Research Article

Products of Sulfide/Selenite Interaction Possess Antioxidant Properties, Scavenge Superoxide-Derived Radicals, React with DNA, and Modulate Blood Pressure and Tension of Isolated Thoracic Aorta

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Selenium (Se), an essential trace element, and hydrogen sulfide (H2S), an endogenously produced signalling molecule, affect many physiological and pathological processes. However, the biological effects of their mutual interaction have not yet been investigated. Herein, we have studied the biological and antioxidant effects of the products of the H2S (Na2S)/selenite (Na2SeO3) interaction. As detected by the UV-VIS and EPR spectroscopy, the product(s) of the H2S-Na2SeO3 and H2S-SeCl4 interaction scavenged superoxide-derived radicals and reduced ⋅cPTIO radical depending on the molar ratio and the preincubation time of the applied interaction mixture. The results confirmed that the transient species are formed rapidly during the interaction and exhibit a noteworthy biological activity. In contrast to H2S or selenite acting on their own, the H2S/selenite mixture cleaved DNA in a bell-shaped manner. Interestingly, selenite protected DNA from the cleavage induced by the products of H2O2 interaction. The relaxation effect of H2S on isolated thoracic aorta was eliminated when the H2S/selenite mixture was applied. The mixture inhibited the H2S biphasic effect on rat systolic and pulse blood pressure. The results point to the antioxidant properties of products of the H2S/selenite interaction and their effect to react with DNA and influence cardiovascular homeostasis. The effects of the products may contribute to explain some of the biological effects of H2S and/or selenite, and they may imply that a suitable H2S/selenite supplement might have a beneficial effect in pathological conditions arisen, e.g., from oxidative stress.
1. Introduction

Exogenously added and endogenously produced H$_2$S affects many physiological and pathological processes [1–3]. Accumulating evidence supports the involvement of H$_2$S in the regulation of cardiovascular homeostasis [4]. It has mostly beneficial effects during oxidative stress by reacting with reactive oxygen and nitrogen species, i.e., hydrogen peroxide (H$_2$O$_2$), superoxide anion radical (O$_2^−$), hypochlorite (HOCI), or peroxynitrite (ONOO$^−$) [5–8]. However, several effects of H$_2$S on cells are toxic [9–11].

Selenium (Se) is a relatively rare but an essential trace element for humans, plants, and microorganisms. Se, which exerts multiple and complex effects on human health, is known as an antioxidant due to its presence in 25 selenoproteins in the form of selenocysteine amino acid. Both beneficial and detrimental effects of Se deficiency and/or supplementation are well known. The biological effects of Se compounds (selenite, selenate, selenocysteine, and selenomethionine) on cardiac oxidative damage, heart disease, cancer prevention, immunity, diabetes, neuroregeneration, or dementia have been reported [12–17]. However, the beneficial effect of Se supplementation for men’s health is still a controversial issue [18–23]. Selenite, which is a common Se supplement, is considered as a promising anticarcinogen [24–26]. It can induce apoptosis in cancer cells through the production of reactive oxygen species (ROS) leading to oxidative stress [27, 28]. However, Se compounds were also found to damage DNA in healthy cells [29] and therefore may not be considered as a suitable protective agent against cancer and/or other chronic diseases. Actually, they can cause or advance some kinds of cancers [30, 31]. The exact mechanisms of the beneficial and toxic effects of Se are not yet fully understood, giving rise to further uncertainty about its potential use in nutrition supplements and/or clinical treatment.

Se and H$_2$S are present in living organisms, and each one has beneficial and/or toxic effects through its interaction mostly with ROS [1, 2, 30–33]. However, the biological effects of products of the H$_2$S/seHenate interaction are not yet known, namely, their involvement in the production and/or inhibition of ROS, reaction with DNA, or influence on cardiovascular system. Therefore, we have studied the effects of products of the H$_2$S/seHenate interaction on O$_2^−$ and cPTIO radicals, DNA cleavage, tension of isolated aortic rings, and rat blood pressure (BP). We found that the products have significant biological effects that differ from those caused by H$_2$S or by selenite on their own. These results may contribute to the understanding of possible coupled biological effects of H$_2$S and Se.

2. Material and Methods

2.1. Chemicals

2.1.1. Selenium Compounds. Stock solutions of sodium selenite (Na$_2$SeO$_3$, 10 or 40 mmol·L$^{-1}$, Merck K34993707-542 or Sigma 214485), selenium tetrachloride (SeCl$_4$, 10 mmol·L$^{-1}$, Aldrich 323527), and sodium selenate (Na$_2$SeO$_4$, 10 mmol·L$^{-1}$, Sigma S0882) were prepared freshly in deionized H$_2$O, stored at 23°C, and used within 5 h. Na$_2$SeO$_3$ dissociates in solution to yield mostly H$_2$SeO$_3$ at acidic pH, HSeO$_3^−$ at neutral pH, and SeO$_3^{2−}$ at alkaline pH. For simplicity, the term SeO$_3^{2−}$ is employed as representative expression to encompass the total mixture of these different (de)protonation states.

2.1.2. Hydrogen Peroxide. Hydrogen peroxide (H$_2$O$_2$) (14.7 mol·L$^{-1}$; Sigma-Aldrich 85321), according to a particular experiment, was diluted in H$_2$O or in 100 mmol·L$^{-1}$ sodium phosphate buffer, supplemented with 200 or 50 μmol·L$^{-1}$ DTPA, pH 7.4, 37°C before application.

