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Biochemistry of Redox-Active Sulfur Compounds in Mammalian Cells and Approaches to Their Detection (A Review)

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Abstract—The discovery of new classes of regulatory molecules in human and animal metabolism always leads to a large-scale study of their properties in the context of biochemistry, physiology, and pharmacology. About 20 years ago, hydrogen sulfide (H_2S) and its derivatives called reactive sulfur species (RSS), such as persulfides, polysulfides, nitrosothiols, sulfenic acids, and others became one of such classes of molecules. The participation of RSS in a variety of physiological and pathological processes, including the regulation of vascular tone, inflammation, long-term potentiation in the central nervous system, etc., has been shown. Changes in RSS levels or patterns of modification of their targets are associated with a wide range of cardiovascular, oncological, neurodegenerative, and other pathologies. For a part of these processes, mechanisms have been studied that involve direct modification of regulatory (NF-κB, Keap1) or effector (GAFD, eNOS, TRPA1) proteins through reactions of cysteine residues and metal-containing centers with APS. The presence of different regulated enzymatic systems producing RSS and numerous molecular targets allows us to consider H_2S and its derivatives as an important class of small regulatory molecules. H_2S is belongs to the class of so-called "gasotransmitters," along with nitric oxide (II) and carbon monoxide. Over the last 20 years, a vast amount of data on the biochemistry of these compounds and approaches to their study has been accumulated.

Keywords: H_2S, polysulfides, persulfides, reactive sulfur species (RSS), persulfidation, detection of RSS

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CONTENTS

1. INTRODUCTION

About 20 years ago, researchers began to closely study the biological role of reactive sulfur species (RSS) such as hydrogen sulfide (H_2S) and its derivatives, the main of which are polysulfides, persulfides, and nitrosothiols (Fig. 1). This class of compounds has been proven to have a signaling function inside the cell, which is due to their reactive capacity with a large number of intracellular targets [1–3].

Over two decades, hundreds of research paper on the biochemical, physiological, and potentially therapeutic properties of RSS have been published. However, due to the extremely vast scope of participation of these compounds in the functioning of the organism, there are still many blind spots in this area of research [4–8]. RSS have been shown to be involved in numerous global processes, such as reducing the oxidative stress caused by various biologically important oxidants, including superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and others [9]. H_2S directly interacts with reactive compounds and reduces their concentration. It also participated in the regulation of expression of several genes encoding proteins of the antioxidant systems [10]. Some of the first physiological effects attributed to H_2S were its cardioprotective [11] and vasodilating [2, 12] effects, the mechanism of which currently remains unclear. Moreover, RSS have been shown to have an antiapoptotic [13, 14] effect on the cells, as well as non-linear, concentration-dependent anti- and proinflammatory functions [15]. Changes in the levels of intracellular RSS are associated with a large number of pathological properties, including Huntington's and Alzheimer's disease, ischemic stroke, cancer, sepsis, and others [16–19].

2. CHEMICAL PROPERTIES AND METABOLISM OF RSS

Hydrogen sulfide (H_2S) is a toxic gas with a strong unpleasant odor of rotten eggs. H_2S is highly soluble and water and is able to penetrate through the lipid bilayer.

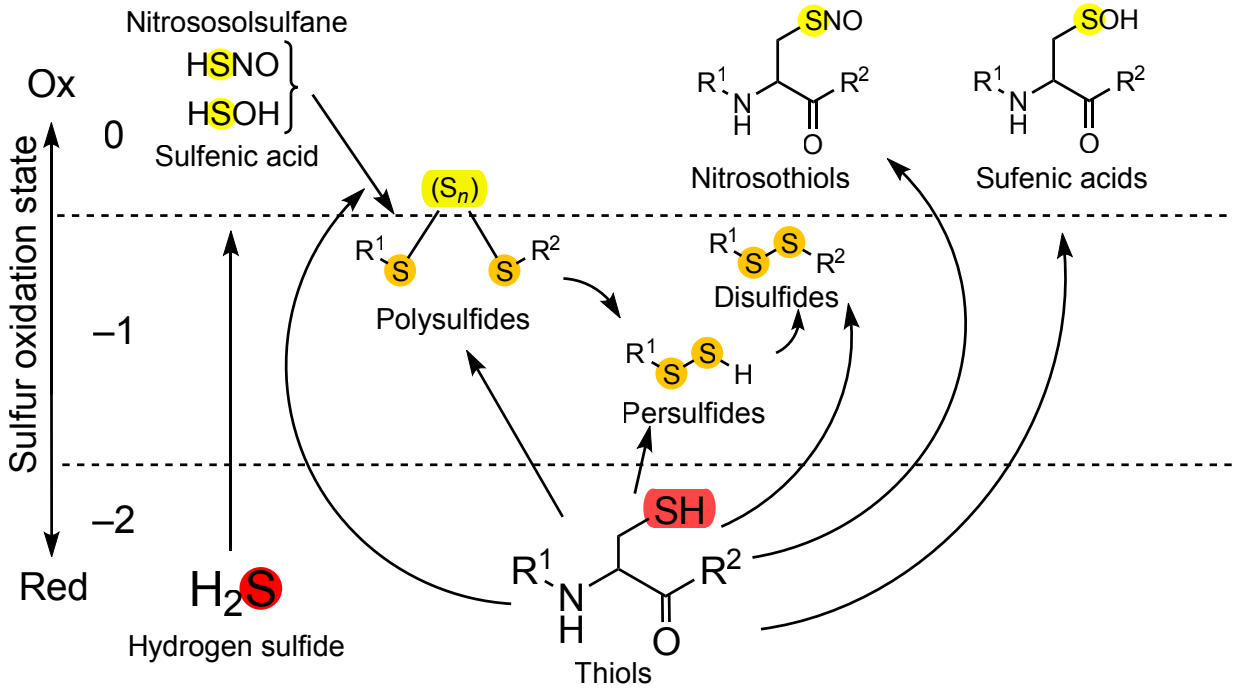

Fig. 1. Reactive sulfur species, ordered by formal oxidation states of sulfur atoms. Arrows indicate genealogical connections discussed further in the text.

In aqueous solution, it is a weak dibasic acid (reactions (1) and (2)):

$$H_2S \leftrightharpoons HS^- + H^+, \quad (1)$$

$$HS^- \leftrightharpoons S^{2-} + H^+. \quad (2)$$

The pK_{a1} value for the first reaction is 6.84 at 37°C [20], while the pK_{a2} was determined by various researches to be in the range from 12.5 [21] to 19 [22] at 25°C. At physiological pH values (7.4) and at 37°C, the ratio between the HS^- and H_2S forms is ~4 : 1, while the concentration of the S^{2-} form is extremely low (10^{-12} M) [23]. Further in the text, we will designate both the H_2S and the HS^- forms as "H_2S." H_2S (especially in the HS^- form) is a potent nucleophile due to the localized negative charge, high polarizability, and relatively low electronegativity of the sulfur atom. However, unlike thiolate anions in which the RS^- group is bonded to carbon, after the first reaction with an electrophile (reaction (3)), an ionization reaction can occur again, leading to subsequent interaction with another electrophile (reaction (4)):

$$HS^- + E_1^+ \rightarrow E_1\text{-}SH, \quad (3)$$

$$E_1S^- + E_2^+ \rightarrow E_1\text{-}S\text{-}E_2. \quad (4)$$

Thus, the reaction of H_2S with disulfides is approximately an order of magnitude slower than the same reaction of thiolates (thiol-disulfide exchange), which is due to a stronger positive inductive effect, higher polarizability, and solvation effect of the latter [24]. In H_2S, sulfur is in the –2 oxidation state. In the cell, H_2S is a potent two-electron reducing agent. Its redox potential $E^{o\prime}$ (HS_2^-, H^+ /2HS^-) against the hydrogen electrode is –0.23 V, which is comparable with the redox potentials of cysteine and glutathione [25]. However, its one-electron redox potential ($S^{\cdot-}$, H^+/HS^-) against the hydrogen electrode is +0.91 V [24], which is comparable to the corresponding values for thiols ($E°(RS^{\cdot}$, $H^+/RSH) = +0.96$ V). One-electron oxidation occurs efficiently due to the high rate of the subsequent reactions with the resulting sulfanyl radical $HS^{\cdot}$, which result in the formation of persulfides (H_2S_2) [27]. H_2S can oxidate to various products, including sulfates (SO_4^{2-}), sulfites (SO_3^{2-}), thiosulfates ($S_2O_3^{2-}$), polysulfides (RSS_nSR), and elemental sulfur (S_n) (Fig. 1). Recently, crosstalk between signaling pathways mediated by H_2S and nitrogen oxide (NO) has attracted increasing interest from researchers [28]. The interaction of H_2S and NO leads to the formation of stable nitrosothiols (HS–NO) and nitroxyls (HNO), with the latter appearing as a result of the decomposition of HS–NO [9]. HNO, as a donor of NO^+ particles, has a different reactivity pattern from NO: for instance, nitroxyl can react directly with thiols to form sulfines and disulfides [29].

