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Evidence for the formation of a novel nitrosothiol from the gaseous mediators nitric oxide and hydrogen sulphide ☆

Matthew Whiteman [a,*], Ling Li [b], Iouri Kostetski [c], Siew Hwa Chu [a], Jia Ling Siau [a], Madhav Bhatia [b], Philip K. Moore [b]

[a] *Cardiovascular Biology Research Group, Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Republic of Singapore 117597*

[b] *Cardiovascular Biology Research Group, Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Republic of Singapore 117597*

[c] *Division of Bioengineering, Faculty of Engineering, National University of Singapore, Republic of Singapore 119260*

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Abstract

The gaseous mediators hydrogen sulphide (H_2S) and nitric oxide (·NO) are synthesised in the body from L-cysteine and L-arginine, respectively. In the cardiovascular system, ·NO is an important regulator of vascular tone and its over- or under-production has been linked to a variety of diseases. The physiological significance of H_2S is not yet clear but, like ·NO, it exhibits vasodilator activity and may play a part in septic and haemorrhagic shock, hypertension, regulation of cardiac contractility, and in inflammation. To date, there have been no reports of a chemical interaction between H_2S and ·NO. Here we show that incubation of the H_2S donor, sodium hydrosulphide, with a range of ·NO donors and ·NO gas in vitro leads to the formation of a nitrosothiol molecule as determined by a combination of techniques; electron paramagnetic resonance, amperometry, and measurement of nitrite. We further show that this nitrosothiol did not induce cGMP accumulation in cultured RAW264.7 cells unless ·NO was released with Cu^{2+}. Finally, using liver homogenates from LPS treated rats we present evidence for the endogenous formation of this nitrosothiol. These findings provide the first evidence for the formation of a novel nitrosothiol generated by reaction between H_2S and ·NO. We propose that generation of this nitrosothiol in the body may regulate the physiological effects of both ·NO and H_2S.

Keywords: Reactive oxygen species; Hydrogen sulphide; Nitric oxide; Peroxynitrite; Cysthionine-β-synthase; Cystathionine-γ-synthase

H_2S is a well-known and pungent toxic gas and its toxicology has been extensively studied (thoroughly reviewed in [1]). It is produced endogenously in mammalian tissues from the amino acids cysteine and homocysteine by pyridoxal-5′-phosphate-dependent enzymes such as cystathionine-γ-lyase (CSE; E.C. 4.4.1.1) and cystathionine-β-synthetase (CBS; E.C. 4.2.1.22) (reviewed in [2,3]). CBS converts homocysteine to cystathionine and hydrolyses cysteine to equimolar amounts of serine and H_2S whereas CSE converts cystathionine to cysteine yielding pyruvate, NH_3, and H_2S. H_2S may also be formed in vivo from the enzymatic desulphuration of β-mercaptopyruvate derived from cysteine transamination [4]. Since the active form of H_2S (H_2S, HS^- or S^{2-}) in vivo has yet to be determined, the term 'hydrogen sulphide' has been used to encompass these three species.

In sharp contrast to other gaseous mediators (i.e., nitric oxide and carbon monoxide; reviewed [5,6]), very little

☆ *Abbreviations:* CBS, cysthionine-β-synthase; CSE, cystathionine-γ-synthase; cGMP, cyclic guanosine monophosphate; DMEM, Dulbecco's modified Eagle's medium; DD1, 3-bromo-3,4,4-trimethyl-3,4-dihydrodiazete 1,2-dioxide; DEA-NONOate, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide-diethylammonium salt; DETA-NONOate, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; EPR, electron paramagnetic resonance; H_2S, hydrogen sulphide; $HgCl_2$, mercuric chloride; LPS, lipopolysaccharide; ·NO, nitric oxide; NO_2^-, nitrite; $ONOO^-$, peroxynitrite; NaHS, sodium hydrosulphide; SNP, sodium nitroprusside; SIN-1, 3-morpholinosydnonimine.

* Corresponding author.
E-mail address: bchwml@nus.edu.sg (M. Whiteman).

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information exists on the mechanisms by which H_2S influences cell function but in the cardiovascular system CSE appears to be the more important of the two known H_2S synthesising enzymes [7,8]. Physiological levels of H_2S in rat serum vary from 30 to 45 μM [9–12] and in mouse serum, ~23 μM [13], and within this concentration range H_2S induces relaxation of aorta tissue and induces transient blood pressure reduction [11,14]. Vascular effect of H_2S may also be mediated by a direct stimulation of K_{ATP} channels with subsequent hyperpolarization of rat aortic vascular smooth muscle in mesenteric artery, aorta, and portal vein [11,15]. Consistent with this, a reduced CSE expression/activity and decreased H_2S concentration contribute to the pathophysiology of pulmonary hypertension in rodents [18] whereas CBS deficiency leads to hyperhomocyst(e)inemia, increased blood pressure, and endothelial dysfunction [19]. In rodents, CBS/CSE deficiency induced by genetic deletion [18] or chronic treatment with DL-propargylglycine (a CSE inhibitor; [19]) results in a severe hypertension and severe loss of endothelial function. Therefore, it is emerging that H_2S is an additional and important physiological mediator in the cardiovascular system.

