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CAPTOPRIL PARTIALLY DECREASES THE EFFECT OF H_2S ON RAT BLOOD PRESSURE AND INHIBITS H_2S-INDUCED NITRIC OXIDE RELEASE FROM S-NITROSOGLUTATHIONE

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Short title: H_2S and captopril and on blood pressure

Summary

We studied the effects of the H_2S donor Na_2S on the mean arterial blood pressure (MAP) and heart and breathing rates of anaesthetised Wistar rats in the presence and absence of captopril. Bolus administration of Na_2S (1-4 μmol/kg) into the right jugular vein transiently decreased heart and increased breathing rates; at 8-30 μmol/kg, Na_2S had a biphasic effect, transiently decreasing and increasing MAP, while transiently decreasing heart rate and increasing and decreasing breathing rate. These results may indicate independent mechanisms by which H_2S influences MAP and heart and breathing rates. The effect of Na_2S in decreasing MAP was less pronounced in the presence of captopril (2 μmol/l), which may indicate that the renin-angiotensin system is partially involved in the Na_2S effect. Captopril decreased H_2S-induced NO release from S-nitrosoglutathione, which may be related to some biological activities of H_2S. These results contribute to the understanding of the effects of H_2S on the cardiovascular system.

Keywords: H_2S, nitric oxide, captopril, blood pressure, heart rate

Introduction

The H_2S endogenously produced in mammals affects many of their physiological and pathological functions including the modulation of the NO signalling pathway and cardiovascular system. H_2S has been reported to influence vascular smooth muscle, myocardial ischemia, ischemia/reperfusion injury, pre-conditioning and post-conditioning (rewieved in Liu *et al.* 2012, Tomaskova *et al.* 2011, Wang 2012). These findings are currently facilitating detailed research into the molecular mechanisms of H_2S interactions to exploit its positive effects in the treatment of several diseases (Predmore *et al.* 2010).

Captopril (CAP), an angiotensin-converting enzyme inhibitor that influences cardiovascular function, is widely used in the treatment of cardiovascular diseases like hypertension, inhibiting the progression of atherosclerosis and reducing mortality in congestive cardiac failure (Aberg and Ferrer 1990, Napoli *et al.* 2004, Repova-Bednarova *et al.* 2013, Hrenak *et al.* 2013, Bencze *et al.* 2013). Because H_2S has been reported to inhibit renin synthesis and release, we studied whether this inhibition is important to the mechanism

of H_2S effects on blood pressure. Therefore, we studied the effects of H_2S on blood pressure in the presence and absence of CAP.

S-nitrosoglutathione (GSNO) is the carrier and/or storage form of NO *in vivo* and thus serves as a reservoir for NO bioactivity (Stamler 1994). Several factors, including the H_2S donor Na_2S, modulate GSNO decomposition resulting in the formation of NO and/or NO-derivatives (Filipovic *et al.* 2012, Ondrias *et al.* 2008, Grman *et al.* 2013, Cortese-Krott *et al.* 2014). These molecules have several biological effects, including smooth muscle relaxation and decrease blood pressure (reviewed in Cacanyiova 2011, Liu *et al.* 2012, Tomaskova et al. 2011, Wang 2012, Cortese-Krott *et al.* 2014). In our previous study, we observed that low molecular thiols, such as cysteine, N-acetylcysteine or glutathione, modulate H_2S induced NO release from GSNO (Grman *et al.* 2013). Because CAP also has a thiol moiety and interferes with the NO pathway (Pecháňová 2007, Zandifar *et al.* 2012, Zhang *et al.* 2012), we were interested in determining whether CAP influences H_2S-induced NO release from GSNO and could potentially be involved in H_2S-NO interactions.

Methods

Chemicals

Na_2S was obtained from Alfa Aesar (England). The anaesthetics, Narkamon and Rometar, were from Zentiva (Czech Republic), and Zoletil 100 was from Virbac (France). All other chemicals were purchased from Sigma-Aldrich. Na_2S was used as a H_2S donor that dissociates in solution and reacts 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-}.