2.1.3. Radicals. 5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide (BMPO, 100 mmol·L$^{-1}$, Dojindo B568-10, Japan) was dissolved in deionized H$_2$O, stored at −80°C, and used after thawing. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 10 mmol·L$^{-1}$, Cayman 81540 or Sigma C221) was dissolved in deionized H$_2$O and was stored at −20°C for several weeks.

2.1.4. Sulfide. Na$_2$S (100 mmol·L$^{-1}$ stock solution, Dojindo SB01, Japan) was dissolved in argon degassed deionized H$_2$O, stored at −80°C, and used immediately after thawing. Na$_2$S dissociates in aqueous solution and reacts with H$^+$ to yield H$_2$S, HS$^-$, and a trace of S$^{2−}$. We use the term H$_2$S to describe the total mixture of H$_2$S, HS$^-$, and S$^{2−}$ forms. The stock concentration was checked by UV-VIS spectroscopy: by the absorbance of 1000x diluted stock solution at 230 nm (ε$_{230 \text{nm}}$ = 7700 mol$^{-1}$·L$^{-1}$·cm$^{-1}$, diluted by deionized water) and also by the reduction of 100 μmol·L$^{-1}$ DTNB by 2000x diluted stock solution (1 H$_2$S molecule generates 2 TNB$^−$ equivalents, ε$_{412 \text{nm}}$ = 14,100 mol$^{-1}$·L$^{-1}$·cm$^{-1}$, measured in 1 mmol·L$^{-1}$ phosphate buffer), according to Nagy et al. [34].

2.1.5. Buffers. 100 mmol·L$^{-1}$ sodium phosphate buffer supplemented with 100 μmol·L$^{-1}$ diethylenetriaminepentaacetic acid (DTPA), pH 6.5, 7.0, 7.4, 8.0, and 9.0, 37°C, was employed for UV-VIS experiments. 50 and 25 mmol·L$^{-1}$ sodium phosphate buffer, supplemented with 100 and 50 μmol·L$^{-1}$ DTPA, pH 7.4, 37°C, was used for electron paramagnetic resonance (EPR) and plasmid DNA (pDNA) cleavage studies, respectively.

2.2. UV-VIS of cPTIO. To obtain 1 mL of the working solution, 10 or 100 μL of stock solution of the compounds studied was added to the appropriate volume (990 or 900 μL, respectively) of 100 mmol·L$^{-1}$ sodium phosphate buffer (at given pH, 37°C) containing the final concentrations of 100 μmol·L$^{-1}$ cPTIO and 100 μmol·L$^{-1}$ DTPA. UV-VIS absorption spectra (900-190 nm) were recorded every 30 s for 20 to 40 min with a Shimadzu 1800 (Kyoto, Japan) spectrometer at 37°C. The cPTIO extinction coefficient of 920 mol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 560 nm was used. The reduction of the cPTIO radical was determined as the decrease of the absorbance at 560 nm (absorption maximum of cPTIO in VIS range) or at 358 nm after subtracting the absorbances at 730 or at 420 nm, respectively [5, 35].
To study the involvement of O$_2$ in the H$_2$S/SeO$_3^{2-}$-induced reduction of the cPTIO radical, 10 mmol L$^{-1}$ Na$_2$S in H$_2$O, 10 mmol L$^{-1}$ Na$_2$SeO$_3$ in H$_2$O, and 102 μmol L$^{-1}$ cPTIO in the 100 mmol L$^{-1}$ sodium phosphate buffer, supplemented with 100 μmol L$^{-1}$ DTPA (pH 7.4; 37°C), were deaerated with argon for 10 min at 37°C. The compounds were mixed in a closed UV-cuvette, and the UV-VIS spectra were recorded. The O$_2$ concentration in the deaerated samples was 3-5%, confirmed with an oxygen electrode (OXELP, SYS-ISO2, WPI, USA). In all UV-VIS experiments, H$_2$O was used as a blank.

2.3. EPR of the BMPO Adducts. To study the ability of H$_2$S/SeO$_3^{2-}$ to scavenge the O$_2^-$ radical or its derivatives produced in DMSO/KO$_2$ solution, sample preparation and EPR measurements were conducted in accordance with previously reported protocols [5]. The solution (final concentrations of BMPO (20 mmol L$^{-1}$) DTPA (100 μmol L$^{-1}$) in sodium phosphate buffer (50 mmol L$^{-1}$, pH 7.4) was incubated for 1 min at 37°C; an aliquot of the compound was added, followed by saturated KO$_2$/DMSO solution (10% v/v DMSO/final buffer) 3 s later. The sample was mixed for 5 s and transferred to a standard cavity aqueous EPR flat cell. The first EPR spectrum was recorded 2 min after the addition of KO$_2$/DMSO solution at 37°C. The sets of individual EPR spectra of the BMPO spin adducts were recorded as 15 sequential scans, each 42 s, with a total time of 11 min. Each experiment was repeated at least twice. EPR spectra of the BMPO spin adducts were measured on a Bruker EMX spectrometer, X-band ~9.4 GHz, 335.15 mT central field, 8 mT scan range, 20 mW microwave power, 0.1 or 0.15 mT modulation amplitude, 42 s sweep time, 20.48 ms time constant, and 20.48 ms conversion time at 37°C. Intensities of the BMPO adducts in the EPR spectra were reproducible, when the KO$_2$/DMSO stock solution was stored at 5°C for 1 day or at 23 ± 1°C for 4 h.