Levels of H_2S are markedly increased up to 160 μM by pro-inflammatory mediators such as bacterial lipopolysaccharide [12], carrageenan [14], and haemorrhagic shock [9]. Interestingly these same stimuli also increase the production of a more well-known and well-characterised pro-inflammatory mediator, nitric oxide (•NO) [5], leading to the suggestion of a possible biochemical interaction or 'cross talk' between H_2S and •NO [2,3]. However, the precise nature of any such interaction has proved difficult to define. For example, the •NO donor, sodium nitroprusside (SNP), upregulates H_2S production in rat vascular tissues suggesting that •NO augments the expression of H_2S synthesising enzymes such as CSE and CBS [20]. At a different level, H_2S may either enhance [21] or attenuate [22] the relaxant effect of •NO in the rat aorta and •NO has been reported to induce H_2S release [7].

Recently, we demonstrated that H_2S was a potent scavenger of peroxynitrite ($ONOO^-$) [23] strongly suggesting that H_2S may react with •NO and other reactive nitrogen species in vivo.With these latter observations in mind, we hypothesised that H_2S would similarly quench •NO and in the process form a novel nitrosothiol compound. Therefore, using biochemical assays for NO_2^- (Griess assay with the Saville reaction), amperometric detection (for •NO), and electron paramagnetic resonance (for •NO), we also investigated the interaction between •NO and H_2S in isolated RAW264.7 cells by examining cGMP production (an assay for •NO) and examined endogenous nitrosothiol formation in liver homogenates from rats treated with LPS, as an agent known to elevate •NO and H_2S synthesis [12]. Our study shows for the first time that •NO and $ONOO^-$ react with H_2S to form a novel nitrosothiol and we propose that generation of this nitrosothiol in the body has the potential to regulate the physiological effects of both •NO and H_2S.

Materials and methods

Materials. Sodium nitroprusside (SNP), DD1, SIN-1, and spermine-, DEA-, and DETA-NONOates were purchased from the Alexis Corporation (Lausen, Switzerland). •NO gas was obtained from Soxal Pte. Ltd. (Jurong Town, Singapore). Distilled water passed through a Millipore water purification system was used for all purposes. Cyclic guanosine monophosphate (cGMP) kits (#RPN226 EIA) were purchased from Amersham Biosciences (Science Park 1, Republic of Singapore). All other chemicals were AnalaR grade and obtained from Sigma Ltd (St. Louis, MO, USA) and were prepared fresh on the day of the experiment, stored on ice throughout.

Cell culture. The murine RAW264.7 macrophage cell line was purchased from the American Type Culture Collection (Rockville, MD, USA), cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin, 10% foetal bovine serum, and grown at an atmosphere containing 5% CO_2:95% O_2 with ~95% humidity to ~85% confluency in 75 cm^2 culture dishes before use as described [24].

Synthesis of peroxynitrite and generation of H_2S. Hydrogen peroxide-free peroxynitrite was synthesised and quantified as described [25] and hydrogen peroxide removal confirmed using a commercial kit (Amplex Red, Molecular Probes, OR, USA). NaSH was used to generate readily controlled amounts of H_2S since it dissociates to Na^+ and HS^- in solution and then HS^- associates with H^+ to produce H_2S [23,26]. We herein use the term H_2S to reflect the sum of the species H_2S, HS^- or S^{2-} present at physiological pH.

Spectrophotometric measurement of nitrite and amperometric determination of •NO release. •NO donors (SNP, DD1, SIN-1 or DEA-, DETA-, and spermine-NONOates) or NaHS or a combination of NaHS ± •NO donors (all 1 mM) was prepared in 100 mM KH_2PO_4- K_2HPO_4 buffer (pH 7.4) and incubated for up to 1 h at 25 °C. Formation of nitrite (NO_2^-) was determined spectrophotometrically by Griess assay as described [27] using a Molecular Devices SpectraMax190 microplate reader (λ543 nm). To analyse nitrosothiol (RSNO) formation, $HgCl_2$ (1 mM) was added to the incubate to convert RSNO to NO_2^- prior to addition of Griess reagent and NO_2^- determination [27]. NO_2^- concentrations were then determined from a standard curve of $NaNO_2$. Amperometric detection of •NO release from RSNO formed from •NO donors and NaHS (33 μM each) was also measured directly by using an Apollo 4000 Free Radical Analyser (World Precision Instruments, Sarasota, FL, USA) equipped with an ISO-NOP3020 •NO-selective electrode after the addition of $HgCl_2$ (33 μM) as described [28] and concentrations of •NO were determined using KNO_2, KI, and H_2SO_4 as described elsewhere [29].

