Persulfidation (S-sulfhydration) and H$_2$S

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Abstract
The past decade has witnessed the discovery of hydrogen sulfide (H\textsubscript{2}S) as a new signalling molecule. Its ability to act as a neurotransmitter, regulator of blood pressure, immunomodulator or anti-apoptotic agent, together with its great pharmacological potential, is now well established. Notwithstanding the growing body of evidence showing the biological roles of H\textsubscript{2}S, the gap between the macroscopic descriptions and the actual mechanism(s) behind these processes is getting larger. The reactivity towards reactive oxygen and nitrogen species and/or metal centres cannot explain this plethora of biological effects. Therefore, a mechanism involving modification of protein cysteine residues to form protein persulfides is proposed. It is alternatively called S-sulfhydration. Persulfides are not particularly stable and show increased reactivity when compared to free thiols. Detection of protein persulfides is still facing methodological limitations, and mechanisms by which H\textsubscript{2}S causes this modification are still largely scarce. Persulfidation of protein such as K\textsubscript{ATP} could contribute to H\textsubscript{2}S-induced vasodilation, while S-sulfhydration of GAPDH and NF-\kappaB inhibits apoptosis. H\textsubscript{2}S regulates endoplasmic reticulum stress by causing persulfidation of PTP-1B. Several other proteins have been found to be regulated by this posttranslational modification of cysteine. This review article provides a critical overview of the current state of the literature addressing protein S-sulfhydration, with particular emphasis on the challenges and future research directions in this particular field.

Keywords
Hydrogen sulfide • Polysulfides • Sulfenic acids • Persulfidation • S-sulfhydration • S-nitrosation

1 H\textsubscript{2}S as a Signalling Molecule

In order to maintain life, nature actually uses a limited number of chemical reactions, one of which is sulfur-based chemistry, mainly exploited for the control of intracellular redox homeostasis and redox-based signalling. Hydrogen sulfide (H\textsubscript{2}S) is the simplest of the thiols found in the cells, and ever since the first report of its potential physiological role (Abe and Kimura 1996), there has been a growing literature on the subject of H\textsubscript{2}S signalling. Very fast, H\textsubscript{2}S joined the other two gases, nitric oxide (NO) and carbon monoxide (CO), as the third gasotransmitter (Wang 2002; Li et al. 2009; Mustafa et al. 2009a).

Numerous are the physiological functions assigned to be exclusively or partly regulated by H\textsubscript{2}S, some of which are vasodilation (Yang et al. 2008; Mustafa et al. 2011), neurotransmission (Abe and Kimura 1996; Kimura et al. 2005), angiogenesis (Papapetropoulos et al. 2009; Szabó and Papapetropoulos 2011), inflammation (Li et al. 2005; Whiteman and Winyard 2011), hypoxia sensing
(Olson et al. 2008; Peng et al. 2010), etc. In addition, H₂S showed a tremendous pharmacological potential in preventing ischemia–reperfusion injury (Calvert et al. 2009, 2010). Furthermore, H₂S is able to induce suspended animation-like state in mice (Blackstone et al. 2005). Several pharmacological donors of H₂S have been developed with hope of their eventual use in disease treatment (Sparatore et al. 2008; Zhou et al. 2012; Szczesny et al. 2014; Zhao et al. 2013).

H₂S is produced by the action of at least three enzymes, cystathionine beta synthase (CBS), cystathionine gamma lyase (CSE) and mercaptopyruvate sulfurtransferase (MST) (Kabil et al. 2014; Kabil and Banerjee 2014). Differently expressed in different tissues (and even cellular compartments), these enzymes control H₂S production with different efficiencies. How are these enzymes regulated to maintain spatio-temporal production/distribution of H₂S is still unclear.

The intracellular levels of H₂S are also a matter of debate, with values spanning from nondetectable to >100 μM, although it seems more probable that the steady-state levels are at low micromolar and/or submicromolar levels, depending on the tissue (Olson 2012; Olson et al. 2014; Kabil et al. 2014). Conversely, the flux of H₂S production is huge, almost as that of glutathione, suggesting that the removal of H₂S is an efficient and tightly regulated process (Vitvitsky et al. 2012; Kabil et al. 2014; Kabil and Banerjee 2014).