2.4. Plasmid DNA Cleavage. pDNA cleavage assay with the use of pBR322 plasmid (New England BioLabs Inc., N3033L) was performed as reported previously [5, 36]. In this assay, all samples contained 0.2 μg pDNA in sodium phosphate buffer (25 mmol L$^{-1}$ sodium phosphate, 50 μmol L$^{-1}$ DTPA, pH 7.4, 37°C). After addition of compounds, the resulting mixtures were incubated for 30 min at 37°C. All concentrations listed in the section were final in the samples. After incubation, the reaction mixtures were subjected to 0.6% agarose gel electrophoresis. Samples were electrophoresed in TBE buffer (89 mmol L$^{-1}$ Tris, 89 mmol L$^{-1}$ boric acid, and 2 mmol L$^{-1}$ EDTA) at 5.5 V cm$^{-1}$ for 2 h; gels were stained with GelRed™ Nucleic Acid Gel Stain and photographed using a UV transilluminator. Integrated densities of all pBR322 forms in each lane were quantified using the TotalLab TL100 image analysis software to estimate pDNA cleavage efficiency (Nonlinear Dynamic Ltd., USA).

2.5. Guide for the Use and Care of Laboratory Animals

2.5.1. Isolated Thoracic Aorta. Procedures were performed in accordance with the Institutional Guidelines of the Eth-}

ical Committee on the Ethics of Procedures in Animal, Clinical and other Biomedical Experiments (permit number: EC/CEM/2017/4) of the Institute of Normal and Pathological Physiology, Centre of Experimental Medicine and were approved by the State Veterinary and Food Administration of the Slovak Republic and by an Ethical Committee according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Directive 2010/63/EU of the European Parliament. The Institute of Normal and Pathological Physiology provided veterinary care.

2.5.2. Rat Blood Pressure. All procedures were approved by the State Veterinary and Food Administration of the Slovak Republic (No.: Ro-1545/15-221) according to the guidelines from Directive 2010/63/EU of the European Parliament. Experiments were carried out according to the guidelines laid down by the animal welfare committee of the Institute of Normal and Pathological Physiology of the Slovak Academy of Sciences and conformed to the principles and regulations, as described in the editorial by Grundy [37].

2.6. Functional Study of Isolated Thoracic Aorta. Normotensive Wistar Kyoto (WKY) rats (307 ± 4.3 g) were killed by decapitation after a brief anesthetization with CO$_2$, and the thoracic aorta was isolated as described in our previous study [38]. The changes in isometric tension were measured by the electromechanical transducers (FSG-01, MDE, Budapest, Hungary). The resting tension of 1 g was applied to each ring and maintained throughout a 45 to 60 min of equilibration period until stress relaxation no longer occurred. Changes in thoracic aorta tension were followed by noradrenaline (NA; 1 μmol L$^{-1}$) precontracted arterial rings after a stable plateau was achieved.

2.7. Functional Study of Rat Blood Pressure. Male Wistar rats (n = 10; 350 ± 40 g) were from the Department of Toxicology and Laboratory Animal Breeding at Dobra Voda, Slovak Academy of Sciences, Slovakia. The rats were housed under a 12 h light-12 h dark cycle, at a constant humidity (45-65%) and temperature (20-22°C), with free access to standard laboratory rat chow and drinking water. The Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine, Slovak Academy of Sciences, provided veterinary care. The tranquilizer xylazine (Rometar) was purchased from Zentiva (Czech Republic), and the anesthetic combination of tiletamine+zolazepam (Zoletil 100) was acquired from Virbac (France). All other chemicals were purchased from Sigma-Aldrich. Experiments were carried out as previously described [39]. Rats were anesthetized with Zoletil 100 (tiletamine+zolazepam, 80 mg kg$^{-1}$, i.p.) and Rometar (xylazine, 5 mg kg$^{-1}$, i.p.). During the anesthesia, BP, heart rate, and reflex responses to mechanical stimuli were monitored. The animals were under anesthesia during the whole experiment and were euthanized with an overdose of Zoletil via jugular vein at the end of the surgical procedure. All experiments were supervised and performed under the same experimental conditions.
2.8. Blood Pressure Measurement. The right jugular vein was cannulated for administering compounds under anesthesia as described above. The left artesia carotis communis was cannulated for inserting the fiber optic microcatheter pressure transducers (FISO LS 2F Harvard Apparatus, USA). The analog signal was digitalized at 10 kHz, filtered at 1 kHz, and recorded by DEWESoft 6.6.7 (GmbH, Austria). The signal was evaluated 5 s before and 10 min after compound administration. After stabilization of BP (10-20 min), the compounds were administered into the right jugular vein as a bolus of 500 μL kg⁻¹ over 15 s period. The solution of the H₂S/SeO₃²⁻ mixture (10/5 in mmol L⁻¹) was prepared as follows: to 123.5 μL of 100 mmol L⁻¹ phosphate buffer, 100 μmol L⁻¹ DTPA, 14 μL of 1 mol L⁻¹ HCl was added, followed by 62.5 μL of 40 mmol L⁻¹ Na₂SeO₃ in 0.9% NaCl, and finally 50 μL of 100 mmol L⁻¹ Na₂S in H₂O was added. The pH of the buffer mixture was 7.4. The mixture was incubated for 40 ± 10 s at 23°C before i.v. administration. Unbuffered H₂S/SeO₃²⁻ mixture (10/5 in mmol L⁻¹) was prepared, when 0.9% NaCl was used instead of phosphate buffer and HCl. The pH of the unbuffered mixture was ~11 measured by a pH paper indicator.