Signaling and paracrine regulation involving H_2S are due to its ability to permeate the membrane because of its high hydrophobic phase/water distribution coefficient. The membrane permeability of hydrogen sulfide increases at lower pH levels [24]. Transmembrane transfer of H_2S occurs at high speed (11.9 cm/s) and, apparently, without the participation of transporters and channels [30]. Due to its high membrane permeability and the large amount of potential targets for interaction, which, as described below, determines the regulation of physiological effects, H_2S is considered an important molecule from the group of gasotransmitters along with nitrogen oxide (II) and carbon monoxide (CO) [31].

Formation of H_2S in human and mammalian cells involves several enzymes that participate in the metabolism of sulfur-containing amino acids: pyridoxal-5′-phosphate-dependent cystathionine-β-synthase (CBS, EC 4.2.1.22), cystathionine γ-lyase/cystathionase (CSE, EC 4.4.1.1), and pyridoxal-independent 3-mercaptopyruvate sulfurtransferase (MST, EC 2.8.1.2) (Fig. 2). These enzymes are characterized by different tissue expression profiles and diverge in the efficiency of H_2S synthesis, substrate specificity, and mechanisms of activity regulation, all of which together allow to fine-tune the levels of intracellular H_2S [32].

CBS exhibits the greatest activity in the brain, especially in the astrocytes [33], but also in vascular endothelium and blood plasma [34]. As a substrate for the synthesis of H_2S, this enzyme can use either two cysteine molecules a cysteine–homocysteine pair. CBS has a regulatory heme-containing domains that is able to interact with other gasotransmitters, such as NO or CO, which significantly reduces CBS activity and

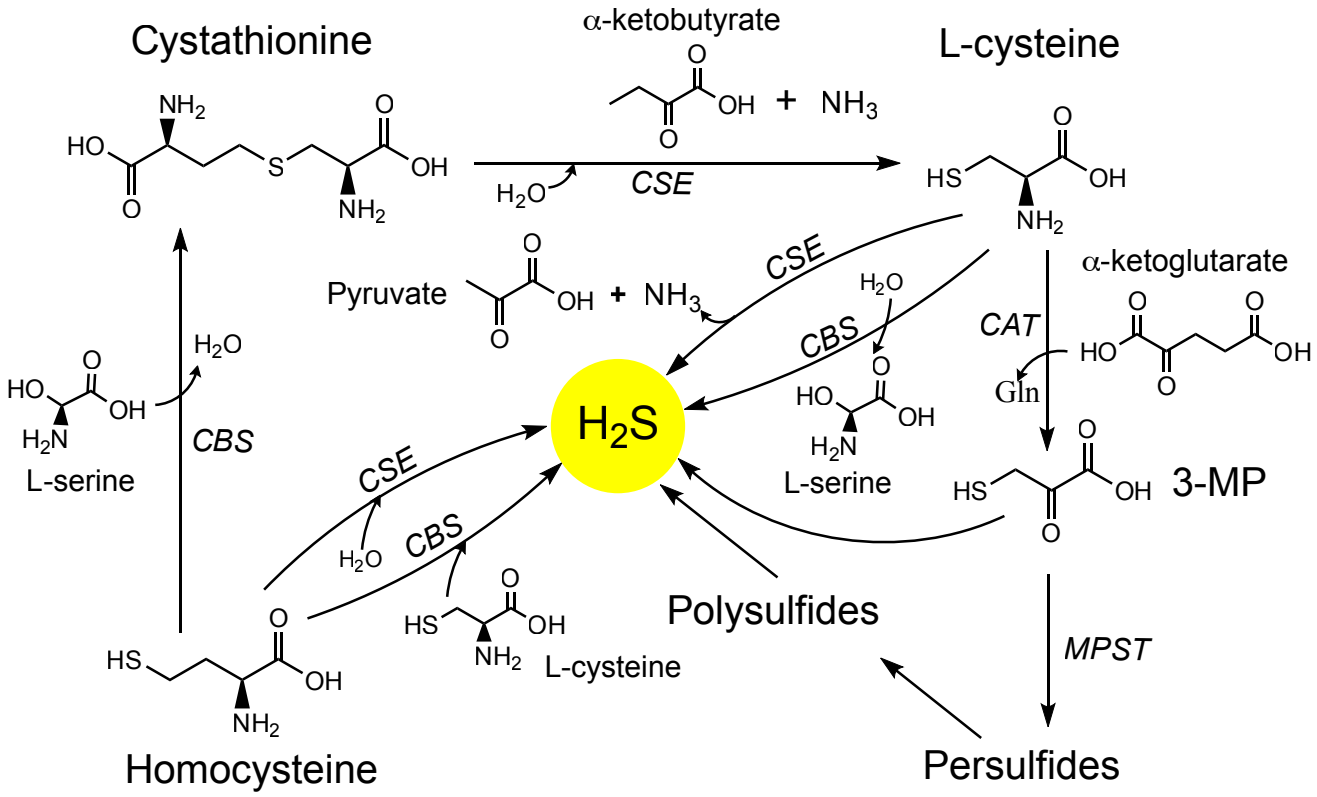

Fig. 2. Main pathways of H_2S biosynthesis in mammalians. CBS, cystathionine-β-synthase; CSE, cystathionine γ-lyase; CAT, cysteine aminotransferase; 3-MP, 3-mercaptopyruvate; MPST, 3-mercaptopyruvate sulfur transferase.

affects signaling [35]. On the contrary, the binding of *S*-adenosylmethionine (SAM), a metabolite of methionine and one of the main methylation coenzymes, increases the catalytic activity of CBS [36]. However, at the same time, the affinity of heme to CO and NO is increased, which accelerates their binding and inhibition of the enzyme [36]. Under oxidative stress, glutathionylation of CBS is observed. This modification increases the activity of the enzyme [37], which increases cysteine production and enhances glutathione synthesis.

CSE is an enzyme that demonstrates the highest H_2S-producing activity in the heart, liver, vessels, and blood plasma [38] and uses primarily homocysteine or cysteine for the synthesis of H_2S [39] (Fig. 2). CSE activity is increased in the presence of the Ca^{2+}–calmodulin complex [49]. Generally, CSE and CBS are characterized by substrate promiscuity, since these enzymes catalyze several reactions. Apparently, this is due to the peculiarities of the pyridoxal-dependent catalysis. The majority of reactions catalyzed by CSE and CBS is accompanied by the release of H_2S. However, due to high K_M for substrates and low concentrations of some substrates, not all of these reactions make a significant contribution to the overall production of H_2S.

MST, which is localized both in the mitochondrial matrix and cytoplasm, catalyzes the formation of H_2S from 3-mercaptopyruvate. The highest H_2S-producing MST activity has been shown in the brain, kidneys, and some immune cells [41]. 3-Mercaptopyruvate is formed by transamination of cysteine by cysteine aminotransferase (CAT, EC 2.6.1.3) or by direct oxidative deamination of D-cysteine by D-amino acid oxidase (DAAO, EC 1.4.3.3), which also produces H_2S. The reaction catalyzed by MST involves the transfer of the sulfur atom for 3-mercaptopyruvate to the cysteine residue of the enzymes. During the reaction, stable MST persulfide and pyruvate are formed, and the enzyme regenerates when a nucleophile attacks the persulfide.

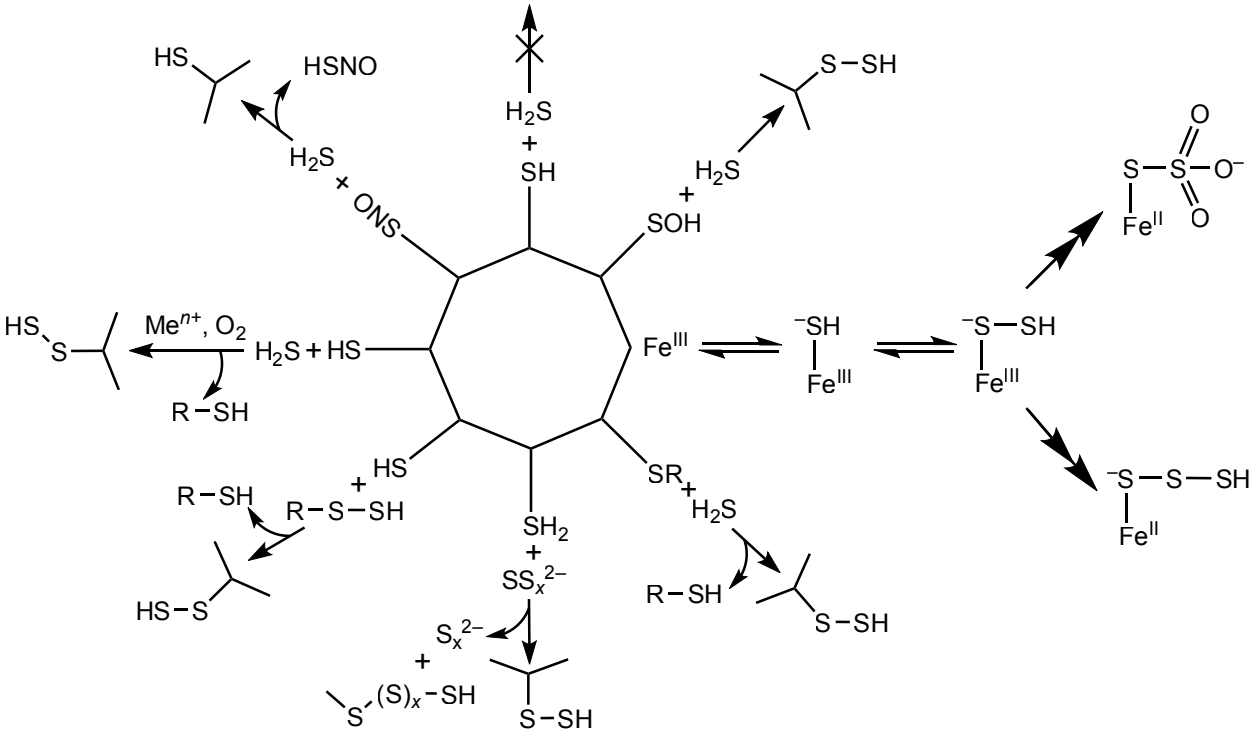

Fig. 3. Main RSS-mediated protein modifications, see comments in the text.