Hydrogen sulfide is a weak acid and immediately ionizes in aqueous solution reaching the equilibrium between H₂S/HS⁻/S²⁻ species:

\[ \text{H}_2\text{S} \rightleftharpoons \text{H}^+ + \text{HS}^- \rightleftharpoons 2\text{H}^+ + \text{S}^{2-} \]

The pKₐ₁ is around 6.9, while pKₐ₂ is estimated to be >12, which means that under physiological conditions, approximately two thirds of H₂S are in the form of bisulfide (hydrogen(sulfide)(1-)), with negligible amounts of sulfide anion (sulfide(2-)). H₂S diffuses freely through the membranes (Mathai et al. 2009; Cuevasanta et al. 2012). Although the diffusion coefficient profile of H₂S is systematically lower than that of H₂O, the differences in the transmembrane Gibbs energy profiles are more dominant. Because of its hydrophobicity, H₂S experiences no barrier to permeation, so it can partition into the interior of the membrane readily (Riahi and Rowley 2014).

Sulfur atom in H₂S is in −2 oxidation state, but sulfur is very versatile in its ability to accept or donate electrons. It can cycle between −2 and +6 oxidation state, due to the six valence electrons and completely empty 3d orbital. Although the standard redox potential often cited in the literature for the two electron oxidation of H₂S to sulfur is 0.144 V (which makes H₂S a weaker reducing agent than cysteine or glutathione), another standard redox potential could be found for the alkaline conditions, which suggests that H₂S is a stronger reducing agent:

\[ \text{S}^{(s)} + 2e^- + 2\text{H}^+ \rightarrow \text{H}_2\text{S}^{(aq)} \quad E^0 = 0.144 \text{ V} \]
\[ \text{S}^{(s)} + 2e^- + \text{H}_2\text{O} \rightarrow \text{HS}^- + \text{OH}^- \quad E^0 = -0.476 \text{ V} \]

H₂S does not react readily with oxygen; however, the solutions of H₂S undergo oxidation, just like the solutions of other thiols (such as cysteine and glutathione).
This process is believed to be catalysed by the traces of metal ions present in the solution (Kotronarou and Hoffmann 1991); therefore, the thorough cleaning of solutions from heavy metals and removal of oxygen can keep H$_2$S solution stable for a while (Wedmann et al. 2014). Oxidation products of H$_2$S in solution are polysulfides, sulfites, thiosulfites and eventually elemental sulfur.

In biological systems, reactivity of H$_2$S could be divided in three groups of reactions: (1) reaction with/scavenging of reactive oxygen (ROS) and reactive nitrogen species (RNS), (2) binding to and/or subsequent redox reactions with metal centres and (3) reaction with proteins, herein called persulfidation (alternatively S-sulfhydration) (Fig. 1).

1.1 Reactions with ROS and RNS

H$_2$S reacts readily with hypochloric acid (HClO), produced by neutrophils, leading to the formation of polysulfides (Nagy and Winterbourn 2010). H$_2$S also reacts with peroxynitrite (Carballal et al. 2011; Filipovic et al. 2012a), in a reaction that generates thionitrate (HSNO$_2$) isomer, which can decompose and serve as an NO donor (Filipovic et al. 2012a). In addition, H$_2$S can also scavenge superoxide (Wedmann et al. 2014). Although the rates of these reactions are higher than those found for cysteine or glutathione, they are not so much higher that can overcome the difference in concentration (particularly when compared to glutathione which is present in millimolar steady-state levels) suggesting that H$_2$S cannot really serve as an antioxidant. Nonetheless, H$_2$S has been shown to have antioxidant and immediate protective effects in the cells exposed to ROS and RNS, something that cannot simply be explained by the modulation of proteins and gene expression. Since soluble macromolecules occupy a significant fraction of the total cell volume (Fulton 1982), within such crowded medium relative size and
shape of a molecule and probability of its successful diffusion, placement and
effective contact with a potential target become crucial factors that significantly
alter its reaction rates (Minton 1998). This speaks in favour of hydrogen sulfdide,
when compared to GSH, despite the difference in steady-state concentrations
(Filipovic et al. 2012a).

H₂S reacts with NO signalling pathways as well (Whiteman et al. 2006; Ali
et al. 2006; Yong et al. 2010, 2011; Filipovic et al. 2012b, 2013; Eberhardt
et al. 2014). With protein S-nitrosothiols, H₂S reacts to form the smallest
S-nitrosothiols, thionitrous acid (HSNO), which can freely diffuse through the
membrane and serve as trans-nitrosating agent (Filipovic et al. 2012b). More
importantly, H₂S can react directly with NO giving nitroxyl (HNO), the one-
electron-reduced sibling of NO which possesses the signalling properties of its
own (Eberhardt et al. 2014). 2 μM combination of NO and H₂S gives the same rate
of HNO generation as ~1 mM Angeli’s salt, the commonly used pharmacological
source of nitroxyl. Eberhardt et al. (2014) recently showed that co-localization of
H₂S and NO production facilitate intracellular generation of HNO which then
activates transient receptor potential A1 channel (TRPA1) leading to the Ca
²⁺
influx and to the release of calcitonin gene-related peptide (CGRP), the strongest
known vasodilator.