Electron paramagnetic resonance. •NO release from •NO donors and NaHS was further assessed by electron paramagnetic resonance (EPR) analysis using a Bruker Elexsys E540 spectrometer (Bruker Biospin GmbH, Germany) coupled with rectangular (TE_{102}) SuperX cavity. Briefly, SNP (1.5 mM) was incubated with NaHS (1.5 mM) in water for 30 s in open flask at 25 °C. After this time, 1.5 mM of $HgCl_2$ was added and the solution was loaded into a standard flat cell placed into the cavity for EPR measurements [30].

Measurement of intracellular cyclic GMP. RAW264.7 cells were washed three times with warm (37 °C) EBSS treated with either SNP (100 μM) or NaHS (100 μM) added separately or SNP + NaHS (100 μM) added together in the presence or absence of 10 μM $CuCl_2$ for 30 min at 37 °C. After this time, cyclic GMP (cGMP) was measured using a commercial kit (Amersham Biosciences; #RPN226 EIA) following the manufacturer's instructions [31].

Lipopolysaccharide treatment of rats. All experiments were approved by the Animal Ethics Committee of National University of Singapore and carried out in accordance with established Guiding Principles for Animal Research. Male Sprague–Dawley rats (300–350 g) were maintained in the Animal Housing Unit of this University in an environment with controlled temperature (20–23 °C) and lighting (12:12 h light–darkness cycle). Standard laboratory chow and drinking water were provided ad libitum. A period of 3 days was allowed for animals to acclimatise before any

experimental manipulations were undertaken. Briefly, bacterial endotoxin lipopolysaccharide (LPS, *Escherichia coli*, serotype O127:B8; 10 mg/kg, i.p.) was administered to conscious rats. Control animals received saline (1 ml/kg, i.p.) as described [12]. Animals were killed 6 h thereafter and livers were removed and homogenized for spectrophotometric assay of NO_2^- formation by Griess assay as described [12]. Aliquots of incubate were either mixed with an equal volume of $HgCl_2$ (to convert nitrosothiol to NO_2^-) [27] or phosphate buffer prior to NO_2^- assay [12]. In parallel experiments, some homogenates were incubated with pyridoxal phosphate (100 μM) and L-cysteine (1 mM) as substrate and co-factor for endogenous H_2S synthesis [2,3] for 1 h prior to Griess assay. Where appropriate, additional samples were incubated with 100 μM NaHS (to generate H_2S) prior to Greiss assay ± $HgCl_2$. Data are expressed as nitrite formation per milligram protein (determined using the Bradford reagent) [12].

Data analysis. Data are expressed as means ± standard deviation of the mean (SD) of separate experiments ($n \geqslant 6$). For significance testing, ANOVA was used (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) followed by post hoc Tukey test using SPSS 12.0 software.

Results

Biochemical evidence for nitrosothiol formation: nitrite determination

We initially characterised nitrosothiol formation by incubating ·NO donors with the H_2S donor, sodium hydrosulphide (NaHS), at pH 7.4, 25 °C and measuring nitrite (NO_2^-) formation by the Griess assay in the presence and absence of $HgCl_2$, an established and extensively used method for determining nitrosothiol (RSNO) formation [27]. Fig. 1A shows the time-dependent release of NO_2^- upon addition of NaHS to SNP and, intriguingly, the substantial increase in NO_2^- formation after treatment with $HgCl_2$, indicative of nitrosothiol formation from the reaction between SNP with NaHS. This effect was not restricted to SNP since incubation of NaHS with additional ·NO donors (SIN-1, DD1, and spermine-, DEA-, and DETA-NONOates) also led to a significant increase in NO_2^- formation after addition of $HgCl_2$ (Fig. 1B).