The thioredoxin system reacts most effectively with the MST persulfide. Cyanide ion, dihydrolipoate, cysteine, homocysteine, and glutathione also interact with the MST persulfide with decreasing effectiveness [42]. Currently, little is known about the regulation of the MST activity.

Gut microbiota also serves as a source of H_2S in the mammalian body. In particular, bacteria from the *Desulfovibrio* genus use sulfate as a final electron acceptor in the respiratory chain and reduce it to H_2S [18]. H_2S can be formed during the breakdown of thiosulfates, which are products of the oxidation of sulfur compounds in the mitochondria [43].

The main reactions of H_2S with biomolecules include binding to and/or redox reactions with metal-containing centers, cross-signaling with reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as redox reaction with cellular cysteine derivatives with the formation of persulfides.

H_2S interacts with metal-containing and heme centers [44] (Fig. 3). Firstly, H_2S is able to directly coordinate to a metal ion. Secondly, H_2S is able to reduce the metal ion with the formation of $HS^{\cdot}$ radicals and subsequent products of oxidation. Thirdly, H_2S is able to covalently modify heme porphyrins [45]. For instance, at low concentrations, H_2S binds to the Fe^{3+} ion in one of the centers of cytochrome *c* oxidase (complex IV of the respiratory chain, EC 1.9.3.1) and reduces it, which increases its affinity to oxygen and enhances tissue respiration [46, 47]. At higher intracellular concentrations, H_2S covalently binds heme centers of cytochrome *c* oxidase and reduces the Cu^{2+} ions, which leads to its inhibition and uncoupling of the respiratory chain [46, 48]. Moreover, upon binding to heme proteins under aerobic conditions, H_2S is oxidized with the formation of persulfides and thiosulfates (Fig. 4) [49].

For disposal of excess H_2S and biosynthesis of oxidized sulfur compounds, intracellular enzymatic systems of H_2S catabolism exist, the main one of which is the

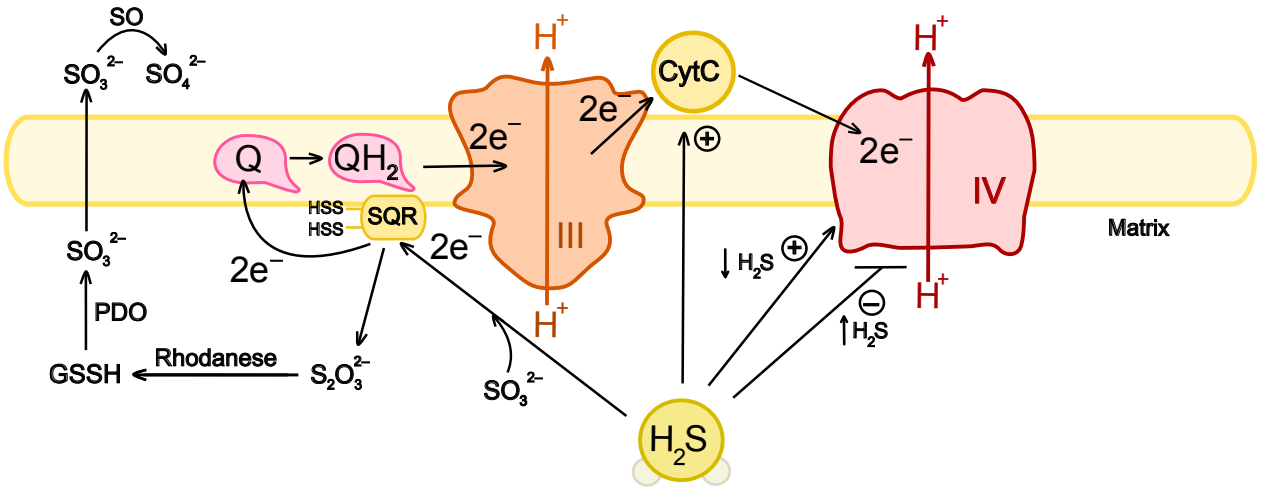

Fig. 4. Mitochondrial H_2S metabolism and its interaction with respiratory chain components. CytC, cytochrome *c*; Q, ubiquinone; QH_2, ubiquinol; PDO, persulfide dioxygenase; SQR, sulfide:quinone reductase; SO, sulfite oxidase; III, complex III of the respiratory chain (ubiquinol:cytochrome *c*-reductase); IV, complex IV of the respiratory chain (cytochrome *c* oxidase).

mitochondrial pathway of H_2S oxidation (Fig. 4). The first reaction is catalyzed by sulfide : quinone reductase (SQR, EC 1.8.5.4), an enzyme of the inner mitochondrial membrane, which performs two-electron oxidation of H_2S to thiosulfate or glutathione persulfide (GSSH) depending on the acceptor (sulfite or glutathione (GSH)) and reduction of ubiquinone to ubiquinol [50]. During this process, electrons enter the ETC, thus making sulfide the first discovered inorganic electron doner in the mammalian respiratory chain [50]. Next, if the reaction product is thiosulfate, it enters into a rhodanese reaction and forms GSSH. Then, GSSH is oxidized by persulfide dioxygenase (PDO, EC 1.13.11.18) to sulfite ion and GSH. The sulfite ion is then oxidized to sulfate by sulfite oxidase (SO, EC 1.8.3.1) [51].

The main mechanism responsible for the participation of H_2S in the cellular signaling is thought to be based on oxidative post-translational modification of cysteine residues, which is called persulfidation (Fig. 3) [33]. Persulfidation occurs under physiological conditions and is increased under oxidative stress. Similar to other oxidative modifications of protein thiol groups, persulfidation can both activate and inactivate individual proteins, thus potentially adapting their functions to changing conditions. Moreover, persulfidation can prevent the irreversible oxidation of thiols. However, the exact mechanism of this process has not yet been discovered [52]. It is assumed that there are two main mechanisms for non-enzymatic protein persulfidation. First, some protein thiol groups can enter into two subsequent reactions: first with hydrogen peroxide (H_2O_2), then with H_2S, which leads to the formation of persulfides. Secondly, a range of proteins is able to interact directly with H_2S via a disulfide bond with the formation of persulfides [53]. However, both reactions are very slow and cannot explain the fact that many proteins are found in the persulfidated state even in the absence of oxidative stress. It is also known that low-molecular-weight persulfides, such as glutathione and cysteine persulfides (GSSH and Cys-SSH) formed in the cells, are able to transfer singular sulfur atoms to thiols [54], although their participation in persulfidation has not yet been confirmed. It is also assumed that post-translational protein persulfidation is performed enzymatically by one or several sulfur transferases. Recently, experimental confirmation of this assumption has been obtained [52]. It has been shown that MST, an enzyme that is generally associated with H_2S production and, as previously thought, is able to transfer sulfur atoms onto thiol groups of only two proteins, thioredoxin and MOCS3/Uba4 for subsequent thiolation of tRNA and urmilation of proteins [55], actually possesses a persulfidating acti-

vity towards a much wider range of proteins. For instance, in *in vitro* experiments, as well as in yeast culture studies, it has been shown that even the fluorescent protein roGFP and bovine serum albumin are sulfur atom acceptors for MST. In *in vitro* experiments, it has also been demonstrated that this enzyme is not the main producer of inorganic polysulfides, which reinforces the idea that direct transsulfuration is the predominant mode of MST-mediated protein persulfidation. Moreover, in human cell culture it has been shown that depletion of MST pool leads to a general decrease in the protein persulfidation levels [52]. The above data, together with previous observations that overexpression of MST increases the content of intracellular "bound" sulfane sulfur (S0), potentially indicating a direct role of this enzyme in the general protein persulfidation [56], allow us to conclude that MST indeed has the ability to persulfidate various proteins under physiological conditions.