1.2 Reactions with Metal Centres

One of the main biological targets for H₂S would be metal centres. H₂S can
coordinate and then additionally reduce the metal centre. In haemproteins, polar
active site favours the reduction, while nonpolar centres favour coordination only
(Pietri et al. 2009). Indeed, the presence of positive charges around the haem centres
leads to a complete change in the haems’ reactivity, allowing the design of efficient
catalysts for H₂S removal (Ivanovic and Filipovic 2012).

H₂S binds to both haem a₃ and Cu₈ centres of cytochrome c oxidase, with Kᵢ
being 0.2 μM for the purified enzyme (Hill et al. 1984; Nicholls et al. 2013). This
suggests that cytochrome c oxidase should be permanently inhibited under physio-
logical conditions (considering the micromolar steady-state concentrations of H₂S),
which is obviously not the case. In fact H₂S shows biphasic effects on whole cell
respiration, stimulating it at low doses and then completely inhibiting it at higher
concentrations (Koenitzer et al. 2007). Binding of H₂S to haem has an important
function in mollusc, *Lucina pectinata*, where H₂S binds to haemoglobin I and is
then transported to the symbiotic chemoautotrophic bacteria living in their gills
(Ríos-González et al. 2014).

Reaction of oxyhaemoglobin with H₂S leads to the green pigment,
sulphaemoglobin, which is in fact a chemically modified porphyrin centre. This
subsequently affects oxygen-binding capacity of that haemoglobin. H₂S also binds
and reduces cytochrome c, a reaction that in the presence of oxygen leads to
superoxide production (Wedmann et al. 2014). In addition, Pálinkás et al. (2014)
have recently investigated interactions of H₂S with human myeloperoxidase
(MPO), a major contributor to inflammatory oxidative stress, to show that \( \text{H}_2\text{S} \) inhibits the enzyme by reducing iron centre and by binding to the reduced \( \text{Fe}^{2+} \).

It is still, however, unclear to which extent is the coordination and/or reduction of metal centres involved in signalling by \( \text{H}_2\text{S} \). Miljkovic et al. (2013) demonstrated that metal centres in mitochondria are responsible for the \( \text{H}_2\text{S} \)-stimulated haem centre-catalysed reduction of nitrite, a reaction which can explain the use of nitrite as an antidote for acute \( \text{H}_2\text{S} \) poisoning.

### 1.3 Protein Persulfides

The third way of direct \( \text{H}_2\text{S} \) signalling would be modulation of proteins by modification which is named S-sulfhydration, although the more correct term should be persulfidation. Protein persulfides add up to the list of oxidative posttranslational modifications (oxPTMs) of cysteine, such as S-nitrosation, S-sulfenylation and S-glutathionylation (Fig. 2). Modification of proteins by \( \text{H}_2\text{S} \) could explain the plethora of effects that \( \text{H}_2\text{S} \) exhibit and several proteins have been identified to be indeed controlled by this modification (Mustafa et al. 2009a, b, 2011; Paul and Snyder 2012). However, this field of research is at its beginning, and it is still facing difficulties/challenges such as the proper choice of detection method, understanding of the mechanism(s) by which persulfidation takes place and the actual impact it has on the cellular functions.

Based on the calculation of the bond energies of GSSG and GSSH, the latter has ~18 kJ mol\(^{-1}\) lower bond energy (Filipovic et al. 2012b) so due to their inherent instability, there is limited information about persulfide reactivity to date. Francoleon et al. (2011) were among the first who reported preparation of the glutathione and papain persulfides, while Pan and Carroll (2013) successfully prepared persulfide on glutathione peroxidase 3. Zhang et al. (2014) also reported

**Fig. 2** Oxidative posttranslational modifications of protein cysteine residues known to regulate protein structure/ function
facile preparation of persulfide of bovine serum albumin (BSA), which can be used as a model to study protein persulfide reactivity.