MAP, heart and breathing rate measurements

All procedures were approved by the State Veterinary and Food Administration of the Slovak Republic. For the experiments with Na_2S, 240±20 g Wistar rats were used, and for the experiments where Na_2S and CAP were applied, 400±30 g Wistar rats were used. The Wistar rats were anaesthetised with Zoletil 100 at a dose of 4.0 mg/100 g body weight (b.w.), and Rometar (Xylazine) was used at a dose of 0.5 mg/100 g b.w. administered intra-muscularly (i.m.). The right jugular vein was prepared and cannulated, and heparin sulphate (25 IU) was administered immediately. The right carotid artery was prepared, cannulated and connected to a Statham P32 Db pressure transducer. After stabilisation of MAP within 15-25 min, Na_2S or CAP dissolved in 100 μl physiological solution was prepared and injected into the jugular vein over a 10-second period. Stock solutions of CAP (20 mmol/l) and Na_2S (100 mmol/l)

were prepared only at the time of measurement and used within a few hours. The recorded analogue signal, low-pass-filter at 1 kHz, was digitalised at 5 kHz (USB-6221, National Instrument, USA) and stored on a PC computer using the DeweSoft 6.6.7 programme for data acquisition and further analysis. Heart rate was evaluated using a fast Fourier transform (FFT) of the digitalised data. From the digitalised data, the frequency of ~70 Hz of FFT was assigned to the breathing rate. The data represent the means ± S.E.M. A paired *t-test* was used to determine the significance of the effect of Na_2S, where $P<0.05$ was considered statistically significant.

Vasoactivity measurements

In *ex-vivo* experiments, the isolated rings of the thoracic aorta were prepared from male Wistar rats and mounted to record isometric tension changes in pneumoxid-oxygenated Krebs–bicarbonate solution as in our previous study (Ondrias et al. 2008). The basal tension was set to 1 g before the addition of the drug. The effects of relaxants were given relative to the difference of tension induced by 1 μmol/l noradrenaline and the original tension. This difference was set at 100%. The precontracted rings were first relaxed by a single addition of GSNO (250 nmol/l) or acetylcholine (30 nmol/l) for 4 min. Then, agents were removed by washing for 20 min. The aorta was precontracted again by 1 μmol/l noradrenaline, Na_2S (40 μmol/l) was applied, and GSNO or acetylcholine were added after 3 min. We also evaluated the involvement of K_{ATP} channel activation (smooth muscle hyperpolarisation) in the effects of Na_2S. The noradrenaline-precontracted rings were first relaxed by the cumulative addition of Na_2S (20, 40, 80, 100 μmol/l). Then, agents were removed by washing for 20 min, the K_{ATP} channel inhibitor glibenclamide (100 μmol/l) was applied 10 min before the repeated addition of noradrenaline and cumulative addition of Na_2S. The vasoactive effects of Na_2S were evaluated as the changes in noradrenaline-induced tone before and after application of glibenclamide (g). The data represent the means ± S.E.M. A paired *t-test* was used to determine the significance of the effect of Na_2S.

CAP and H_2S induced GSNO decomposition

To study the effect of CAP on H_2S-induced NO release from GSNO, the Griess assay and UV-VIS spectrometry were used essentially as described in our previous studies (Ondrias *et al.* 2008, Grman *et al.* 2013). GSNO (100 μmol/l), Na_2S (100 μmol/l) and CAP (100-800 μmol/l) were mixed in buffer (in mmol/l) of 160 KCl, 1 MgCl2, 0.1 diethylenetriaminepentaacetic acid (DTPA), 50/25 HEPES/Tris, at pH 7.4 and 23±1°C, and incubated for 10 min. The Griess reagent was then added to quantify the NO oxidation

product, nitrite (NO_2^-) by absorption (ABS) at 540 nm. To study the effects of CAP on the time dependence of H_2S-induced GSNO decomposition/NO release, CAP (100-400 μmol/l) and GSNO (200 μmol/l) were included in the buffer (in mmol/l), 100 sodium phosphate, 0.01 DTPA, at 7.4 pH, and after adding H_2S (200 μmol/l), the kinetics of the GSNO decomposition/NO release were measured using UV-VIS spectrophotometry for 30 minutes at 23±1°C. The NO release from GSNO was signified by a decrease in absorbance at 334 nm and by the formation of an unknown product at 412 nm, as in our previous study.

Results

Na_2S treatment transiently increased breathing rate and decreased heart rate at 3 μmol/kg. At 8-30 μmol/kg, Na_2S biphasically influenced the breathing rate, transiently decreased the heart rate, transiently decreased MAP for ≤30 s and increased MAP for ≤120 s (Fig. 2).