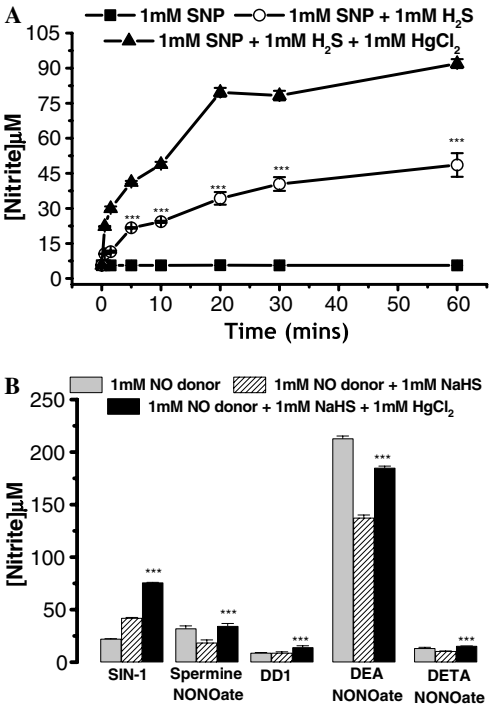

Fig. 1. Biochemical evidence for the in vitro formation of a nitrosothiol: measurement of nitrite. (A) SNP (1 mM) was incubated in phosphate buffer, pH 7.4, in the presence or absence of the H_2S donor NaHS (1 mM) for up to 1 h. Formation of NO_2^- was measured by Griess assay and formation of nitrosothiol was determined by Griess assay after the addition of 1 mM $HgCl_2$. (B) ·NO donors (1 mM each) were incubated in phosphate buffer in the presence or absence of NaHS (1 mM) for 1 h and NO_2^- levels were determined by Griess assay with or without added $HgCl_2$. Concentrations of NO_2^- were determined using a freshly prepared standard curve of $NaNO_2$. Experiments were conducted as described in Materials and methods. Data are expressed as means ± SEM of at least six separate determinations. ***$p < 0.001$ compared to ·NO donor + H_2S without $HgCl_2$.

Biochemical evidence for nitrosothiol formation: amperometric determination of ·NO release

The reaction between SNP with thiols has previously been shown to yield NO_2^- but intermediate formation of ·NO was not investigated in this study [30]. Since nitrosothiols can form NO_2^- without generation of ·NO under certain experimental conditions, we also investigated ·NO release directly in these experiments by amperometry using a ·NO-selective electrode [29]. Fig. 2A also shows that ·NO was not detected from SNP alone (Fig. 2A, i) and small quantities of ·NO were released when SNP was mixed with NaHS (Fig. 2A, ii). However, upon addition of $HgCl_2$, extensive ·NO release was subsequently detected (Fig. 2A, iii). Additional control experiments revealed that addition of $HgCl_2$ to SNP alone did not result in detectable ·NO release (Fig. 2A, iv). Further ·NO donors, as well as ·NO gas, were also examined by amperometry (Fig. 2B). Incubation of DD1, SIN-1, and DEA- and spermine-NONOates with PBS led to small amounts of ·NO release, as expected (Fig. 2B, i–iv, respectively). However after the addition of NaHS, ·NO detection was halted presumably due to a ·NO–H_2S interaction and nitrosothiol formation since addition of $HgCl_2$ led to further ·NO release. Further confirmation of a NO-H_2S interaction was obtained by mixing ·NO gas with NaHS; residual ·NO was measured initially but after addition of $HgCl_2$ substantial ·NO was released (Fig. 2B). In addition to releasing ·NO, SIN-1 also releases superoxide ($O_2^{\cdot -}$) to form peroxynitrite ($ONOO^-$); an additional reactive nitrogen species capable of reacting with thiols to form nitrosothiols [32]. Consequently, the addition of freshly synthesised $ONOO^-$ [25] to NaHS led to a small immediate increase in ·NO detection which subsided over time (Fig. 2B, vi). However, upon addition substantial ·NO was detected, suggesting that $ONOO^-$ also reacted with H_2S to form a nitrosothiol (Fig. 2B, vi). Control experiments showed that the addition of $ONOO^-$ to PBS did not significantly alter the pH of the buffer or result in any detectable ·NO (data not shown).