Persulfides, along with polysulfides, are the most extensively studies RSS. Together, they are called sulfane sulfur compounds or simply sulfane compounds. There are several pathways of their formation. Some of them have already been described above, while the others are equally important in the biochemistry of RSS. Thus, H_2S can react directly with disulfides, especially in the compartments where their concentration is elevated due to oxidative conditions, such as in the lumen of endoplasmic reticulum [57]. Another persulfide formation pathway is the reaction of H_2S with sulfenic acid residues (–SOH) in proteins and low molecular weight compounds formed in the cell by oxidation by hypohalic acids and/or peroxides [24]. The third biosynthesis pathway is the reaction of H_2S with nitrosothiol residues (RSNO) in proteins and low molecular weight compounds [58].

The important role of co-translational incorporation of cysteine persulfides, generated during the catalysis of cysteinyl-tRNA formation by cysteinyl-tRNA synthetases (EC 6.1.1.16), has been shown. Cysteine persulfide can be considered a proteogenic amino acid, as it is incorporated in the polypeptide chain already at the stage of translation instead of corresponding cysteines [59]. This may play the following physiological role: formation of cysteine sulfones is irreversible under cellular conditions and is observed under pronounced or local oxidative stress; in persulfidated residues, the distal sulfur atom is oxidized and can be detached by cellular antioxidant systems along with the reduction of intact cysteine residue. Thus, in a persulfidated state, proteins are better protected from oxidative stress [60].

Per- and polysulfides are more reactive than H_2S. In similar reactions, they exhibit more pronounced nucleophile properties and are regarded as direct participants of H_2S signaling. This is due to their lower pK_a values relative to thiols and H_2S, which leads to the high availability of the anionic form, but at the same time to the presence of the α-effect [24, 61–63]. Interestingly, sulfane compounds exhibit electrophile properties that are almost absent in H_2S and thiols. For instance, polysulfides may act as electrophiles in reaction with low-molecular-weight thiols and protein cysteine residues with the formation of polythiolated cysteine residues (R-$(S)_n$-SH) with varied numbers of sulfur atoms. Subsequently, if there are spatially close cysteine residues or other thiols, di-, tri-, tetra-, and pentasulfide bonds can be formed [64]. H_2S is not able to directly produce such modifications, although similar effects have been observed in experiments with its exogenous addition, which is likely due to the use of pre-oxygenated H_2S donors [61].

For persulfidation as a regulatory modification, mechanisms of its removal have also been shown. Two enzymatic antioxidant systems are involved in this process: the thioredoxin and glutaredoxin-glutathione reductase systems [65, 66]. They perform NADPH-dependent depersulfidation of CysSSH residues and glutathione persulfides with the formation of H_2S and the corresponding thiols. However, presently, the regulation of these processes and their efficiency for various protein targets remain poorly understood.

3. MAIN PHYSIOLOGICAL EFFECTS AND TARGETS OF RSS

Hydrogen sulfide and its derivatives, mainly poly- and persulfides, are signaling molecules that actively participate in the regulation of physiological and biochemical processes not only in cells and tissues of mam-

mals, but also of bacteria and plants. First studies on the regulatory role of H_2S began at the end of the XX century in the context of its effect on neuromodulation [1]. In mammals, endogenous H_2S controls a range of physiological processes and participates in the regulation of pathogenesis of various diseases, such as hypertension, atherosclerosis, myocardial infarction, and others [16]. H_2S and its derivatives have been shown to possess antiapoptotic activity, which is important when studying the action of H_2S as an effector molecule in a model of ischemia-reperfusion and inflammation. For instance, H_2S-mediated persulfidation of the Cys-38 residue of the p65 subunit of NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells) leads to its translocation to the nucleus and to the activation of expression of TNF receptor associated factors, c-FLP, and cellular inhibitors of apoptosis [8, 67]. TNFR (tumor necrosis factor receptor) expression also leads to the increased expression of CTH, which enhances H_2S production [14]. H_2S also persulfidates the Cys-151 residue of Keap1 (Kelch-like ECH-associated protein 1), which, according to a yet unknown mechanism, leads to the translocation of Nrf2 (NF-E2-related nuclear factor 2) to the nucleus and activates the expression of cytoprotective proteins, such as glutathione *S*-transferase, ferritin, epoxide hydrolase [40, 68]. Moreover, H_2S persulfidates the Cys-341 residue of MEK1 and induces subsequent phosphorylation of ERK1/2 with further activation of PARP1 (poly ADP-ribose polymerase 1), which generally activates DNA reparation pathways [69]. Therefore, RSS affects the main signaling pathways that function in various body systems.

3.1. Effect of RSS on the Cardiovascular System

It has been established that RSS affect the cardiovascular system: in particular, they reduce the damaging effect of ischemia-reperfusion on the myocardium, stimulate angiogenesis, cause relaxation of smooth muscles, and participate in the regulation of arterial pressure [10]. One of the first biological activities of H_2S is vasodilation [7], so the effect of H_2S on the state of vessels is an important factor for the investigation of mechanisms of development of such pathological conditions as arterial hypertension and ischemia-reperfusion. This property of H_2S is used in the development of drugs for the treatment of hypertension; for instance, H_2S donors are being investigated as antihypertensive drugs [4]. However, the mechanism of H_2S-mediated dilatation has not yet been fully studied. It is known that H_2S stimulates vasodilatation through the activation of the ATP-dependent potassium channel due to persulfidation, which leads to the hyperpolarization of smooth muscle cells in the vascular walls [70]. On the other hand, H_2S action on Ca^{2+}-dependent K^+ channels causes their blockage, which, possibly due to different concentration dependencies and degrees of enrichment, does not lead to vasoconstriction [71]. It should be noted that H_2S acts synergistically with ·NO. Therefore, there is a direct interaction between H_2S and either ·NO or enzymes of ·NO synthesis. The combined action of H_2S and ·NO is also linked to the formation of nitrosothiols in neurons that regulate the vascular tone [72]. Nitrosothiols activate TRPA channels and increase intraneural calcium levels. This results in the exocytosis of vesicles containing CGRP (calcitonin gen-related peptide), which, in turn, activates the G-protein-coupled receptor on the smooth muscle cells in the vascular walls [73]. This event promotes the activation of adenylate cyclase, cAMP synthesis, and activation of protein kinase A. Protein kinase A is potentially capable of enhancing the activity of endothelial ·NO synthase [73]. Persulfidation of the Cys-443 residue of ·NO synthase enhances enzyme activity, which increases ·NO production and, in turn, leads to the activation of the soluble guanylate cyclase. It is known that H_2S promotes the binding of ·NO by the guanylate cyclase [74]. In this case, the synthesis of cGMP is activated, while its breakdown is suppressed due to the binding of H_2S to Zn^{2+} ions in the phosphodiesterase. The described effects lead to vasodilatation (Fig. 5) [75].

Various endothelial signals, such as the VEGF (vascular endothelial growth factor) signaling that is carried out with the participation of NADPH oxidases and the formation of H_2O_2 as a signaling molecule, lead to the increased expression and activity of CTH. In turn, CTH is the most important and active enzyme that performs H_2S synthesis in the cardiovascular