To study the involvement of the renin-angiotensin system in the effects of Na_2S, we compared the effect of 8 and 16 μmol/kg Na_2S on MAP and heart and breathing rates before, during and after CAP application (2 μmol/kg) (Fig. 3). An example of the time-dependent changes observed is shown in Fig. 3A,B,C. Before CAP application, Na_2S exerted a biphasic effect on MAP and breathing rates and transiently decreased heart rate. CAP decreased MAP, but did not significantly influence heart or breathing rates. Na_2S was less effective in decreasing MAP in the presence of CAP, but its effect on the heart and breathing rates did not produce a significant difference from their rates in the absence of CAP (Fig. 2D,E,F). Acetylcholine (0.1 or 1 μmol/kg) in the presence of CAP additionally decreased MAP (data not shown). When the effect of CAP wore-off and MAP returned to the control MAP condition, the Na_2S effect was similar to that prior to the CAP application (Fig. 3).

The effects of H_2S on smooth muscle tonus were studied by changes in the vasoactivity of the thoracic aorta. The application of the NO donor GSNO (250 nmol/l) or the NO-synthase activator acetylcholine (30 nmol/l) induced the vasorelaxant effect caused by the ability of noradrenaline to precontract the thoracic aorta. Acute pretreatment with Na_2S (40 μmol/l) significantly increased the vasorelaxant effect of GSNO; however, it did not affect the vasorelaxation induced by acetylcholine (Fig. 4A). The application of increasing doses of Na_2S on the noradrenaline-precontracted thoracic aorta induced a biphasic effect: the lower concentrations (20, 40 μmol/l) induced vasoconstriction (Fig. 4B), and the higher concentrations (80, 100 μmol/l) induced vasorelaxation (Fig. 4B). Acute pretreatment with the K_{ATP} channel inhibitor glibenclamide (100 μmol/l) did not affect the vasoconstrictor effect induced by lower Na_2S doses (20 and 40 μmol/l); however, it inhibited the vasorelaxant effect

induced by higher doses of Na_2S (8 and 100 μmol/l), and vasoconstriction was observed instead of vasorelaxation (Fig. 4B).

We studied whether CAP can interfere with the H_2S-induced NO release from GSNO. Two approaches were applied. As detected by the Griess assay, 100 μmol/l Na_2S (but not CAP alone at 100-800 μmol/l) released NO from GSNO (100 μmol/l). However, increased CAP concentrations (100-800 μmol/l) decreased the NO release induced by Na_2S (Fig. 5A). Similar results were obtained using UV-VIS measurement of time dependent Na_2S-induced NO release (ABS at 334 nm). The rate of Na_2S-induced NO release from GSNO decreased in the presence of increased CAP concentrations (100-400 μmol/l) at pH 7.4 (Fig. 5B). The rate decrease indicates an inhibition of H_2S-induced NO release. The kinetics and amount of unknown product formation detected at 412 nm were also inhibited by CAP (Fig. 5C, 7.4 pH). Additionally, the rate of Na_2S-induced NO release was slow at pH 6.0, but CAP (100-400 μmol/l) increased the rate of NO release from GSNO (Fig. 5D).

Discussion

The transient and biphasic effects of H_2S on MAP

In our study, we observed that effects of the H_2S donor Na_2S on blood pressure, heart and breathing rates were transient. Considering that a 250 g rat has a blood volume of approximately 17 ml and a cardiac output of approximately 17 ml in 10 seconds, the bolus application of 3, 15 and 30 μmol/kg for 10 seconds delivered 44, 220 and 441 μmol/l Na_2S in the blood, respectively. This treatment is in the range of Na_2S concentrations that release NO from GSNO (Fig. 5). We presume that free H_2S was rapidly eliminated from the rat's blood resulting in transient H_2S effects.

Intravenously injected Na_2S at 8-30 μmol/kg exerted a biphasic transient effect on blood pressure. The K_{ATP} channel has been reported to be a major molecular target of the vasorelaxant and vasodepressor effects of H_2S (Zhao *et al.* 2001). We confirmed this finding using glibenclamide, a K_{ATP} channel inhibitor that significantly inhibited the vasorelaxation induced by higher doses of H_2S (Fig. 4B). Because the transient increase in MAP was observed at lower H_2S concentration than the biphasic effects, we assume that K_{ATP} channels are not involved in the transient MAP increase. Moreover, in *ex vivo* experiments, we showed that the acute pretreatment with glibenclamide did not affect the contractile responses induced by lower doses of H_2S (Fig. 4B).