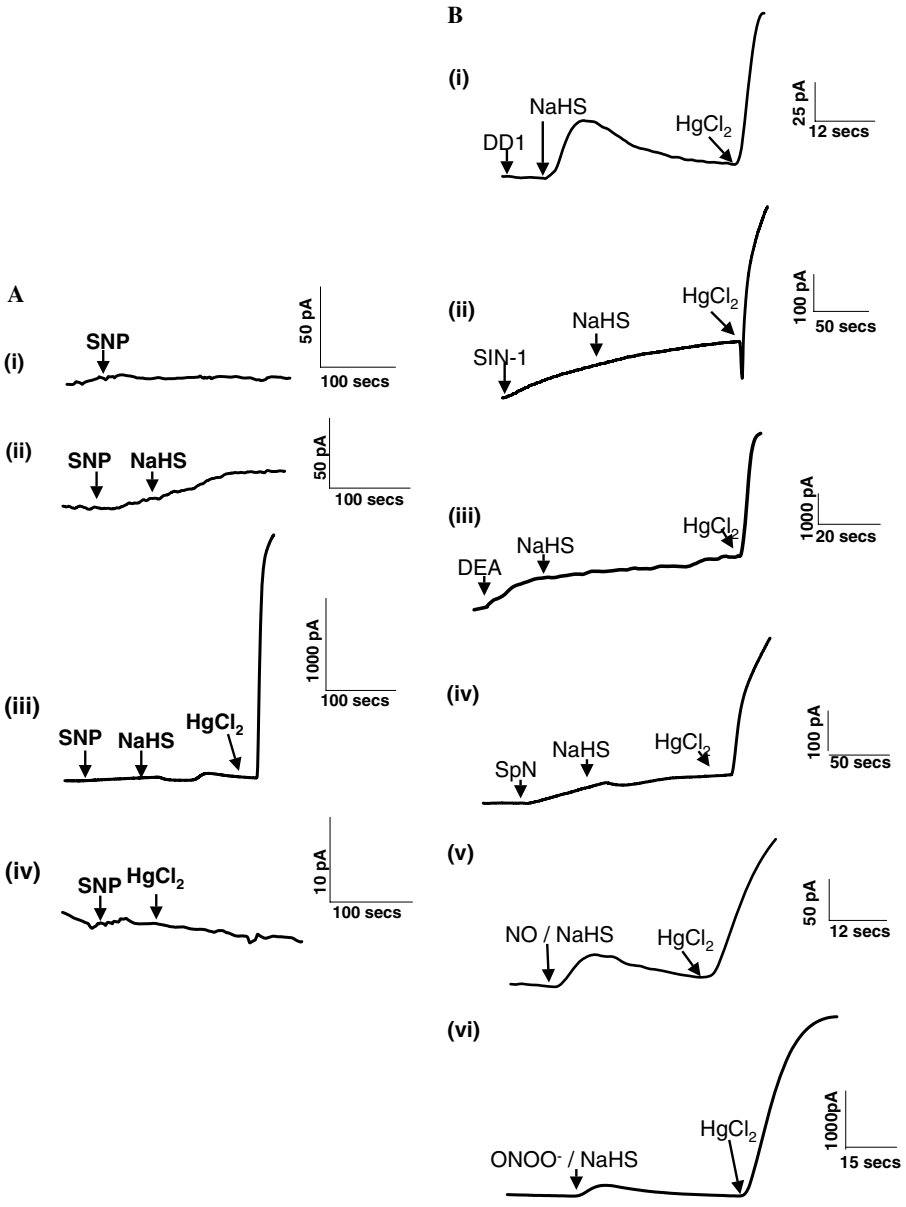

Fig. 2. Biochemical evidence for the in vitro formation of a nitrosothiol: amperometric determination of nitric oxide (•NO) release. (A) •NO release from nitrosothiol formation by SNP: (i) SNP (33 μM) was incubated in phosphate buffer, pH 7.4, alone; (ii) with phosphate buffer and NaHS (33 μM); (iii) with phosphate buffer and NaHS (33 μM) with $HgCl_2$ (33 μM) added at the time stated; or (iv) with phosphate buffer and $HgCl_2$ (33 μM) alone. (B) •NO release from nitrosothiol formation by other •NO donors (33 μM) and NaHS (33 μM); (i) DD1, (ii) SIN-1, (iii) DEA-NONOate, (iv) spermine-NONOate, (v) •NO gas or (vi) $ONOO^-$ solution. Experiments were conducted as described in Materials and methods, and •NO was detected using Apollo 4000 Free Radical Analyser equipped with an ISO-NOP3020 • NO-selective electrode in the presence or absence of added $HgCl_2$ (33 μM). Graphs are representative of six or more separate determinations.

Biochemical evidence for nitrosothiol formation: EPR determination

Nitrosothiol formation was further characterised by EPR. Fig. 3A shows a typical EPR spectra of •NO formed under our experimental conditions by the •NO donor spermine-NONOate ($g = 2.0057$, $a_N = 16.25$ G) whereas SNP (Fig. 3B), NaHS (Fig. 3C), and $HgCl_2$ (Fig. 3D) were EPR silent. Immediately upon addition of NaHS to SNP, an EPR spectrum characteristic of $[Fe(CNO_3)NO]^{3-}$ was detected ($g = 2.0290$, $a_N = 14.95$ G; Fig. 3E) [27] which dissipated after 30 s to give an EPR silent species (Fig. 3F), consistent with previous observations of thiols with SNP [27]. However, as not previously reported, the addition of $HgCl_2$ 30 s after mixing NaHS with SNP led to •NO release ($g = 2.0068$, $a_N = 13.12$ G) and return of $[Fe(CNO_3)NO]^{3-}$ (Fig. 3G), further demonstrating that the reaction of •NO with H_2S led to the formation of a nitrosothiol capable of •NO release.