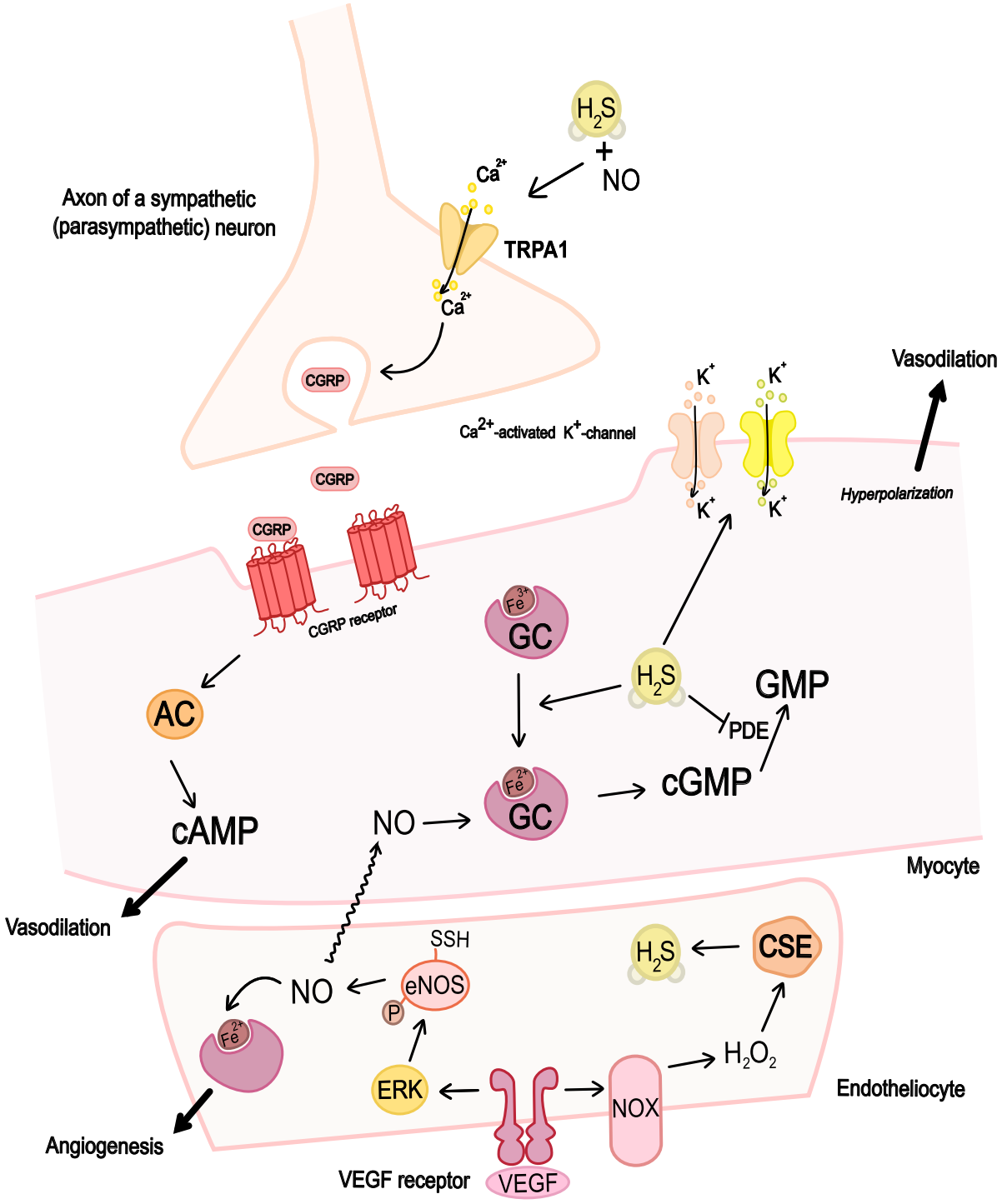

Fig. 5. Regulation of vasodilation by H_2S and ·NO. CGRP, calcitonin gene-related peptide; K_{ATP}-channel, ATP-dependent K^+ channel; AC, adenylate cyclase; GC, guanylate cyclase; CSE, cystathionine γ-lyase; VEGF, vascular endothelial growth factor; eNOS, endothelial nitric oxide synthase; NOX, NADPH oxidase.

system [76, 77]. Increased levels of H_2S and RSS enhance the activity of endothelial nitrogen oxide synthase and guanylate cyclase according to the above-described mechanism, which triggers subsequent cascades leading to the proliferation and differentiation of endothelial cells, thus stimulating angiogenesis [72]. Moreover, H_2S is able to dose-dependently increase the number of myoepithelial cells, affect their migration and the formation of myotubes through the Akt signaling pathway. In CSE knockout mice, the formation of microcapillaries was suppressed, while H_2S promoted angiogenesis through the MAPK (mitogen-activated protein kinase) signaling pathway. As a molecular switch, H_2S specifically breaks the disulfide bond Cys-1045–Cys-1024 in VEGFR2 (vascular endothelial growth factor receptor 2), which changes its conformation, thus stimulating angiogenesis [78]. Moreover, H_2S enhances STAT3 (signal transducer and activator of transcription 3) activity, as well as increases the phosphorylation of mTOR (mammalian target of rapamycin) through the VEGFR2 pathway and stimulates the proliferation of endothelial cells [79].

H_2S has a cardioprotective effect. It has been shown that both endogenous and exogenous H_2S prevents the development of atherosclerosis, arrythmia, hypertrophic cardiomyopathy, myocardial infarction, and others. This cardioprotective effect is achieved by increasing the level of phosphorylation of the serine/threonine protein kinase RACα, as well as by translocating the NRF-1 (nuclear respiratory factor 1) an Nrf2 (nuclear factor erythroid-like 2), which activates antiapoptotic signaling, inhibits apoptosis, and increases mitochondrial biogenesis [80]. Therefore, RSS (H_2S in particular) are important regulators of cardiovascular metabolism.

3.2. Effect of RSS on the Nervous System

As described above, CBS, one of the main enzymes ensuring the endogenous H_2S synthesis, is expressed in the nervous system. Studies involving immunohistochemistry have shown that this enzyme is localized in astrocytes and microglia, as well as in some neurons, such as the Purkinje cells and hippocampal neurons [81]. CBS-mediated H_2S production is linked to the activation of the Ca^{2+}/calmodulin signaling pathway and is performed after neuronal excitation. RSS and H_2S in particular affect the nervous system through modulation of neurotransmission [40]. For instance, it is known that glutamate is one of the main neuromodulators involved in learning, memory formation, long-term potentiation. Glutamate acts on NMDA (*N*-methyl-D-aspartate) receptors. H_2S increases the currents associated with this receptor by activating the adenylate cyclase and downstream cAMP/protein kinase A cascades. Persulfidation of the NR2A subunit of the NMDA receptor has been shown that is, apparently, responsible for the effects described above [82]. Moreover, RSS are able to influence glutamate secretion directly, enhancing it, which leads to neuronal death as a result of glutamate-mediated excitotoxicity [83]. Another example of the effect of H_2S on neurotransmitter receptors is its effect on the GABA (γ-aminobutyric acid) receptor. GABA is the main inhibitory neurotransmitter; deficiency in the GABAergic inhibition causes febrile seizures and increased neuronal excitability. H_2S reduces hippocampal damage caused by repeated seizures by enhancing GABAergic inhibition. This effect is realized not due to an increase in the amount of neurotransmitter, but due to an increase in the number of receptors, which has been shown both on the mRNA and protein levels [84]. This possibly occurs as a result of an increase in intracellular calcium levels due to the activation of TRPA channels, T- and L-type Ca^{2+} channels, as well as due to the stimulation of calcium-dependent transcription [85, 86]. Moreover, in the experiments involving microglia and astrocyte cell cultures, it has been shown that Cl^-/HCO_3^- and Na^+/H^+ exchangers are the targets of H_2S, and its action on them causes acidosis [87]. Another important function of H_2S in the nervous system is the inhibition of oxidative stress. For instance, H_2S prevents the HOCl-mediated inactivation of α1-antitrypsin, protein oxidation, cytotoxicity, and lipid peroxidation [88]. H_2S also protects brain endothelial cells from methionine-induced oxidative stress [89].

During the development of various neurodegenerative diseases, changes in the rates of endogenous production of RSS can be observed. Reduced H_2S levels have been

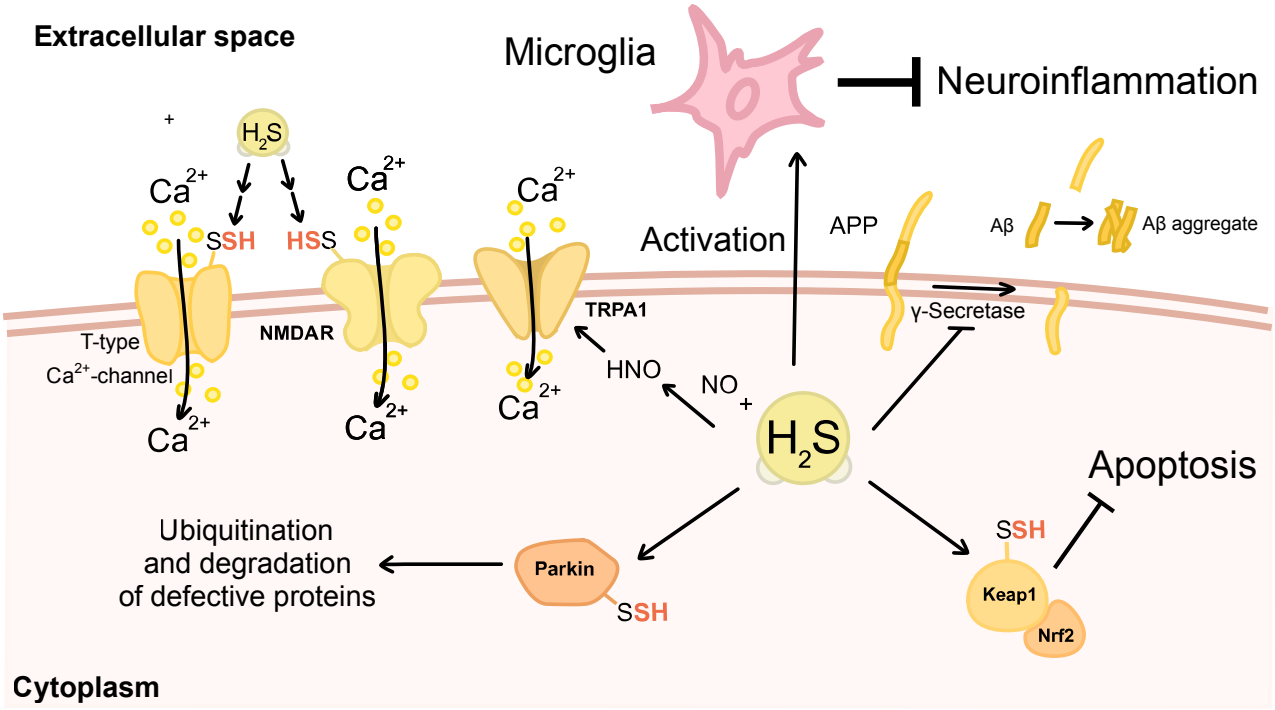

Fig. 6. RSS-mediated regulation in neurons, see comments in the text. NMDAR, ionotropic NMDA receptor; APP, amyloid precursor protein; TRPA1, transient receptor potential cation channel subfamily A member 1; Nrf2, nuclear E2 related factor 2; Keap1, Kelch-like ECH-associated protein 1.

registered in patients with Alzheimer's disease [90]. In experiments involving neuron-like cells, it has been shown that H_2S, with the participation of the PI3K/Akt signaling pathway, reduces BACE-1 expression, which is responsible for the synthesis of β-amyloid [91]. When modeling Alzheimer's disease using primary cell culture and BV-2 cell line, it has been demonstrated that H_2S is able to protect microglia from β-amyloid-induced (Aβ-induced) damage, suppressing the production of inflammatory molecules, such as NO and TNF-α, in cells treated with Aβ [92]. It is also supposed that H_2S suppressed Aβ production in neurons by inhibiting the glycosylation of the precursor protein and decreasing the activity of γ-secretase, which cleaves Aβ from the precursor protein [93] (Fig. 6).