It our previous studies, we showed that H_2S causes the release of NO from NO donors and that the effects of NO donors on aortic ring relaxation were enhanced in the presence of

H_2S donors (Ondrias *et al.* 2008, Bertova et al. 2010). In the present study, the H_2S-induced MAP decrease was observed at transient Na_2S blood concentrations, which released NO from GSNO (Fig. 5). Moreover, we confirmed that Na_2S releases NO from the NO donor in *ex vivo* experiments because it significantly augmented the vasorelaxant effect of GSNO (Fig. 4A). However, pretreatment with Na_2S did not affect the vasorelaxant effect of acetylcholine, an NO-synthase activator. Therefore, we assume that H_2S increases NO concentrations *in situ* by releasing NO from endogenous NO donors and not by the activation of the NO-synthase pathway. Thus, the ability of H_2S to decrease MAP may include K_{ATP} channels and NO release from endogenous NO donors.

Biphasic responses of MAP to H_2S could be associated with a sympathetic reflex response, at least the increased phase of the MAP, in accordance with reports for several vasoactive substances such as endothelin, urotensin and apelin (King *et al.* 1990, Gardiner *et al.* 2004, Charles *et al.* 2006). This finding is also in agreement with those of Gines et al. (1994), who suggested the sympathetic reflex response issued from the pressure receptor stimulation after detecting aortic hypotension after intravenous acetylcholine injection. The increase of MAP and reduced heart rate observed in our study (Fig. 2) indicate that baroreceptors are also involved in the H_2S effects. Because baroreceptors include channels and H_2S was found to influence membrane channels, we could suppose that H_2S influences baroreceptors through the membrane channels (Chapleau et al. 2007, Malekova *et al.* 2009, Peers *et al.* 2012).

The effect of H_2S on heart and breathing rate

We observed that H_2S (3-30 μmol/kg) applied i.v. transiently decreased the heart rate. This finding is supported by the observation of a negative chronotropic effect of H_2S, where the effect of H_2S on membrane channels of pacemaker cells in SA nodes was implicated. In our study, the Na_2S-induced transient decrease in heart rate was accompanied by a decrease and increase in MAP; therefore, we assume that the influence of H_2S on MAP and heart rate results from different H_2S targets and different molecular mechanisms.

We observed that intravenous H_2S influences breathing rate. Our results are supported by the observation that intraperitoneal or peripheral injection of NaSH affected breathing (Kombian *et al.* 1988, Almeida and Guidotti 1999) and that inhalation of H_2S-induced a suspended animation-like state in house mice, which included a sharp decrease in the animal's breathing rate, from 120 to 10 breaths/min (Blackstone *et al.* 2005). Because H_2S was found to influence membrane channels involved in membrane excitability and electrical signal

transmission (Malekova *et al.* 2009, Peers *et al.* 2012), we assume that the effect of H_2S on these channels is involved in its effect on heart and breathing rates.

Effect of H_2S in the presence of CAP

We observed that H_2S activity on MAP was partially decreased in the presence of CAP. Because acetylcholine additionally decreased MAP in the presence of CAP, we assume that a change in the MAP baseline after CAP addition is not responsible for the weaker H_2S effect. The effect of H_2S on MAP in the presence of CAP can be explained by the reported H_2S-inhibition of renin synthesis and release (Lu et al., 2010). However, because the effect of H_2S was reduced but not blocked by CAP, we assume that the renin-angiotensin system was only partially involved. Moreover, the associations among H_2S, the baroreflex mechanism and the autonomic nervous system should be taken into account. Increases in the angiotensin II level in the central nervous system have been shown to affect arterial baroreflex control and increase sympathetic outflow (Gao et al. 2005). Because administration of CAP results in the inhibition of angiotensin II synthesis, this compound could mask the possible partial baroreflex-mediated effects of H_2S.