Electronegativity of sulfur is almost identical to that of carbon; therefore in R-S-SH, the sulfur atom covalently bound to carbon could be considered as sulfane sulfur. Sulfane sulfur is sulfur with six valence electrons and a formal charge 0, often represented by S\(^0\). This sulfane sulfur could be a good target for nucleophilic attack, while the other sulfur atom, which is formally −1, could react with electrophiles. The pK\(_a\) of persulfides is lower than that of corresponding thiols, suggesting that at physiological conditions, majority of persulfide would be in deprotonated form (R-S-S\(^{−}\)), making the persulfide “super” nucleophilic. This dramatically increases persulfides’ reactivity when compared to the corresponding thiols, as recently demonstrated for the reaction with H\(_2\)O\(_2\) (Ida et al. 2014) and previously for the reduction of cytochrome c\(^{3+}\) (Francoleon et al. 2011).

Artaud and Galardon (2014) synthesized a persulfide analogue of the nitrosothiol SNAP, which opens up new possibilities for examining metastable low molecular weight (LMW) persulfides. The authors clearly demonstrate that spontaneous decay of LMW persulfides does not lead to H\(_2\)S release, but when mixed with other thiols, such as glutathione, an immediate H\(_2\)S release could be observed. Additionally, Bailey et al. (2014) characterized tritylhydrosulfide (TrtSSH), another LMW persulfide, to show that protonated form does not react with nucleophiles, while it readily reacts with electrophiles and reducing agents. Protonation leads to no change in substance stability, while the deprotonation stimulates decomposition with the elimination of elemental sulfur.

Based on the current literature data, the following characteristics of persulfides could be described:

1. Persulfides are metastable species which decompose in solution in a complex manner, leading to the formation of elemental sulfur, among other molecules.
2. Persulfides are much better nucleophiles than corresponding thiols which can explain their better reactivity.
3. Persulfides are better reducing agents than corresponding thiols.
4. Persulfides readily react with electrophiles.
5. Persulfides could transfer sulfane sulfur to other thiols, leading to trans-persulfidation (vide infra).

### 2 Persulfide Formation

Although there is a growing interest for persulfidation of proteins, only a few studies actually addressed the issue of the mechanism(s) behind protein S-sulfhydration. The original misconception (which tends to overtake this whole field) was that thiolate on the protein can react directly with H\(_2\)S to form protein persulfide (Fig. 3a). However, that reaction is impossible due to the thermodynamic constrains. Both sulfur atoms get oxidized in the reaction so the electrons will have to end up on protons, leading to elimination of hydrogen as a gas. Incubation of
proteins, such as GAPDH, BSA or immunoglobulins with H\textsubscript{2}S, led to no detectable protein S-sulfhydration (Zhang et al. 2014; Wedmann et al. 2014) confirming the theory.

So how are the proteins modified by H\textsubscript{2}S? Getting the answer(s) to this question is of the utmost importance for our understanding of H\textsubscript{2}S signalling and also for the interpretation of the vast amount of data accumulated to date.

![Fig. 3 Proposed reaction mechanisms for persulfide formation.](image)

- (a) A direct reaction between protein thiols and H\textsubscript{2}S is not possible, but H\textsubscript{2}S can react with sulfenic acids (b).
- (c) Reaction of S-nitrosated cysteines with H\textsubscript{2}S leads to the formation of HSNO, but depending on the protein environment surrounding the thiol, it is also possible to generate protein persulfides in this reaction.
- (d) H\textsubscript{2}S could react with already existing inter- or intramolecular disulfides, while sulfane sulfur in polysulfides could react directly with protein thiols and give persulfide (e).
- (f) Metal centres could act as oxidants for the formation of protein persulfides from H\textsubscript{2}S and protein thiols.
- (g) Persulfides could serve as carriers of sulfane sulfur and engage in “trans-S-sulfhydration” reaction.
2.1 Enzymatic Generation of Persulfides

Despite the recent interest for protein persulfidation, sparkled by the discovery of H$_2$S as a signalling molecule, protein persulfides formed as intermediates that facilitate sulfur delivery in several biosynthetic pathways have been known for a while (Mueller 2006). Persulfides are found to be formed in sulfurtransferases and cysteine desulfurases. Particularly interesting examples are the two enzymes involved in H$_2$S production and its oxidation, mercaptopyruvate sulfurtransferase (MST) and sulfide–quinone oxidoreductase (SQR), respectively.