Reduced H_2S levels are also characteristic of Huntington's disease. This is due to the fact that mutant huntingtin inhibits SP1, a transcription factor that affects CSE expression. This causes decreased expression of the enzyme, which reduces H_2S production [94].

The cytoprotective role of H_2S in Parkinson's disease has been studied in detail. This disease is associated with a significant reduction in the persulfidation of Parkin, an ubiquitin ligase, which in pathology is modified to a much lesser extent than normally. Normally, persulfidation of Parkin at certain residues increases its ubiquitin ligase activity, thus decreasing the number of defective proteins in the cytoplasm [95]. The balance between the persulfidation and nitrosylation is also important in enzyme modification. For instance, persulfidation activates Parkin, while nitrosylation decreases its activity [95]. It is known that polysulfidation has a neuroprotective action, affecting Keap1 and TRPA1 channels to a greater extent than H_2S [96]. The data described above indicates that regulation of RSS homeostasis is essential for the normal functioning of the nervous system.

3.3. Effect of RSS on the Endocrine System

Among all of the organs of the endocrine system, the pancreas and the effect of RSS on it are the most studied. The release of insulin from the islets of Langerhans is a critical event in the regulation of glucose metabolism and in the pathogenesis of insulin resistance. Exogenous H_2S in physiologically relevant concentrations significantly suppresses glucose-induced insulin release. The same effect of endogenous H_2S on insulin release has been shown in experiments with CSE overexpression. Knockdown of the gene encoding CSE, however, led to an opposite result [97]. Inhibitory effect of H_2S is largely associated with the stimulation of ATP-sensitive potassium channels in β cells [97]. In cultured INS-1E cells, it has been shown that CSE overexpression causes apoptosis and decreases cell viability, as does exposure to exogenous H_2S, due to the inhibition of ERK1/2 and simultaneous activation of p38 MAPK. Proapoptotic effect of H_2S is explained by the activation of the endoplasmic reticulum [98].

Thus, RSS can have an ambiguous impact on various organ systems depending on the concentration, demonstrating both protective and damaging properties.

3.4. Participation of RSS in Inflammation

Inflammation is a physiological protective reaction of the organism that occurs in response to tissue damage or the appearance of a pathogen and is aimed at the removal of products and agents of damage. Inflammatory reactions can be acute and chronic. Chronic inflammation is characteristic of a wide number of diseases, so it is considered the most common cause of mortality [99]. It is known that ROS and RNS are involved in the regulation of inflammatory reactions [100]. Recently, the role of RSS in the pathogenesis of inflammatory conditions has been brought to light. For instance, H_2S has been shown to be beneficial in various cardiovascular, neurodegenerative, and musculoskeletal diseases with an inflammatory component, such as osteoarthritis. It is believed that this effect is associated with persufidation. However, increasing experimental evidence suggests that polysulfides, rather than H_2S, are responsible for this post-translational modification of proteins [101]. It has been shown that polysulfides and persulfides possess antioxidant and anti-inflammatory properties. They are significantly more effective at neutralizing hydrogen peroxide than H_2S. These compounds also inhibit synthesis of TNF-α and IFN-β that are produced by macrophages in response to lipopolysaccharide-induced activation of TLR4 (Toll-like receptor 4) [102]. In experiments with concanavalin A-induced hepatitis, anti-inflammatory activity of polysulfides has been shown. Oral administration of dipropyl polysulfides to research animals led to the decreased expression of inflammatory markers, such as IL-1β, IL-12, and IL-16, while the expression of an anti-inflammatory marker, IL-10, was increased [103].

However, a lot of evidence indicates that H_2S possesses proinflammatory activity as well. For instance, pancreatitis studies have shown that the level of H_2S produced by CBS and CSE is elevated during the acute inflammatory stage of the disease. The use of inhibitors of enzymes that produce H_2S alleviated the progress of disease [104]. Donors that ensure slower release of H_2S also demonstrated an anti-inflammatory action [105]. In liver, the highest constitutive level of CSE expression is observed, which leads to a relatively high base level of H_2S. This is logical considering the fact that liver is the key organ for the production and removal of H_2S [106]. Endogenous H_2S participates in the regulation of the metabolism of glucose, lipids, and xenobiotics in the liver [107]. Changes in the rates of endogenous H_2S production lead to a range of diseases that affect the liver, including fibrosis, cirrhosis, and cancer [108]. Low H_2S concentrations have a cytoprotective effect, while high H_2S levels may be hepatotoxic [109]. Moreover, H_2S participates in the functioning of the kidneys, affecting the parameters linked to the regulation of the water-salt balance, such as the rate of glomerular filtration and reabsorption of sodium and potassium [110]. Enzymes that are responsible for H_2S synthesis (CBS, CSE, MST) are found almost in every compartment of the kidneys, with the proximal tubules having the highest expression of these enzymes [111, 112]. During sepsis, elevated H_2S levels in blood plasma and increased CSE expression can be observed, while the administration of CSE inhi-

bitors decreases the rate of leukocyte infiltration in kidneys [113].

Thus, the role of RSS in inflammation is ambiguous and requires further study. Steroid hormones and immunosuppressants are widely used for the treatment and suppression of inflammatory reactions, but these drugs are characterized by a large number of adverse effects. Evidence obtained during the last several years indicates in favor of the use of RSS donors as potential therapeutic agents, although the rate of RSS release should be taken in the account during their use.

4. DETECTION OF RSS

There are two main approaches to the detection of RSS: (1) direct, during which the concentration of the effector molecule is determined, and (2) indirect, during which RSS-mediated protein modifications are analyzed. In this section, we will discuss the main methods for the determination of levels of H_2S, persulfides, and polysulfides.

4.1. Direct RSS Detection

Methods of this group allow for quantitative of semiquantitative determination of concentration of target compounds in biological samples. In general, these methods can be divided into two groups. The first one includes methods that can be used for the monitoring of intra- and extracellular RSS levels *in vivo*. The second one includes *in vitro* methods that require violation of integrity of the samples during their preparation.

4.2. Methods of Intravital RSS Detection

Various imaging tools, including fluorescent dyes, genetically encoded fluorescent biosensors, and techniques based on the giant Raman scattering principle, are suitable for intravital studies [114, 115].

There are several groups of fluorescent dyes. The mechanism of their action is based on various reaction properties of RSS (Fig. 7), including nucleophilic and redox properties, as well as the ability to precipitate metal ions.

One of the most widely represented group of dyes utilizes the redox properties of H_2S [116]. A large number of probes has been developed that can be reduced in reactions with H_2S. The most common of them contain an azide ($–N_3$, Fig. 7a) or a nitro group ($–NO_2$, Fig. 7b) and are converted into corresponding amino derivatives, thus changing their fluorescent properties [117–119]. However, these dyes react irreversibly and are characterized by relatively low reaction rates with H_2S. Moreover, the degree of interference of other intracellular reducing agents, such as thiols, remains unclear. H_2S is able to undergo double nucleophilic reactions (Reaction (4)), while monosubstituted thiols (glutathione, cysteine), which are more widely represented in the cell, can only undergo a single nucleophilic reaction (Reaction (3)). Based on this fact, fluorescent probes with two electrophilic centers have been developed that are able to distinguish between H_2S and similar compounds. H_2S can react with a more electrophilic part of the dye with the formation of an intermediate product that contains a free SH group, which, in its turn, can undergo Michael addition or spontaneous cyclization with a change in the fluorescence parameters of the probe [120] (Fig. 7c). There are also dyes the mechanism of action of which is based on the addition of H_2S via double carbon-carbon bonds [121, 122], as well as on thiolysis reactions [123]. The third one of the main groups of dyes is based on extremely low solubility products of copper (II) and zinc sulfides ($K_{sp}(CuS) = 6.4 \times 10^{-36}$; $K_{sp}(ZnS) = 1.6 \times 10^{-24}$) [124]. The metal ion in chelated form acts as a fluorescence quencher. During the reaction with H_2S, the corresponding sulfide is formed, which is accompanied by a significant increase in fluorescence (Fig. 7d) [125]. For probes of this type, selectivity patterns are also unclear, as thiols and NO can also chelate or reduce Cu^{2+} ions. Moreover, chelation occurs most effectively with the S^{2-} form, which is the least represented at physiological pH values (pH 7.0–7.5) [126].