The inhibitory effects of CAP on H_2S-induced NO release from GSNO were similar to other low molecular thiols, such as cysteine, N-acetylcysteine or glutathione, thereby indicating that the thiol group of CAP is quite important for this effect (Grman *et al.* 2013). If we assume that transient i.v. H_2S concentrations release NO from endogenous NO-donors and decreases MAP, then the inhibition of NO release should lessen the decrease of MAP. Because CAP inhibits the H_2S-induced NO release from GSNO at pH 7.4 *in vitro*, we hypothesise that this effect may also contribute to the lessened MAP decrease. Interestingly, the influence of CAP on H_2S-induced NO release was reversed at pH 6.0 versus pH 7.4. At pH 6.0, CAP increased the NO release from GSNO in the presence of Na_2S. This may indicate a possible pH-dependent influence of CAP on sulfide-nitroso signalling pathways (Tomaskova *et al.* 2011, Grman et al. 2013). This idea could be supported by the observation that CAP interferes with the NO pathway (Pecháňová 2007, Zandifar *et al.* 2012, Zhang *et al.* 2012). Its thiol group was found to be important to the prevention of spontaneous hypertension (Pecháňová 2007). In addition to its efficacy as an angiotensin-converting enzyme inhibitor, CAP has several other biological functions. For example, the following have been reported: CAP protects against cystamine-induced duodenal ulcers (Saghaei *et al.* 2012), inhibits cell degeneration and growth (Nagano 2006), scavenges reactive oxygen molecules (Goldschmidt and Tallarida 1991), has renoprotective effects in doxorubicin-induced nephrotoxicity (Hrenák *et al.* 2013), influences mitochondrial ATP production in

spontaneously hypertensive rats (Mujkošová *et al.* 2010), and affects potassium and L-type calcium channels (Alvin *et al.* 2011a and 2011b). The diversity of CAP's biological effects have not been fully elucidated. We can assume that some of CAP's numerous biological effects could result from its pH-dependent effects on the H_2S-induced NO release from nitroso-compounds.

Conclusion

The i.v. administration of the H_2S donor Na_2S exerted a transient biphasic effect on MAP, transiently decreased heart rate and influenced breathing rate in anesthetised rats. In addition to activation of K_{ATP} channels, the transient decrease in MAP has been suggested to partially result from the H_2S influence on the renin-angiotensin system and by releasing NO from endogenous NO donors. CAP inhibited the H_2S-induced NO release from GSNO *in vitro*, which could be included in its numerous biological effects. These results contribute to the understanding of the biological effects of H_2S in the cardiovascular system.

Conflict of interest

There is no conflict of interest to declare.

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Figures

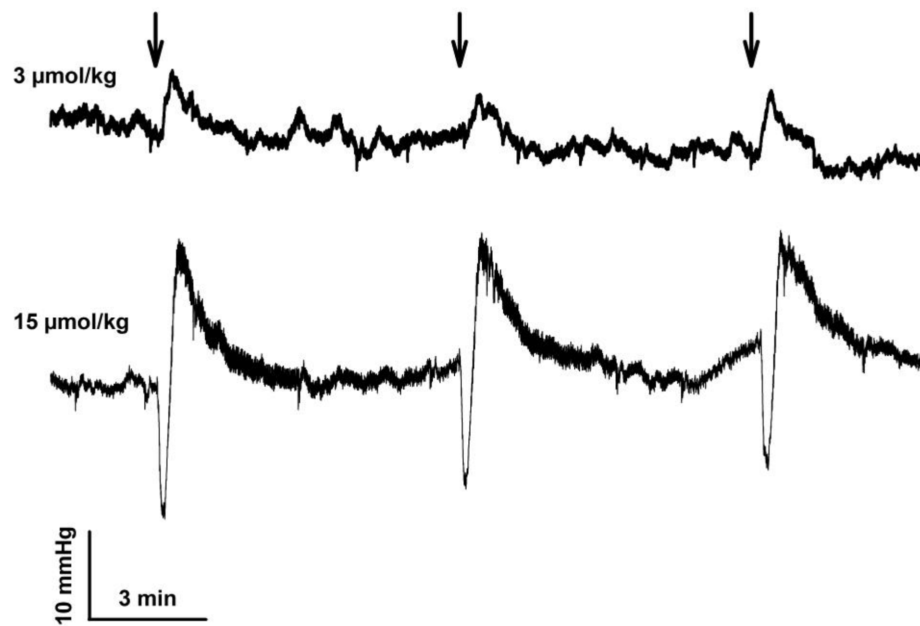

Figure 1

Representative traces of the time-dependent effect of the repeated i.v. application of Na_2S on MAP of the anaesthetised rat. The arrows indicate i.v. application of Na_2S at 3 μmol/kg (upper trace) and 15 μmol/kg (lower trace).