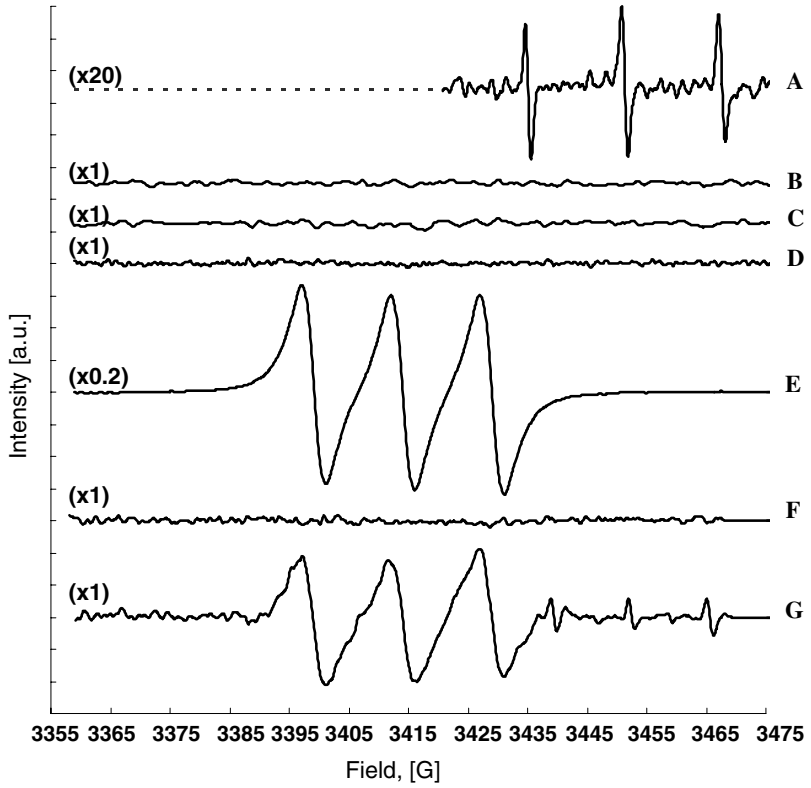

Fig. 3. Biochemical evidence for the in vitro formation of a nitrosothiol: EPR determination of nitric oxide release. ·NO release from SNP (1.5 mM) and NaHS (1.5 mM) after the addition of $HgCl_2$ (1.5 mM) was determined by EPR analysis using a Bruker Elexsys E540 spectrometer (Bruker Biospin GmbH, Germany) coupled with rectangular (TE_{102}) SuperX cavity. SNP was incubated with NaHS in water for 30 s in open flask at 25 °C, $HgCl_2$ added, and the solution loaded into a standard flat cell placed into the cavity. (A) ·NO spectra obtained from spermine-NONOate (5 mM), (B) SNP (1.5 mM) alone, (C) NaHS (1.5 mM) alone, (D) $HgCl_2$ (1.5 mM) alone, (E) NaHS and SNP immediately after mixing, (F) NaHS and SNP at 30 s after mixing, and (G) NaHS and SNP at 30 s after mixing with added $HgCl_2$. Experiments were conducted as described in Materials and methods, and spectra are representative of six or more separate determinations performed on separate days using freshly prepared reagents.

Physiological evidence for nitrosothiol formation: stimulation of cellular cGMP

The major intracellular signalling event subsequent to cellular exposure to ·NO is the elevation of the messenger cyclic guanosine monophosphate (cGMP) through the activation of guanylate cyclase. Fig. 4A shows the effects of exposing RAW264.7 cells to the ·NO donor SNP (100 μM for 30 min) significantly elevated intracellular cGMP levels. In sharp contrast, incubating cells with SNP in the presence of NaHS did not result in a significant increase in cGMP, even when incubated for up to 30 min unless Cu^{2+} was added to release ·NO (Fig. 4A). Preliminary experiments showed that the addition of $HgCl_2$ to cells resulted in rapid cell death (data not shown) so a non-toxic concentration of 10 μM $CuCl_2$ was used instead [33]. Further control experiments showed that neither $CuCl_2$ nor NaHS significantly increased cellular cGMP beyond that of untreated cells (Fig. 4A).

Physiological evidence for nitrosothiol formation: lipopolysaccharide treated rats

Finally, to investigate whether the reaction between H_2S and ·NO was possible in a physiological model where H_2S and ·NO are well documented to be produced [12], we incubated liver homogenates prepared from saline and lipopolysaccharide (LPS)-injected rats with L-cysteine and pyridoxal phosphate (to generate endogenous H_2S) or NaHS (to generate H_2S directly). NO_2^- formation as an index of ·NO formed from endogenous L-arginine was measured. Treatment of the resulting incubates with $HgCl_2$ prior to NO_2^- assay significantly increased the concentration of NO_2^- detected, again indicating the formation of a nitrosothiol formed from the reaction of endogenous ·NO with H_2S during the incubation (Fig. 4B). Interestingly, the addition of pyridoxal phosphate and L-cysteine (added as co-factors for H_2S synthesis) to the homogenates did not significantly increase NO_2^- formation unless $HgCl_2$ was subsequently added. Furthermore, the addition of exogenous H_2S (added as the H_2S donor NaHS) to liver homogenates from LPS treated rats also significantly increased NO_2^- levels further after $HgCl_2$ addition, again suggesting the formation of a nitrosothiol formed from endogenous and enzymatically generated ·NO and H_2S (Fig. 4B).