Probes for the detection of sulfane compounds are based on the reactions of either nucleophilic (Fig. 7e) or electrophilic (Fig. 7f) groups of the dye with the analyte with the formation of covalent adducts and subsequent increase in fluorescence due to the release of

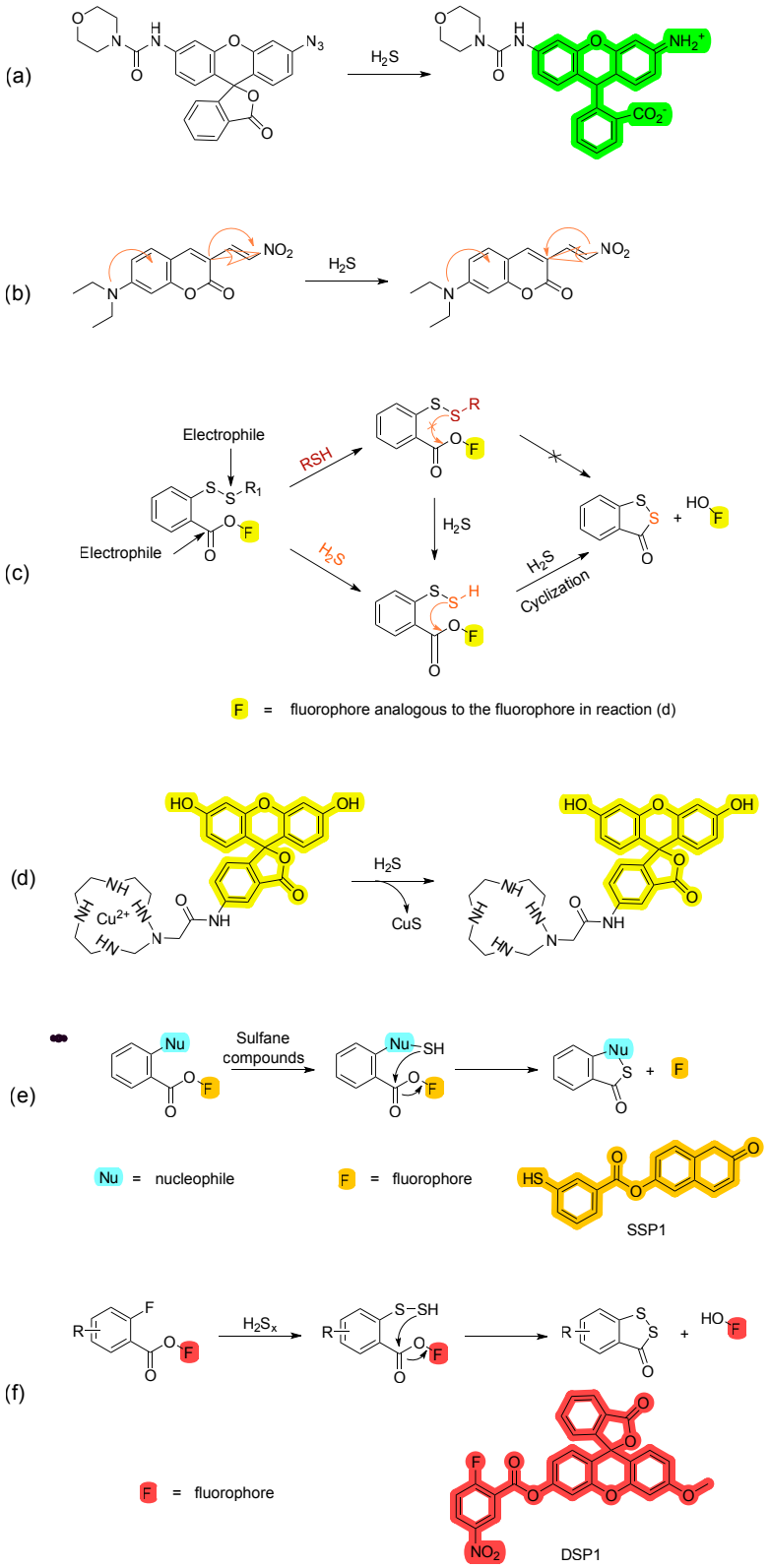

Fig. 7. Fluorescent dyes for the detection of H_2S (a–d) and sulfane compounds (e, f); (a, b) dyes thar are reduced by H_2S to an amino group from an azide (a) or nitro group (b); (c) dyes with two electrophilic groups, discrimination of H_2S from other thiol agents is shown; (d) precipitative dye based on the formation of CuS from Cu^{2+}, which leads to an increase in fluorescence; (e, f) dyes that react with sulfane compounds using nucleophilic (e) or electrophilic (f) groups, which leads to an increase in fluorescence.

the fluorophore part or, conversely, to the weakening of quenching [127–129].

Synthetic dyes have a number of disadvantages that complicate their use in biological systems, including cytotoxicity, low cell membrane permeability, irreversibility (disposability) of the response. From the point of view of practical applications, less than a dozen of described dyes is available commercially. However, the main disadvantage of such dyes is the limitations that are associated with their use *in vivo* or with targeted localization in certain intracellular compartments.

Genetically encoded fluorescent biosensors based on GFP-like proteins can be viewed as a solution to this problem as they can be easily targeted to various cellular compartments, including the nucleus, matrix and intermembrane space of mitochondria, ER). In many cases, such tools represent a single option to study compounds that are highly reactive *in vivo*. To detect H_2S, a protein GFP-based biosensor cpGFP-Tyr66pAzF has been developed, although it contains a chromophore that is synthesized from an unnatural amino acid, *p*-azidophenyltyrosine (pAzF) [130]. This amino acid is inserted in place of the tyrosine residue in the biosensor sequence and participates in the chromophore maturation, thus forming a structure that does not exhibit fluorescent properties in the same way as the azide dyes described above. However, upon reaction with H_2S, the azide group is reduced to an amino group, which leads to a significant increase in fluorescence. The disadvantage of this approach is the irreversibility of this modification, therefore the cpGFP-Tyr66pAzF biosensor can be used only once. This type of sensors has not been widely used in practice, mainly due to the complexity of application not only in *in vivo* models, but also in cell cultures. This is explained by the fact that, to assemble such a biosensor in the cellular system, it is necessary to introduce not only its gene, but also the corresponding amino acid, the aminoacyl-tRNA synthetase gene, and the aminoacyl-tRNA.

Other genetically encoded fluorescent biosensors for the registration of sulfane compounds, psGFP and psRFP (modified mCherry), are based on the insertion of two cysteine residues in the proximity of the fluorophore. These redox-active cysteine residues are placed at such a distance that does not allow the formation of a disulfide bond between them, but is available for the formation of a trisulfide bond upon interaction with persulfides and polysulfides. The formation of a bond between these residues of the fluorescent proteins leads to a change in its spectral characteristics [131, 132]. Biosensors of this type are non-selective as they are able to react with a variety of biologically relevant oxidants [133, 134].

4.3. Biochemical Methods of RSS Detection

There is a wide variety of biochemical approaches for the *ex vivo* analysis of samples or homogenates that allow for accurate quantitative assessment of the studied modifications. At the same time, information on the spatial and temporal dynamics of RSS is lost. Measurement artifacts associated with the release of labile sulfur, such as from iron-sulfur clusters, as well as with the loss of gaseous H_2S from the samples at physiological pH values are possible [135]. A large number of works has been devoted to the analysis of the applications and peculiarities of these methods [6, 125, 136–138]. Among them, approaches for the detection of H_2S using colorimetric methods (e.g. based on methylene blue) with formation of derivatives are the most widespread [139]. The use of high-performance liquid chromatography with direct chemiluminescent detection [140], with detection of fluorescence produced by the derivatives of monobromobimane and H_2S [141], as well as combined with mass spectrometry [64] is also widespread.

To optimize the speed of measurements and make possible the miniaturization of devices to further implant them in living organisms, electrochemical sensors based on ion-selective [142] or polarographic [143] electrodes can be used. However, existing methods are characterized by large differences in sensitivity (by orders of magnitude), so there is still no "golden standard" for detecting H_2S in biological samples yet. When these methods are used to measure similar samples, conflicting estimates of H_2S level can be obtained [144, 145].

To measure the levels of sulfane compounds, cold cyanolysis [24, 146], HPLC of derivatives of the alky-

lating agents (e.g., monobromobimane [64] or iodacetamide [147]) combined with mass spectrometry, as well as isotope dilution mass spectrometry [148] can be used. Most of these methods are characterized by the problem of selectivity towards certain groups of sulfane compounds, which is further complicated during work with complex biological samples (homogenates, biological fluids). Moreover, persulfides exhibit ow stability during sample preparation. Because of this, large discrepancies are observed when assessing the levels of sulfane compounds in similar samples using these methods [24, 146].