2.9. Statistical Analysis. Unless otherwise stated, data are represented as the means ± S.E.M. Statistical significance was determined by Student’s t-test or one-way ANOVA followed by the multiple comparison test. Differences between means were considered significant at *P < 0.05. Data analysis and plot construction were carried out using SigmaPlot 12 (Systat Software GmbH).

3. Results

3.1. H₂S Interacts with Na₂SeO₃ and SeCl₂, but Not with Na₂SeO₄, to Form Initial Reactive Intermediate(s), which Reduce the cPTIO Radical. Since the antioxidant properties of H₂S/SeO₃²⁻ products are unknown, we have used the cPTIO radical to study the reducing properties of the products of H₂S/SeO₃²⁻ interaction. The cPTIO radical is stable in aqueous solution, and its formation and reduction can be monitored by the UV-VIS spectrophotometry at 358 or 560 nm. Even in the presence of up to 100 μmol L⁻¹ H₂S or 100-400 μmol L⁻¹ SeO₃²⁻, the absorbance (ABS) of the radical at 358 and 560 nm decreases only by <7% after 40 min, indicating that neither H₂S nor SeO₃²⁻ on its own reduces this radical (Figure 1). In contrast, once H₂S (25-100 μmol L⁻¹) was added to the cPTIO/SeO₃²⁻ (100/2.5-400 μmol L⁻¹) mixture, or SeO₃²⁻ was added to cPTIO/H₂S, the absorbances at 358 and 560 nm decreased rapidly over the time (≤30 s), indicating a possible formation of strong reducing agent(s) which lastly and efficiently reduced the cPTIO radical (Figures 1 and 2). Similar results were obtained when SeCl₂, but not Na₂SeO₄, was used instead of SeO₃²⁻ (Figures S1 and S2a).

The reduction of cPTIO followed a bell-shaped dependence on the concentration of SeO₃²⁻ at a constant cPTIO/H₂S concentration (Figure 2(a)), with a maximum radical scavenging activity at an H₂S:SeO₃²⁻ ratio of roughly 4:1. The ability of H₂S to reduce cPTIO in the presence of SeO₃²⁻ increased with the increasing H₂S concentration (Figure 2(b)) and followed also a bell-shaped dependence on pH (Figures 3 and S3).

If H₂S and SeO₃²⁻ were preincubated for different periods of time before the addition to cPTIO, it clearly resulted in the highest radical scavenging activity, which was subsequently lost over the time (Figure 4). An H₂S/SeO₃²⁻ (100/100 in μmol L⁻¹) mixture preincubated for ≥1 min prior to cPTIO addition did not reduce cPTIO, demonstrating that later products of the reaction of sulfide with SeO₃²⁻ could not be responsible for the reduction of the radical and that the relevant active species were formed swiftly, in less than 1 min and have a short lifetime, as recently suggested [40]. Notably, formation of these active early intermediates to reduce cPTIO was prolonged with the increase of the H₂S/SeO₃²⁻ ratio (Figure 4(e)). At H₂S/SeO₃²⁻ concentration of 100/25 in μmol L⁻¹ and 5 min preincubation time, the mixture still possessed around 50% potency to reduce cPTIO (Figure 4(e)). This timing, once more, accounts for a rapidly formed selenosulfide intermediate as being the ultimate responsible for this radical scavenging activity, a species also possibly being sensitive to oxidation over prolonged time periods (Figure 4).

While the available evidence is in accordance with the formation of HSSeSH as the main reactive species, there are other chalcogen-based candidates which are good reducing agents, namely, hydrogen selenide (H₃Se), hydroselelenide anion (HSe⁻), selenide (Se⁻²), persulfides, and polysulfides (Sₓ⁻²) [41-43]. Interestingly, O₂ does not seem to play a major role in the H₂S/SeO₃²⁻-induced reduction of the cPTIO radical probably due to slower kinetics of interaction of reactants and/or intermediates with O₂ in comparison to the rate of cPTIO reduction [44-48]. Under argon flushed conditions, reduction of the cPTIO radical was neither enhanced nor suppressed significantly, hence ruling out any major involvement of H₃Se, as this selenium compound is highly sensitive to O₂ (Figure S2b).

3.2. EPR of BMPO-OOH: The Initial Products of the H₂S/SeO₃²⁻ Interaction Also Scavenge Derivatives of Superoxide Anion (O₂⁻) Radical. We aimed to ascertain whether the initial products of the H₂S/SeO₃²⁻ interaction are able to scavenge other radicals, i.e., O₂⁻ or its derivatives. The interactions with O₂⁻ were studied with the EPR spin trap method based on the reaction of this dioxygen radical with BMPO to form the BMPO-OOH adduct [49]. This assay was chosen due to biological and mechanistic reason; O₂⁻ is a simple radical reduced by one-electron transfer.