MST is expressed in both mitochondria and cytoplasm and could be found in kidney cells, liver and cardiac cells, neuroglial cells, etc. (Kabil and Banerjee 2014; Kimura 2014). Recent studies suggest that MST is an important source of H$_2$S in some organisms and tissues (Mikami et al. 2011; Mödis et al. 2013). Although it was known for a while that during the reaction protein persulfide is formed in the catalytic site (MST-Cys-S-SH), the mechanism of H$_2$S release has been only recently discovered. Yadav et al. (2013) reported the first crystal structure of MST with its cysteine residue in the form of persulfide. This allowed them to propose a detailed reaction mechanism, while kinetic analysis of the reaction led to conclusion that thioredoxin is likely to be the major physiological persulfide acceptor for MST (Fig. 4a).

The same group, led by Ruma Banerjee, elucidated the full mechanism of SQR (Libiad et al. 2014). The sulfide oxidation pathway begins with SQR, and it also includes a sulfur dioxygenase, rhodanese and sulfite oxidase (Fig. 4b). By consuming H$_2$S and its persulfide products, SQR and sulfur dioxygenase are important switch-off regulators of sulfide signalling (Bouillaud and Blachier 2011). In the first step, SQR catalyses the oxidation of H$_2$S to sulfane sulfur, which remains covalently attached to the enzyme. In the second step, this sulfane sulfur could be transferred to sulfite, to form thiosulfate (Jackson et al. 2012), but recently, Libiad et al. (2014) showed that in fact glutathione is a more probable acceptor of sulfane sulfur. This leads to the generation of glutathione persulfide, which can be consumed by rhodanese to actually form thiosulfate rather than to use it as a substrate (Fig. 4b).

Recent work on reactive cysteine persulfides and S-polythiolation has shaken our understanding of the action of CBS and CSE (Ida et al. 2014). Namely, the authors demonstrated that persulfide formation by CSE and CBS-mediated CysSSCys metabolism are facile and more likely a source of biological persulfides (Fig. 4c). Therefore, the direct enzymatic production of per- and polysulfide that is highly prevalent has been suggested. In fact, the authors question the role of H$_2$S as a major signalling molecule suggesting that Cys-based persulfides may be the actual signalling species (Ono et al. 2014).
2.2 Direct Nonenzymatic Generation of Persulfides by $H_2S$

An obvious similarity between protein S-sulfhydration and S-glutathionylation could be drawn. Based on known mechanisms for the generation of glutathionylated proteins, it is possible to assume that the same reactions could lead to generation of protein persulfides.

2.2.1 Reaction with Sulfenic Acids

Protein S-sulfenylation (Fig. 2) is the reversible oxidation of protein thiols to sulfenic acids (R-SOH) that has been recognized as an important oxidative post-translational modification of cysteines (Paulsen and Carroll 2013; Gupta and Carroll 2014). Most recently, an extensive study was published identifying the
whole cell sulfenylome, i.e. site-specific mapping and quantification of protein S-sulfenylation in the cells (Yang et al. 2014). More than 1000 proteins have been characterized as modified by sulfenylation. Peroxiredoxins are the most abundant antioxidant enzymes in the cytosol, and their catalytic cycle relies on sulfenic acid formation (Poynton and Hampton 2014). Sulfenic acid formation is shown to be involved in H$_2$O$_2$-mediated inactivation of protein tyrosine phosphatases (PTPs) (Paulsen et al. 2011). Also, reversible sulfenylation is shown to switch on or off the activity of transcription factors such as OxyR, OhrR or Orp1-Yap1 (for extensive review, see Paulsen and Carroll 2012). Epidermal growth factor receptor and the phosphatasases SHP2, PTEN and PTP1B were all found to be sulfenylated upon regular signalling by epidermal growth factor (Paulsen et al. 2011).

The biochemistry of sulfenic acid formation, reactivity and functions has been extensively reviewed recently (Paulsen and Carroll 2013; Gupta and Carroll 2014). Sulfenic acids are known to react with thiols to form disulfides. The same could be said for the reaction of sulfenic acids with H$_2$S (Fig. 3b). Namely, in the recent study, Zhang et al. (2014) showed that formation of sulfenic acids on GAPDH and subsequent reaction with H$_2$S do indeed lead to the formation of protein persulfides. Furthermore, they used sulfenic acid of bovine serum albumin (BSA), known as an example of a relatively stable protein sulfenic acid (Carballal et al. 2003), to successfully generate S-sulfhydrated BSA. Although the steady-state concentration of H$_2$S is orders of magnitude lower than that of glutathione (Olson et al. 2014; Kabil et al. 2014), which makes it difficult for H$_2$S to compete for the reaction with protein sulfenic acids, the high flux of H$_2$S generation (Vitvitsky et al. 2012), its free diffusion (Mathai et al. 2009; Cuevasanta et al. 2012) and therefore its ability to reach deeper parts of the proteins suggest that the reaction of H$_2$S with sulfenic acids could still be a major source of protein persulfide formation (Fig. 3b). Indeed, Zhang et al. (2014) find that intracellular persulfidation co-localizes with endoplasmic reticulum, an organelle rich with sulfenic acids.