Discussion

Nitric oxide (·NO) has been extensively studied over the last decade where it has been found to mediate blood

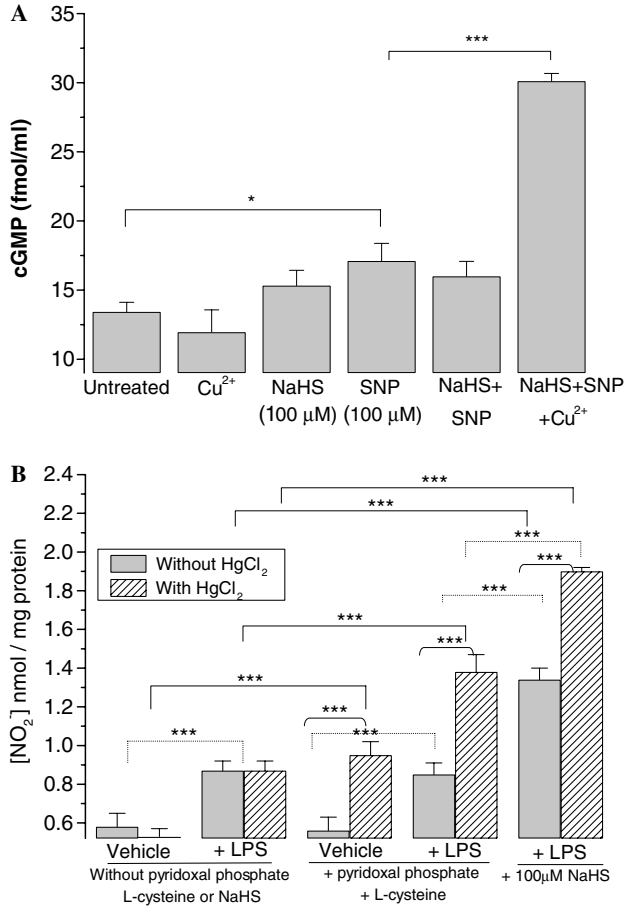

Fig. 4. Physiological evidence for the formation of a nitrosothiol: intracellular cGMP measurement and nitrite determination in tissue homogenates. (A) Measurement of intracellular cyclic guanosine monophosphate (cGMP). Cultured RAW264.7 cells were treated for 30 min in EBSS in a cell culture incubator at 37 °C with either 10 μM $CuCl_2$, 100 μM NaHS, 100 μM SNP, NaHS + SNP or NaSH + SNP with $CuCl_2$ and intracellular cGMP was determined using a commercial kit (Amersham, #RPN226 EIA) following the manufacturer's instructions. (B) Endogenous formation of nitrosothiol. Bacterial endotoxin lipopolysaccharide (LPS, *Escherichia coli*, serotype O127:B8; 10 mg/kg, i.p.) was administered to conscious rats. Control animals received saline (1 ml/kg, i.p.). Animals were killed 6 h thereafter and livers were removed for spectrophotometric assay of NO_2^- formation by Griess assay. Aliquots of incubate were either mixed with an equal volume of $HgCl_2$ (to convert nitrosothiol to nitrite) or phosphate buffer prior to nitrite assay. Where appropriate, samples were incubated at 37 °C for 1 h with pyridoxal phosphate and L-cysteine or 100 μM NaHS prior to NO_2^- determination with or without $HgCl_2$. Data are expressed as nmol NO_2^- formation per mg protein (determined using the Bradford reagent). $^*p < 0.05$. $^{***}p < 0.01$.

pressure, learning and memory, immune and inflammatory response, and host-defence [5]. In comparison, unravelling the physiology of H_2S is in its infancy. However, it is clear from current studies that H_2S appears to be involved in similar processes, i.e., learning and memory [26], mediating blood pressure [8,11,15,16,19,21], chronic inflammation [12,13], oedema [14], and shock [9,12]. Given the high basal concentrations of ·NO and H_2S, and much higher levels reached under pathological conditions in animals and humans [12], the possibility of 'cross talk' between H_2S and ·NO has been suggested [2,3] but not demonstrated.

To examine the possibility of a chemical 'cross talk' between ·NO and H_2S, we initially investigated the effects of the ·NO donor SNP and measured the formation of NO_2^- by Greiss assay in the presence and absence of added $HgCl_2$ as an established assay for nitrosothiol formation [27]. The addition of SNP to NaHS, used to generate H_2S [23,26], resulted in a significant increase in NO_2^- compared to SNP alone, suggesting that H_2S induced ·NO release from SNP (Fig. 1A). This phenomenon has been previously reported with other low molecular weight compounds [34] but not H_2S. However, the addition of $HgCl_2$ to mixtures of SNP and NaHS resulted in a substantial and significant time-dependent increase in NO_2^- compared to SNP or SNP with NaHS alone, suggesting a nitrosothiol compound was formed from SNP and H_2S (Fig. 1A). When incubated with NaHS, additional ·NO donors were shown to also result in nitrosothiol formation (Fig. 1B). For example, incubation of spermine-, DEA- or DETA-NONOates, alone for 1 h, resulted in NO_2^- formation which was markedly decreased in the presence of NaHS. After the addition of $HgCl_2$, further NO_2^- formation was observed, suggesting that H_2S sequestered ·NO release from these drugs, which was subsequently released after $HgCl_2$ addition. In contrast, when SIN-1 was used as the ·NO donor, significantly more NO_2^- formation was observed in the presence of NaHS than in its absence (Fig. 1B). A probable explanation for this observation is that when SIN-1 decomposes to release ·NO, superoxide ($O_2^{\cdot -}$) is also released resulting in the formation of peroxynitrite ($ONOO^-$) [35,36] which also reacts with H_2S to form a nitrosothiol compound which subsequently formed NO_2^- after $HgCl_2$ addition (Fig. 2B, vi).