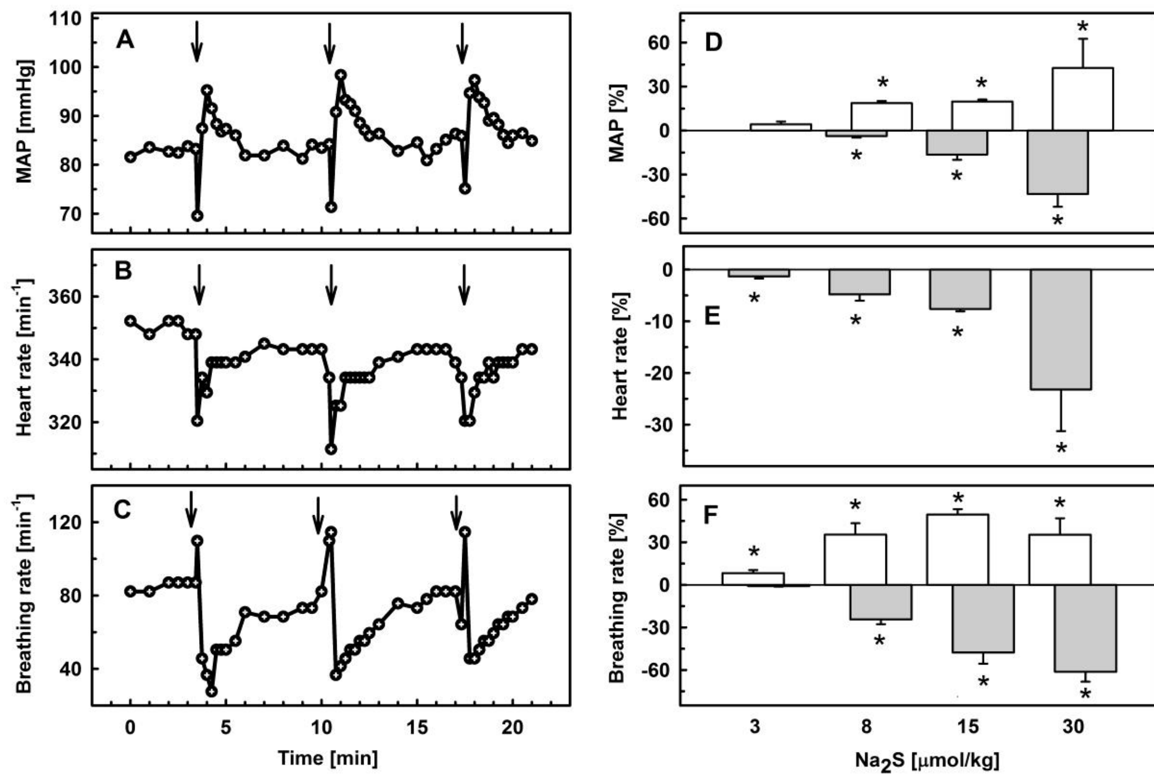

Figure 2

Time-dependent effect of the repeated i.v. application of 15 µmol/kg Na_2S on the MAP (A), heart (B) and breathing rates (C) of the anaesthetised rat. The arrows indicate i.v. application of Na_2S. The concentration-dependent effect of Na_2S on the MAP (D), heart (E) and breathing rates (F) of the anaesthetised rat. The control MAP, heart and breathing rates were 82±2 mm Hg, 298±12 min^{-1}, and 77±2 min^{-1}, respectively. Error bars represent the S.E.M. (n=4-8; * $p < 0.05$ vs. Control).

