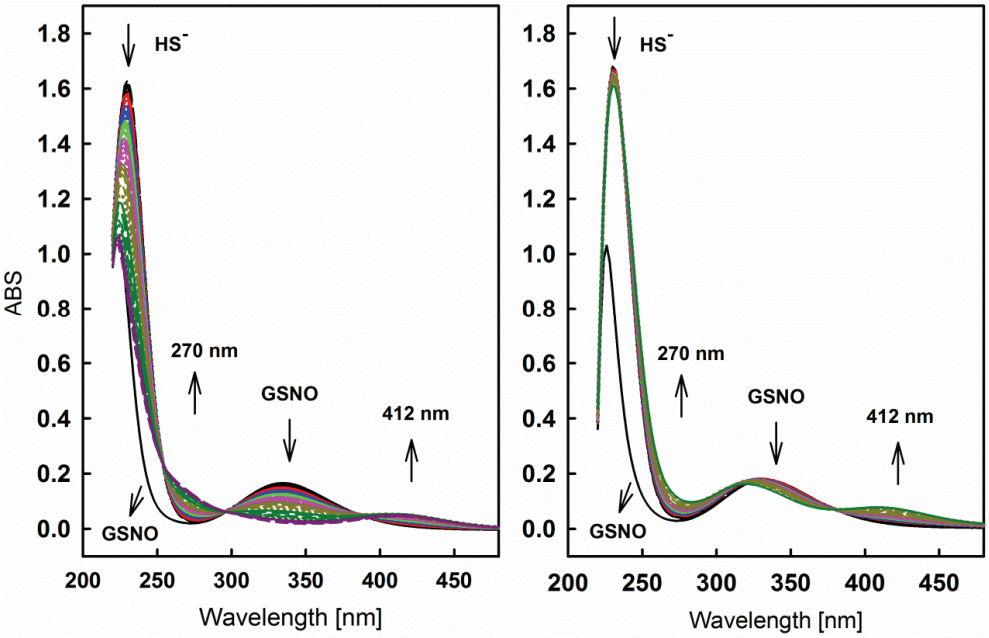

Figure S6. Representative spectra of the comparison of the time dependent UV-VIS spectra of the S-nitrosoglutathione (GSNO)-Na_2S mixture at pH 9.0 (left) and 12.0 (right). Absorbance spectra of 200 μmol/l GSNO before (black line marked by GSNO) and after addition of 200 μmol/l Na_2S. The arrows indicate changes of the spectral intensities at HS^--232, 270, GSNO-334 and 412 nm during 10 min incubation. The 100 mmol/l phosphate buffers at pH 9.0 and 12.0 were used.