O₂⁻ was dissolved in phosphate buffer (pH 7.4, 37°C) and trapped by BMPO. Under these conditions, the relative intensity of the BMPO-OOH adduct decreased slowly over the time and was comparable to the values reported under physiological conditions (Figures S5(a1)–S5(a3)) [49]. The addition of SeO₃²⁻ (25 and 50 μmol L⁻¹) did not significantly interfere with the BMPO-OOH adduct formation, its concentration, and rate of decay (Figures S5(b1)–S5(b3), S4b1-b3). In contrast, H₂S (25 and 50 μmol L⁻¹) decreased the BMPO-OOH concentration and increased the rate of the decay, and this “antioxidant” activity of H₂S was
Figure 1: Interaction of \( \cdot \text{cPTIO}/\text{SeO}_3^{2-} \) with H\(_2\)S. (a) Time resolved UV-VIS spectra of the interaction of 100 \( \mu \text{mol L}^{-1} \) cPTIO with 100 \( \mu \text{M} \) \( \text{SeO}_3^{2-} \) (3 times repeated every 30 s, black) and subsequent addition of 100 \( \mu \text{mol L}^{-1} \) H\(_2\)S. Spectra were collected every 30 s for 15 min; the first spectrum, indicated by the solid red line, was measured 15 s after addition of H\(_2\)S. Top inset: kinetics of changes in absorbance at 560 nm after addition of 100 \( \mu \text{mol L}^{-1} \) H\(_2\)S into \( \cdot \text{cPTIO}/\text{SeO}_3^{2-} \) (100/100 in \( \mu \text{mol L}^{-1} \)) solution at time 0 s. Bottom inset: details of the time resolved spectra of the \( \cdot \text{cPTIO}/\text{SeO}_3^{2-} \) (100/100 in \( \mu \text{mol L}^{-1} \)) interaction before (black) and after addition of H\(_2\)S (100 \( \mu \text{mol L}^{-1} \), the first spectrum is indicated by the solid red line, which is followed each 30 s by: long dash red, medium dash red, short dash red, dotted red, solid blue line, long dash blue, medium dash blue, etc.). (b, c, d, e) H\(_2\)S (100 \( \mu \text{mol L}^{-1} \)) was added to 100 \( \mu \text{mol L}^{-1} \) solution of \( \cdot \text{cPTIO} \) containing different concentrations of \( \text{SeO}_3^{2-} \) (0–400 \( \mu \text{mol L}^{-1} \), see legend). The kinetics of the reduction of 100 \( \mu \text{mol L}^{-1} \) cPTIO before and after addition of 100 \( \mu \text{mol L}^{-1} \) H\(_2\)S (marked by arrow) was monitored as a decrease of the absorbance at 358 nm minus the absorbance at 420 nm (b, d) and also as a decrease of the absorbance at 560 nm (c, e).
increased further in the presence of SeO$_2^{-}$ (more than three times at a 50/25 in μmol L$^{-1}$ H$_2$S/SeO$_3^{2-}$ ratio). This indicates that H$_2$S/SeO$_3^{2-}$ products scavenge O$_2^{-}$ and/or its derivatives. The effects of H$_2$S/SeO$_3^{2-}$ were less pronounced when KO$_2$/DMSO was added 5 min after addition of H$_2$S/SeO$_3^{2-}$, suggesting that the reducing intermediate had already decomposed at this stage (Figure 5, S4). Similar results were obtained when H$_2$S was incubated with SeCl$_4$ (Figure S5). This indicates that the production of highly active species, products of the H$_2$S/SeO$_3^{2-}$ interaction, was time-dependent: they appeared within a few seconds after addition of SeO$_3^{2-}$ to the H$_2$S solution and their effects diminished after a few minutes of interaction.

From our previous studies of O$_2^{-}$ reaction with BMPO [5], we know that the EPR spectra of the BMPO adducts were superposened on the BMPO-OH/SeOH radicals with minor contribution from the BMPO-C radical. Therefore, we simulated the spectra using hyperfine coupling constants for BMPO-OH, BMPO-OH, and BMPO-C (derived from DMSO) radicals. The means of hyperfine coupling constants used were as follows: BMPO-OH1 (black) \( a_N = 13.30 \pm 0.03 \) G, \( a_H = 11.7 \pm 0.1 \) G; BMPO-OH2 (red) \( a_N = 13.24 \pm 0.03 \) G, \( a_H = 9.4 \pm 0.1 \) G; BMPO-OH1 (green) \( a_N = 13.7 \pm 0.3 \) G, \( a_H = 12.3 \pm 0.4 \) G; BMPO-OH2 (yellow) \( a_N = 13.6 \pm 0.2 \) G, \( a_H = 15.3 \pm 0.1 \) G; and BMPO-C (blue) \( a_N = 15.2 \pm 0.1 \) G, \( a_H = 21.5 \pm 0.1 \) G. The constants are similar to those reported by Zhao et al. [49]. The simulation revealed that O$_2^{-}$ was trapped in the control and in the presence of SeO$_3^{2-}$ (Figures 5(a) and 5(b)), since the EPR spectra of BMPO-OH were only observed. However, BMPO-OH component was present in the samples containing H$_2$S alone or with SeO$_3^{2-}$ (Figures 5(c)–5(h)). The results may indicate that H$_2$S alone or with SeO$_3^{2-}$ decomposed BMPO-OH to BMPO-OH and/or OH was formed as a result of compound presence. Since the first spectrum was recorded 110 ± 15 s after sample preparation, we cannot exclude a possibility of trapping of other radicals with lifetimes shorter than 110 s.