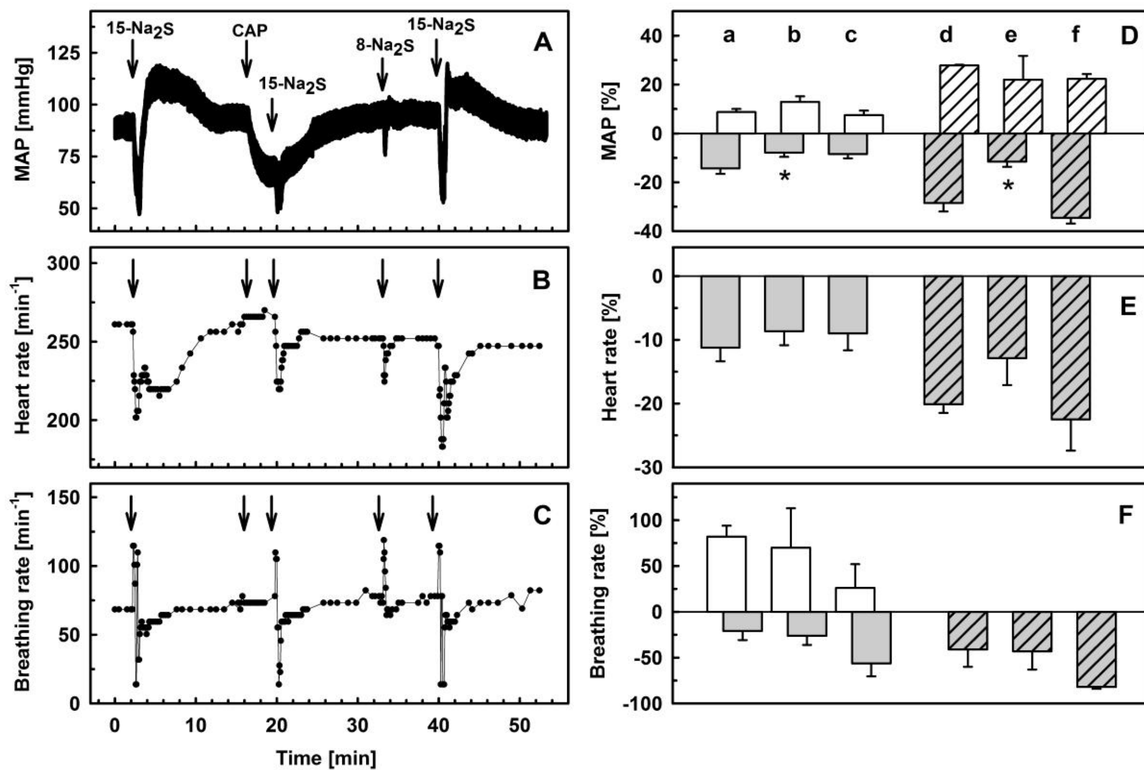

Figure 3

The time-dependent effects of the sequence of application of Na_2S, CAP, Na_2S in the presence of CAP, and Na_2S on MAP (A, trace), heart (B) and breathing (C) rates. The arrows indicate i.v. application of the drugs. The effects of Na_2S (a, d), Na_2S in the presence of CAP (b, e) and Na_2S after CAP (c, f) on MAP (D), heart (E) and breathing (F) rates. 2 µmol/l CAP; 8 µmol/l Na_2S (open columns); 15 µmol/l Na_2S (hatched columns). The control MAP, heart and breathing rates were 98±3 mm Hg, 271±4 min^{-1} and 74±7.5 min^{-1}, respectively. The error bars represent the S.E.M. (n=4-8 at 8 µmol/kg; n=3 at 15 µmol/kg; * $p < 0.05$ vs. Control).

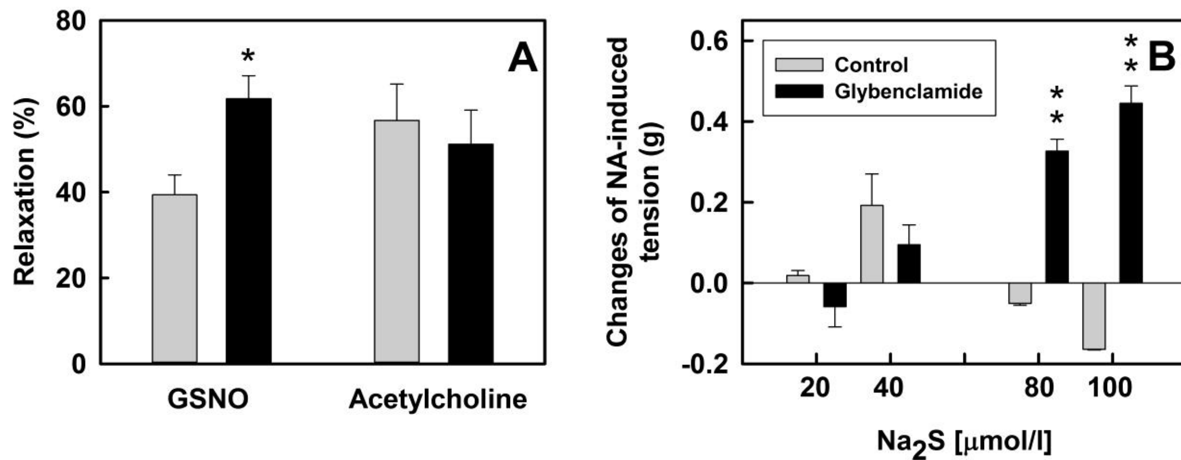

Figure 4.