4.4. Detection of RSS-Mediated Protein Modifications

Methods for the analysis of end products of the reactions involving RSS-modified proteins that alter their properties or activity in the cell are widely used. Among these modifications, persulfides and polysulfides of cysteine residues are of interested. Despite the fact that these approaches often allow to identify protein regulatory targets and even specific modified residues, it remains impossible to directly tie changes in the RSS levels to changes in the modification patterns. These methods are not always quantitative and they cannot be used to monitor processes with high time resolution (minutes and even hours). Direct determination of the presence of such modifications is complicated, firstly, due to the instability of the persulfide group, including during mass spectrometry; secondly, due to the extreme similarity in $\Delta m/z$ in persulfidation and oxidation to sulfinic acid residues [149]. A popular approach is the use of covalent tags that specifically react with RSS-modified cysteine residues. It is important that such tags do not interact with alternative modifications of cysteine residues. One of the first such approaches was the method involving *S*-methyl methanethiosulfonate (MMTS) and *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide (biotin-HPDP), which are sequentially added to the sample. As a result, specific biotinylation of the persulfidated proteins occurs, with further isolation of such proteins on a streptavidin scaffold (Fig. 8a). Then, the fraction of the obtained persufidated proteins can be analyzed with immunoblotting or mass spectrometry [150]. However, it has been shown that non-modified cysteine residues can also become biotinylated when using this method; intra- and intermolecular disulfide bonds can also be formed [6, 151].

An easy-to-use method is to treat the sample with a fluorescent derivative of *N*-ethylmaleimide (NEM-Cy5) that alkylates both cysteine residues and their persulfidated modifications [14]. After treatment with a reducing agent (DTT), fluorescence of persulfidated proteins decreases due to the dissociation of the fluorescent tag, which can be detected in parallel electrophoretic separations (Fig. 8b). It should be noted that NEM is able to react with amino groups [152], which decreases the selectivity of this method. To optimize this approach in order to obtain higher resolution, biotin-conjugated NEM derivatives are used. They allow to purify the obtained peptides containing persulfidated cysteine residues using HPLC-MS after trypsinolysis (Fig. 8c) [24, 153]. Modifications of this method using isotope tags allow to conduct quantitative proteomic studies. However, analysis can be difficult due to the cross-reactivity of NEM derivatives with cysteine sulfinic acid residues [154]. The problem of this method, associated with underestimating the levels of modified residues, is solved in the methods where only persulfidated residues are adsorbed. In one of the methods, it was proposed to use methylsulfonyl benzothiazole (MSBT) as a blocking agent that modifies both thiol and persulfide groups. However, during the next stage, the adduct of the persulfide and MSBT with increased electrophilicity becomes the target of either fluorescent (CN-Cy3) or biotinylated (CN-biotin) derivatives of cyanoacetic acid (Fig. 8*d*). For the latter, affinity chromatography and mass spectrometry are performed [57]. The disadvantages of this method include the low solubility of MSBT derivatives in aqueous solutions and low stability of the cyanoacetate adducts during mass spectrometry [24, 153]. Authors of this method have proposed a number of changes that facilitate the experimental procedure due to the use of commercially available reagents that are more selective towards persulfides [77].

Data from proteomic studies, mostly performed with the methods described above, differ in their estimates of the content of the persulfide modifications of the

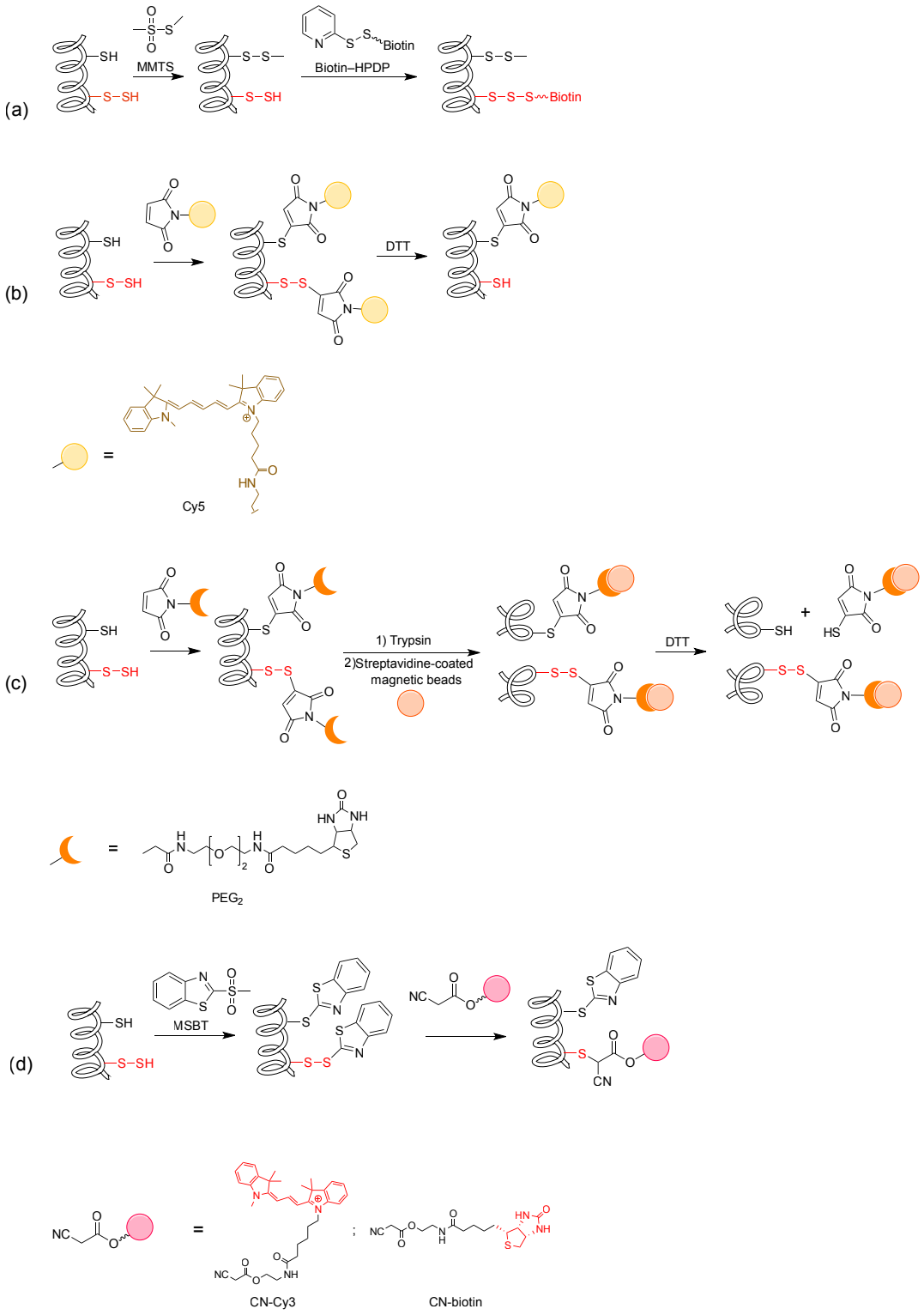

Fig. 8. Approaches for the detection of RSS-mediated modifications of proteins and peptides. MMTS, *S*-methyl methanethiosulfonate; biotin-HPDP, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide; DTT, dithiothreitol; PEG, polyethylene glycol; MSBT, methylsulfonyl benzothiazole. See comment in the text.

cysteine residues. These estimates vary from ~5% (for the proteome of MIN6, a mouse pancreatic cell line) to 0.15% (for the proteome of HEK293). This difference might reflect the real physiological differences between various cell types, but also can be explained by the use of different methods [66, 153]. Therefore, the development of reliable methods for studying RSS dynamics and detecting the modifications of their target remains an urgent task.

5. CONCLUSIONS

Reactive sulfur species are important members of various signaling cascades that regulate both the physiological and pathological processes in living systems. There is convincing evidence that disturbances in the synthesis or catabolism of RSS correlate with the development of various socially significant diseases, such as Alzheimer's disease, lateral amyotrophic sclerosis, Parkinson's disease, diabetes, cardiovascular diseases, and others [155]. Recently, both H_2S donors and RSS themselves are increasingly used as therapeutic agents [156, 167]. However, despite the progress achieved in recent decades in establishing the mechanisms of formation, catabolism, and action of RSS, much remains poorly understood, not least because of the ack of tools that allow for *in vivo* studies with high selectivity and high time resolution. Because of this, the development of methods for RSS detection remains an urgent task both in chemistry and biotechnology. Expanding the palette of dyes and genetically encoded tools will both increase the pool of information on the participation of H_2S and its derivatives in biological processes and allow to select potential targets and drugs for the treatment of various diseases.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies involving patients or animals as test objects.

Informed consent was not required for this article.

CONFLICT OF INTEREST

No conflicts of interest was declared by the authors.

AUTHOR CONTRIBUTION

All authors made an equal contribution to the writing of this article.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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