### 3.3. H$_2$S/SeO$_3^{2-}$ Cleaves pDNA

It was of interest to know if the products of the H$_2$S/SeO$_3^{2-}$ interaction have biological effects in vitro. Therefore, we investigated the direct effects of H$_2$S and SeO$_3^{2-}$ on the cleavage of pDNA in vitro using the Fenton reaction as a positive benchmark control (Figure 6) [5]. Interestingly, neither H$_2$S (1 mmol L$^{-1}$) nor SeO$_3^{2-}$ (0.1 mmol L$^{-1}$) alone significantly cleaved pDNA. In contrast, SeO$_3^{2-}$ in a concentration-dependent manner caused damage to DNA in the presence of 1 mmol L$^{-1}$ H$_2$S (Figures 6(a1) and 6(a2)). The observed cleavage of DNA caused by SeO$_3^{2-}$ and H$_2$S showed a bell-shaped concentration ratio dependence similar to the one observed in the reduction of the cPTIO radical. We can suggest that the intermediate responsible for the action can be a selenopolysulfide with a H$_2$S:SeO$_3^{2-}$ ratio of 4:1. In the presence of H$_2$O$_2$, damage to pDNA by H$_2$S occurs also without SeO$_3^{2-}$, since H$_2$O$_2$ now takes on the role of SeO$_3^{2-}$ as an oxidant, with the simultaneous formation of the OH radicals (Figures 6(b1) and 6(b2)). Further evidence for the involvement of radicals may come from the fact that dimethylsulfoxide (DMSO), a known OH scavenger [50, 51] frequently employed as solvent in biology, is able to interfere with the damage to DNA (Figures 6(c1) and 6(c2)).

### 3.4. H$_2$S/SeO$_3^{2-}$ Modulates Tension of Isolated Thoracic Aorta

As some of our in vitro assays indicated an antioxidant activity of the H$_2$S/SeO$_3^{2-}$ mixture, and H$_2$S is known to promote relaxation of blood vessels [52], the impact of the H$_2$S/SeO$_3^{2-}$ mixture on the isolated thoracic aorta was examined. The thoracic aorta was precontracted by noradrenaline (NA) (1 μmol L$^{-1}$). After a stable plateau of the contraction was reached (Figure 7(a)), SeO$_3^{2-}$ (100 μmol L$^{-1}$) showed only negligible activity, while Na$_2$S (200 μmol L$^{-1}$) significantly relaxed the aortic rings, in agreement with our previous studies [38]. A simultaneous addition of SeO$_3^{2-}$ (100 μmol L$^{-1}$) and H$_2$S (200 μmol L$^{-1}$) resulted once more in a biphasic activity profile, where a minor
relaxation was noticed first, followed by a pronounced contraction (Figure 7). It is supposed that the contraction effect may result also from the antioxidant properties of the mixture, similarly as it has been reported for ascorbate [53].

3.5. H2S/SeO3 2− Modulates Rat Systolic and Pulse Blood Pressure. Since the products of the H2S/SeO3 2− interaction modulated the tension of the thoracic aorta, we subsequently studied whether the products influence blood pressure (BP). Intravenous (i.v.) administration of 5 μmol kg−1 SeO3 2− had only minor effects on BP (Figure 8(g)). The administration of 10 μmol kg−1 of Na2S transiently decreased and increased BP (Figures 8(a) and 8(g)), as observed in our previous study [54]. The stock solution of the H2S/SeO3 2− mixture (20/10 in mmol L−1) prepared in 0.9% NaCl was colorless and had pH ~ 11. However, when the mixture was prepared in solution with pH 7.4, it had orange color with an absorption maximum at 570 nm (Figure S6), indicating formation of the sulfur-selenium complexes [40]. The i.v. administration of the mixture H2S/SeO3 2− (10/5 μmol kg−1, pH ~ 7.4), in comparison to H2S alone, inhibited both BP decrease and increase (Figures 8(b) and 8(g)). The effects of the mixture were less pronounced at pH ~ 11, being the effects at this pH similar to those observed for H2S alone (Figures 8(c) and 8(g)).