The vasoactive effects of GSNO (250 nmol/l) and acetylcholine (30 nmol/l) on noradrenaline (1 μmol/l) precontracted aortic rings before (grey) and after (black) the pretreatment with Na_2S (40 μmol/l) (A). The vasoactive effects of Na_2S on noradrenaline (1 μmol/l) precontracted aortic rings before (grey) and after (black) acute pretreatment with glibenclamide (100 μmol/l) (B). The data are expressed as the mean ± SEM (* $p < 0.05$ and ** $p < 0.01$ vs. Control).

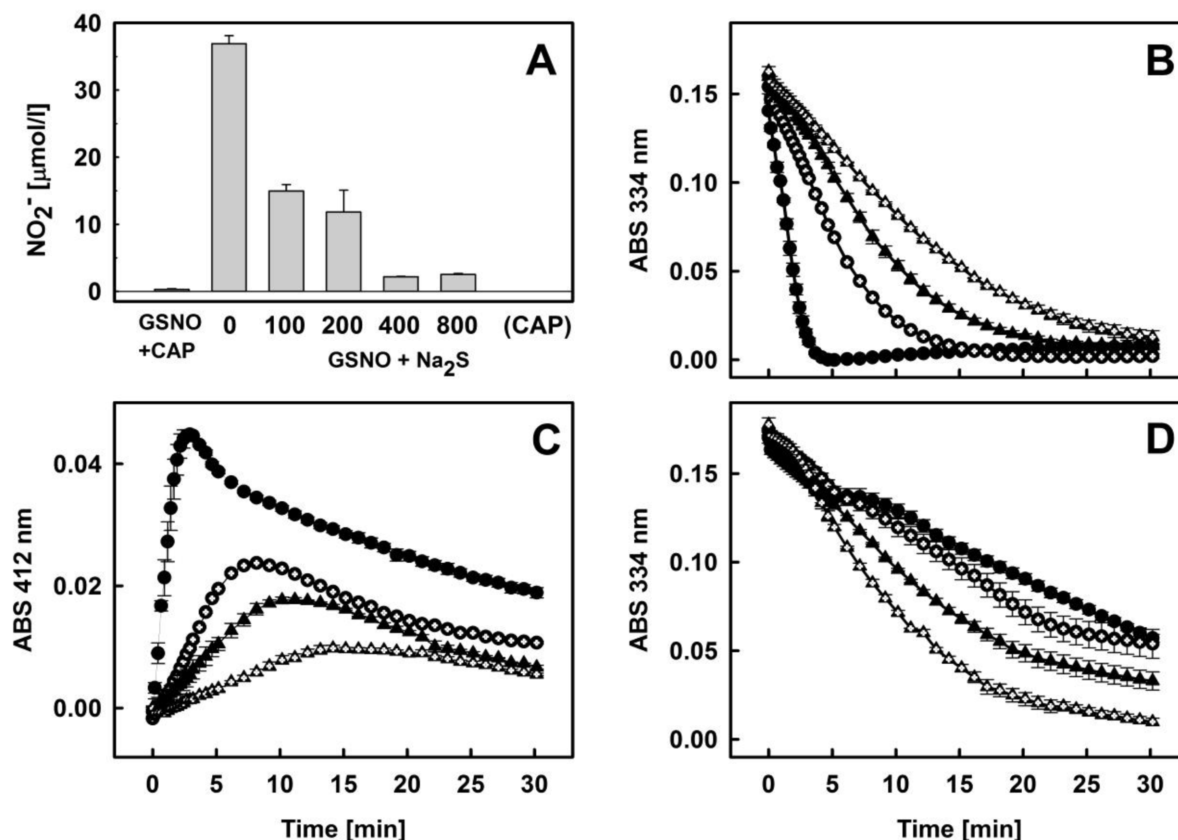

Figure 5.

Concentration-dependent effect of CAP on the release of NO from 100 µmol/l GSNO at pH 7.4 induced by 100 µmol/l Na_2S as indicated by the formation of NO_2^- and monitored by the Griess assay (A). The concentration-dependent effect of CAP (100, 200 and 400 µmol/l) on the time-dependent effect of Na_2S (200 µmol/l) induced GSNO (200 µmol/l) decomposition at pH 7.4, as detected with ABS at 334 nm (B) and 412 nm (C). ABS at 334 nm measured at the samples at pH 6.0 (D). Control 200 µmol/l Na_2S + 200 µmol/l GSNO (full circles), and in the presence of CAP: 100 µmol/l (open circles), 200 µmol/l (full triangles) and 400 µmol/l (open triangles).