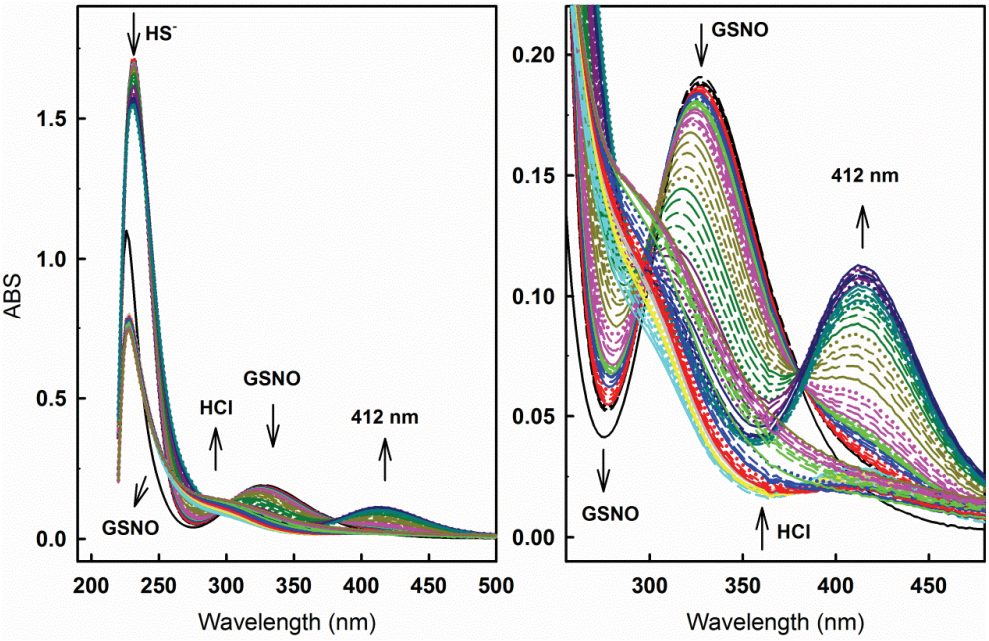

Figure S7. Representative spectra of the comparison of the time dependent UV-VIS spectra of the S-nitrosoglutathione (GSNO)-Na_2S mixture at pH 12.0 and after addition of HCl (pH decreased to pH 5.4), full spectra (left) and details (right). Absorbance spectra of 200 μmol/l GSNO before (black line marked by GSNO) and after addition of 200 μmol/l Na_2S. The arrows indicate changes of the spectral intensities at HS^--232, GSNO-334 and 412 nm at 12.0 pH for 30 min. The HCl-arrows at 290 nm (left) and 360 nm (right) indicate changes of the spectra after addition of HCl (the pH dropped from 12.0 to 5.4). The 100 mmol/l phosphate buffer pH 12.0 was used.

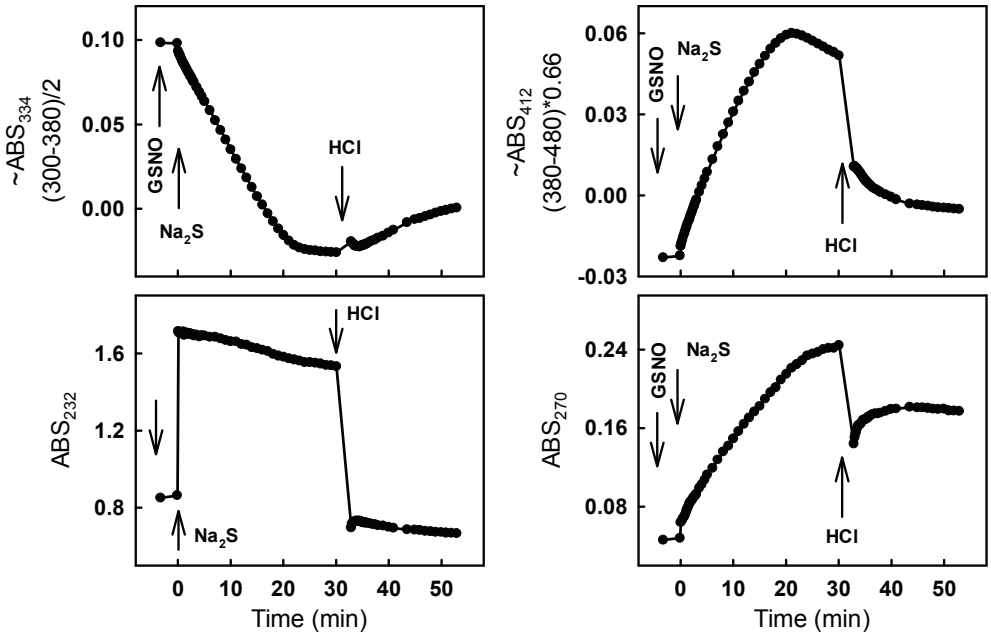

Figure S8. The time dependence of absorbance (ABS) at ~334, ~412, 232 and 270 nm after mixing of 200 µmol/l Na_2S with 200 µmol/l GSNO at pH 12.0 100 mmol/l phosphate buffer, and addition of HCl, which decreased pH to 5.4.