The studied compounds influenced pulse BP, as an important parameter of cardiovascular system reflecting arterial stiffness [55, 56]. The administration of 5 μmol kg−1
Figure 4: Influence of the preincubation time of the H₂S/SeO₃²⁻ mixture on the time-dependent reduction of cPTIO. The reduction of cPTIO (100 μmol L⁻¹) once added into the preincubated H₂S/SeO₃²⁻ mixture (100/50 in μmol L⁻¹, (a, b); 100/100 in μmol L⁻¹ (c, d)) was evaluated as the decrease of absorbance at 358 nm minus 420 nm (a, c) and the decrease of absorbance at 560 nm (b, d), respectively. The preincubation time of the H₂S/SeO₃²⁻ mixture was 0-40 min (see legend). (e) Impact of the preincubation time of the H₂S/SeO₃²⁻ mixture on the reduction of cPTIO. cPTIO (100 μM) was added into preincubated H₂S/SeO₃²⁻ (in μmol L⁻¹, 100/100, circles; 100/50, triangles; 100/25, diamonds) at pH 7.4, 37°C. Data represent the reduction of cPTIO in 1st minute after addition of cPTIO.
Figure 5: EPR spectra of BMPO in the presence of $O_2^-$ are modulated by $H_2S/SeO_3^{2-}$. Representative EPR spectra of the BMPO adducts were monitored in 10% v/v saturated KO$_2$/DMSO solution in 50 mmol L$^{-1}$ sodium phosphate buffer, 0.1 mmol L$^{-1}$ DTPA, pH 7.4, 37°C in the presence of the various investigated chalcogen species and 20 mmol L$^{-1}$ BMPO. Sets of individual EPR spectra of the BMPO adducts monitored upon 15 sequential scans, each 42 s (a1-h1), starting acquisition 2 min after sample preparation in: control 10% v/v KO$_2$/DMSO in the buffer (a1), the KO$_2$/DMSO in the presence of 25 μmol L$^{-1}$ SeO$_3^{2-}$ (b1), 25 μmol L$^{-1}$ H$_2$S (c1), mixture of 25/25 in μmol L$^{-1}$ H$_2$S/SeO$_3^{2-}$ (d1), 25/25 in μmol L$^{-1}$ H$_2$S/SeO$_3^{2-}$ preincubated 5 min before KO$_2$/DMSO stock solution addition (e1), 50 μmol L$^{-1}$ H$_2$S (f1), mixture of 50/25 in μmol L$^{-1}$ H$_2$S/SeO$_3^{2-}$ (g1), and 50/25 in μmol L$^{-1}$ H$_2$S/SeO$_3^{2-}$ preincubated 5 min before addition of KO$_2$/DMSO stock solution (h1). The spectra (a2-h2) show details of the accumulated first ten spectra of the (a1-h1) sets. The intensities of the time-dependent EPR spectra (a1-h1) and detailed spectra (a2-h2) are comparable; they were measured under identical EPR settings. EPR modulation amplitude 0.15 mT. (a3-h3) Comparison of the integral intensity of individual components of simulated BMPO+O$_2^-$ without (control) and with chalcogen species shown in (a1-h1). The first five EPR spectra were accumulated and used for simulation. The data represent the means of n = 2; standard error was ≤10% of the mean value. Simulated relative intensities of the two conformers of the radicals: BMPO-OOH1 (black), BMPO-OOH2 (red), BMPO-OH1 (green), BMPO-OH2 (yellow), and BMPO-C (blue).
SeO$_3^{2−}$ intravenously had minor effects on pulse BP (Figure 8(h)). The administration of 10 μmol kg$^{-1}$ of Na$_2$S transiently increased and later decreased pulse BP (Figures 8(d) and 8(h)) [54]. The i.v. administration of the mixture H$_2$S/SeO$_3^{2−}$ (10/5 in μmol kg$^{-1}$, pH ~7.4), with comparison to H$_2$S alone, eliminated pulse BP increase, but did not affect pulse BP decrease (Figures 8(e) and 8(h)). The effects of the mixture were less pronounced at pH ~11 and were similar to those observed for H$_2$S alone (Figures 8(f) and 8(h)). The inefficiency of the
H\textsubscript{2}S/SeO\textsubscript{3}\textsuperscript{2–} products at pH ~ 11 may be connected with the minor effect of the mixture on the cPTIO radical reduction at high pH (Figures 3(e) and 3(f)). This may imply that the reduction properties of the mixture could be responsible for their effects on systolic and pulse BP. The results confirm that the products of the H\textsubscript{2}S/SeO\textsubscript{3}\textsuperscript{2–} interaction depend on pH and influence differently the cardiovascular system. In conclusion, the reactivity and biological activity of the H\textsubscript{2}S/SeO\textsubscript{3}\textsuperscript{2–} interaction products prepared at pH 7.4 differ from those of Na\textsubscript{2}S alone.

4. Discussion and Conclusions

Overall, our studies demonstrate that the two suspected and commonly used “antioxidants,” H\textsubscript{2}S and SeO\textsubscript{3}\textsuperscript{2–}, are not necessarily typical reducing agents, such as ascorbic acid or tocopherol, when employed on their own. Interestingly, these two chalcogen agents, when added together, rapidly activate each other and form a cascade of considerably more reactive, often reducing species, supposing the involvement of inorganic HSSeSH and polysulfides S\textsubscript{x}–, which may account for some of the observed biological actions. The nature of some of these intermediate reactive sulfur and/or selenium species was suggested in a recently published review [40].

The fast and efficient reduction of the cPTIO radical by H\textsubscript{2}S/SeO\textsubscript{3}\textsuperscript{2–} products (Figures 1 and 4) supports the notion that the initial intermediate(s) formed by the reaction of H\textsubscript{2}S with SeO\textsubscript{3}\textsuperscript{2–} are responsible for this kind of action. The bell shape and the maximum radical scavenging activity of H\textsubscript{2}S:SeO\textsubscript{3}\textsuperscript{2–} at a ratio of ~4:1 (Figure 2) may indicate the suggested formation of (HSS)\textsubscript{2}Se [40]. The kinetics and efficiency of the H\textsubscript{2}S/SeO\textsubscript{3}\textsuperscript{2–} products to reduce cPTIO (Figure 3) point out to complex pH-dependent chemical and radical reactions of the species.

Reactions of H\textsubscript{2}S or polysulfides with SeO\textsubscript{3}\textsuperscript{2–} and/or SeCl\textsubscript{4} were reported [40, 57–59]. They point towards a rapid conversion of SeO\textsubscript{3}\textsuperscript{2–} and SeCl\textsubscript{4} to an intermediate, probably HSSeSH, and a subsequent, slower reductive elimination of this intermediate to elemental (mixed) chalcogen particles and disulfides [40]. However, to our knowledge, there is no information about the formation and detection of HSSeSH in cells or its cytoprotective or other biological effect. The first synthesis of H-S-Se-S-H (1,3-dithiapristane) was reported by Hahn and Klünsch in 1994, but the stability and reactivity in aqueous solution were not investigated. Solid HSSeSH has a melting point at ~40°C. It was one component of a mixture of H\textsubscript{2}S\textsubscript{2}Se\textsubscript{2}, prepared by the interaction of 2 H\textsubscript{2}S with SeCl\textsubscript{4} [60].

The EPR data (Figure 5) once more confirm that SeO\textsubscript{3}\textsuperscript{2–} on its own is not an antioxidant; it becomes activated by reduction, for instance, by H\textsubscript{2}S, which concurrently is activated by oxidation. The mutual redox activation is fast, and, as in the case of the cPTIO radical scavenging, the pristine mixture of H\textsubscript{2}S and SeO\textsubscript{3}\textsuperscript{2–} is most active, with a decrease of activity over the time, pointing once more at simple H\textsubscript{2}S or H\textsubscript{2}S-Se, and notably HSSeSH, but not an S\textsubscript{x}Se\textsubscript{y} as being responsible for this kind of activity.