Since nitrosothiols may produce NO_2^- without ·NO release under certain conditions, we further investigated ·NO release using donors by amperometry with a ·NO-selective electrode (Fig. 2). As expected from the measurements of NO_2^- levels, the addition of NaHS to ·NO donors inhibited ·NO detection but upon $HgCl_2$ addition substantial ·NO was released. Nitrosothiol formation was also observed with authentic ·NO gas and NaHS (Fig. 2B, v), indicating that the nitrosothiol formation was not specific to ·NO donors. Although control experiments ruled out potential artefactual formation in our NO_2^- and ·NO determinations, an additional and complementary method to confirm ·NO release was also performed, EPR (Fig. 3). EPR also showed that the reaction of H_2S with the ·NO donor SNP caused ·NO release only after the addition of $HgCl_2$, further confirming the in vitro formation of a nitrosothiol formed from ·NO and H_2S.

The finding that ·NO and H_2S interact chemically to form a nitrosothiol is potentially of physiological importance. Inhibition of H_2S synthesis using pharmacological agents acting on CSE or CBS has been shown to reduce inflammation [13] and swelling [14]. Similarly, inhibitors of iNOS are also anti-inflammatory and prevent swelling [37]. These studies would imply that both ·NO and H_2S

are pro-inflammatory mediators. Therefore to examine whether endogenous H_2S and endogenous $^{\cdot}NO$ could form a nitrosothiol, we incubated liver homogenates prepared from rats injected with LPS (an agent known to increase $^{\cdot}NO$ and H_2S synthesis) [12], with L-cysteine and pyridoxal phosphate (to generate endogenous H_2S) (2.3) or NaHS and then measured the formation of NO_2^- formed from endogenous L-arginine (Fig. 4B). Treatment of the resulting incubates with $HgCl_2$ prior to NO_2^- assay significantly increased the concentration of NO_2^- detected, again indicating the formation of a nitrosothiol during the incubation. Interestingly, $HgCl_2$ treatment did not increase the NO_2^- concentration when L-cysteine and pyridoxal phosphate or NaHS were omitted from the incubation medium strongly suggesting that endogenous $^{\cdot}NO$ (generated from residual L-arginine in the homogenate) reacted with endogenous H_2S (generated from added L-cysteine) in these homogenates to form a nitrosothiol.

From our current study it is tempting to speculate that the formation of a nitrosothiol (from $^{\cdot}NO$ and H_2S) is a process to inactivate $^{\cdot}NO$ and/or H_2S into a less biologically active molecule. Indeed, the addition of SNP with H_2S (which formed a nitrosothiol; Figs. 1A, 2A, and 3) did not result in the increase in intracellular cGMP, indicative of $^{\cdot}NO$, until Cu^{2+} was added to cleave RS-NO bonds and liberate $^{\cdot}NO$ (Fig. 4A). This was in sharp contrast to other low molecular weight nitrosothiols such as *S*-nitrosocysteine [38,39], *S*-nitrosoglutathione [40,41] which readily induce cellular guanylate cyclase activity resulting in cGMP intracellular accumulation in a variety of cell types. As such, a principal physiological role for H_2S released from the vasculature [11], in the brain [2,3], and other tissues [8–13,11,14–19] may be to aid in the regulation or control of local concentrations of $^{\cdot}NO$ and $ONOO^-$ which are well known to exert cytotoxic effects [36] rather than to dilate blood vessels directly. It is also possible that the converse may exist whereby the role of $^{\cdot}NO$ is to quench H_2S. However, further work is required to substantiate this hypothesis.

Our present study shows that several $^{\cdot}NO$ donor drugs as well as $^{\cdot}NO$ gas and synthetic $ONOO^-$ react with H_2S in vitro to form a novel compound which using currently available technology is biochemically indifferent to that of a novel nitrosothiol. We also show that the nitrosothiol does not induce cGMP formation unless $^{\cdot}NO$ is released and that under conditions of simultaneous $^{\cdot}NO$ and H_2S production (i.e., in LPS treated rats) this nitrosothiol may be formed in vivo. It is possible that the physiological role of H_2S in the body may be to sequester $^{\cdot}NO$ in the form of this nitrosothiol further providing first evidence for the suggested 'cross talk' between these gaseotransmitters. Furthermore, recent reports providing evidence for a pathophysiological role for H_2S in, for example, inflammatory and cardiovascular diseases such as shock and hypertension may need to be re-evaluated in light of the present findings. Current measurements of NO_2^- and H_2S in tissue homogenates or elsewhere (e.g., plasma) may underestimate the presence of both gases due to nitrosothiol formation.

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