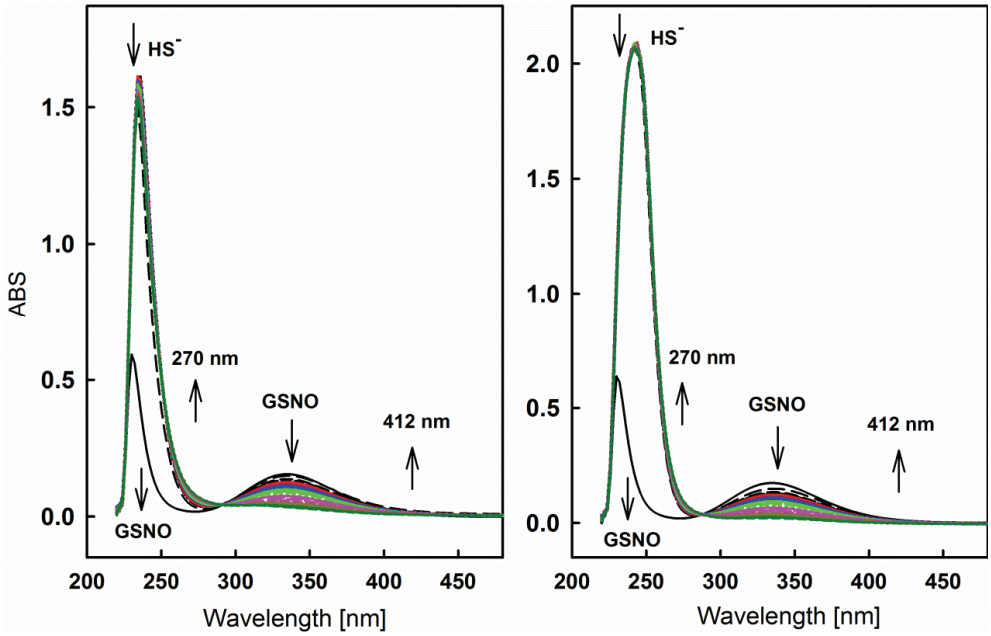

Figure S9. Representative spectra of the comparison of the time dependent UV-VIS spectra of the S-nitrosoglutathione (GSNO) (200 µmol/l) with Na_2S (200 µmol/l) at the presence of 5 mmol/l GSH (left) or 5 mmol/l Cys (right). Absorbance spectra of 200 µmol/l GSNO before (black line marked by GSNO) and after addition of GSH or Cys, and Na_2S. The arrows indicate changes of the spectral intensities at HS^--232, 270, GSNO-334 and 412 nm during 20 min incubation. The 100 mmol/l phosphate buffer pH 7.4 was used.

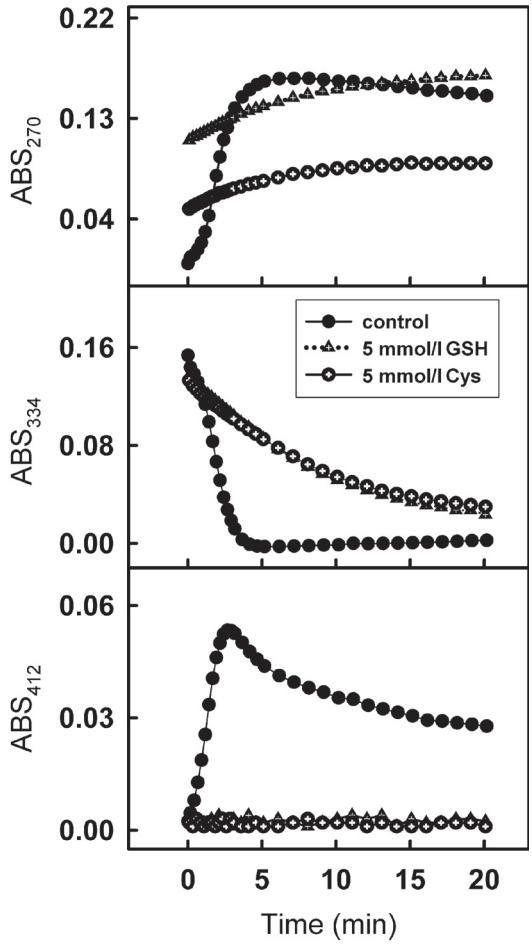

Figure S10. The time dependence of absorbance (ABS) at 270, 334, and 412 nm of 200 μmol/l S-nitrosoglutathione (GSNO) with 200 μmol/l Na_2S in the absence (full circles) and in the presence of 5 mmol/l L-glutathione (GSH) (triangles) and 5 mmol/l L-cysteine (Cys) (open circles). The 100 mmol/l phosphate buffer pH 7.4 was used.