We found that the products of this described H\textsubscript{2}S/SeO\textsubscript{3}\textsuperscript{2–} interaction have several noteworthy biological effects, involving ROS scavenging, modulation of the redox state, reaction with DNA, tensing isolated aorta, and influencing BP and pulse BP (Figures 6–8). These effects obviously need to be investigated further and in considerably more detail and were not present or were less pronounced when H\textsubscript{2}S or selenite acted alone. The properties of the products of the H\textsubscript{2}S/SeO\textsubscript{3}\textsuperscript{2–} interactions significantly depended on the H\textsubscript{2}S/SeO\textsubscript{3}\textsuperscript{2–} molar ratio, pH, and preincubation time. The combination of these variables makes the work with H\textsubscript{2}S/SeO\textsubscript{3}\textsuperscript{2–} very complicated, and these facts should be taken into account at the time of designing in vitro and in vivo experiments. These properties of the products may explain the previously published beneficial and contrasting toxic Se
H₂S is endogenously produced in vivo in most, if not in all, cells, and H₂S donors are commonly used in biological experiments, and they are considered to be applied in medicine. Our results suggest that in biological experiments with selenite, in its nutrition supplement and clinical use, effects of the H₂S/SeO₃²⁻ interaction should be considered. While SeO₃²⁻ is used widely as a nutritional supplement already, one may, in the future, wish to spice it up with some reduced sulfur. Natural spices such as garlic and onions contain suitable sulhide-releasing agents, such as diallyltrisulfide (DATS) and diallyltetrasulfide (DATTS), which both occur naturally in garlic, or dipropyltrisulfide and dipropyltetrasulfide, both present in onions [61]. Our results imply that application research of suitable H₂S/SeO₃²⁻ supplements may lead to the beneficial effects in pathological conditions arising, e.g., from ROS overproduction.

**Figure 8:** The time-dependent effects of SeO₃²⁻, H₂S, and H₂S/SeO₃²⁻ on rat BP and pulse BP. Representative traces of the time-dependent effect of i.v. bolus administration of H₂S (10 μmol kg⁻¹; (a, d)) and its mixture with 5 μmol kg⁻¹ SeO₃²⁻ prepared at pH ~ 7.4 (b, e) and pH ~ 11 (c, f) solution on BP (a, b, c) and pulse BP (d, e, f). Transient changes of rat BP (g) and pulse BP (h) after i.v. bolus administration of SeO₃²⁻ (5 μmol kg⁻¹, empty column), H₂S (10 μmol kg⁻¹, empty coarse column) and their mixture (SeO₃²⁻/H₂S, 5/10 in μmol kg⁻¹) prepared at pH ~ 7.4 (grey column) and pH ~ 11 (grey coarse column). Data are presented as means ± SD; n = 5-10. To test a statistical significance between group differences, we used one-way ANOVA followed by Dunnett’s test for multiple comparisons. Hence, we also observed the biphasic effect of Na₂S in our previous study [54]; we compared a set of “first part” and “second part” effects of SeO₃²⁻, H₂S/SeO₃²⁻ at pH ~ 7.4, and H₂S/SeO₃²⁻ at pH ~ 11.0 to the corresponding effect of Na₂S on systolic or pulse blood pressure. Only the mixture of H₂S/SeO₃²⁻ prepared at pH ~ 11.0 was able to generate similar decrease and subsequent increase or vice versa in systolic blood pressure or pulse blood pressure as the H₂S, respectively. Asterisks mark the statistical significance as follows: **P < 0.01, ***P < 0.001, and ****P < 0.0001.
Abbreviations

ABS: Absorbance
BMPO: 5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide
BP: Blood pressure
cPTIO: 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DTPA: Diethylenetriaminepentaacetic acid
EPR: Electron paramagnetic resonance
H₂S: Hydrogen sulfide
H₂O₂: Hydrogen peroxide
i.p.: Intraperitoneal
i.v.: Intravenous
NA: Noradrenaline
pDNA: Plasmid DNA
SeCl₂: Selenium tetrachloride
SeO₃²⁻: Selenite
UV/VIS: Ultraviolet-visible.

Data Availability

All findings and conclusions are based on the presented figures in the main text or in the supplementary information. Original source files (UV-VIS spectra, EPR spectra, DNA gels, rat blood pressure records, and aorta relaxation records) can be sent from the corresponding author, Dr. Karol Ondrias, upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors’ Contributions

K.O. and M.C. conceived, initiated, and coordinated the study; K.O., M.C, and V.B. designed research; K.O., M.G., A.M., and A.K. performed UV-VIS experiments and analyzed data; V.B., K.O., M.G., and A.M. performed EPR experiments and analyzed data; M.C. and A.M. performed pDNA cleavage experiments and analyzed data; L.T., A.M., K.O., S.C., A.B., and P.B. performed aorta experiments and analyzed data; L.K., A.M., S.C., A.B., and K.O. performed rat in vivo experiments. K.O. wrote the paper; K.O., M.C., V.B., A.K., and E.A-D contributed to analyze data and manuscript writing. Marian Grman and Anton Misak contributed equally to this work. Miroslav Chovanc and Karol Ondrias shared senior authorship.

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Supplementary Materials

The supplementary information’s file (PDF) contains only additional figures with legends to the main manuscript text. (Supplementary Materials)

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