It is worth noting that sulfenic acids can be formed from the thiols just by the presence of traces of metal ions in the buffers and oxygen (Paulsen and Carroll 2013). Considering that R-SOH could react with H$_2$S to form protein persulfides, these reaction steps could be an explanation for the misconception that H$_2$S reacts directly with thiols.

### 2.2.2 Reaction with S-Nitrosothiols

Protein S-nitrosation is considered by some to be the second most important posttranslational modification of proteins. The number of proteins found to be controlled by this modification is constantly increasing. To date, S-nitrosation has been implicated in the regulation of proteins involved in muscle contractility, neuronal transmission, host defence, cell trafficking, apoptosis, etc. (Hess and Stamler 2012; Seth and Stamler 2011; Foster et al. 2009; Lima et al. 2010). S-nitrosation of haemoglobin has been proposed to regulate its ability to release oxygen (Reynolds et al. 2013). S-nitrosation of ryanodine receptors regulates intracellular Ca$^{2+}$ levels (Xu et al. 1998) as does S-nitrosation of transient receptor potential cation channels (TRPs) (Yoshida et al. 2006). The role of S-nitrosation in
controlling the protein function has been extensively reviewed elsewhere (Hess and Stamler 2012; Seth and Stamler 2011).

The mechanism of protein S-nitrosation is still the matter of debate (Broniowska and Hogg 2012). Like in the case of S-sulfhydration, direct reaction of NO with thiols to lead to the S-nitrosothiol formation is thermodynamically unfavourable. The mechanism usually involves either the reaction of thiols with higher nitrogen oxides, such as N2O3, or reactions with metal centre-catalysed one-electron oxidation product of NO (Broniowska and Hogg 2012). Trans-nitrosation, transfer of NO+ moiety, from one protein to another is also a subject of a lively debate. Cysteine and glutathione have been proposed as common carriers. However, H2S also reacts with S-nitrosothiols leading to the formation of the smallest S-nitrosothiol, HSNO (Fig. 3c, Filipovic et al. 2012b). HSNO can freely diffuse through the membranes serving as a carrier of NO+ and conveying further trans-nitrosation. In addition, HSNO is very unstable and highly reactive so it could react further with H2S-forming polysulfides and/or sulfur, which could be a source of protein S-sulfhydration (Fig. 3c). The reaction of S-nitrosothiols with H2S to directly give persulfides and nitroxyl (HNO) is thermodynamically unfavourable (Δrxn1G° = +40 kJ mol−1) (Filipovic et al. 2012b). Although it seems that trans-nitrosation is a generally favoured pathway, a recent computational study pointed out that the surrounding of S–NO bond within a protein could significantly affect the thermodynamic feasibility of the thiolation reaction, making it possible for certain proteins (depending on the surrounding of the cysteine residue) to get directly S-sulfhydrated (Fig. 3c, Talipov and Timerghazin 2013).

2.2.3 Reaction with Disulfides

Reaction of thiols with disulfides normally proceeds until the equilibrium is established (Moriarty-Craige and Jones 2004). Following the same logic, H2S should be able to react with disulfides leading to persulfide formation (Fig. 3d). Protein disulfides are always formed between cysteine residues (of the same or different polypeptide chain), or they appear as mixed disulfides between the cysteine residues of the protein and glutathione (in S-glutathionylated proteins). Based on the calculation of the bond energies of GSSG and GSSH, the latter has ~18 kJ mol−1 lower bond energy (Filipovic et al. 2012b) which makes the reaction of H2S with oxidized thiols very slow. Francoleon and colleagues demonstrated that glutathione persulfide could indeed be formed in a reaction mixture containing oxidized glutathione and H2S, but the product was unstable (Francoleon et al. 2011). Formation of protein persulfides by the H2S-induced reduction of intra- or intermolecular protein disulfides seems highly unlikely in the cells, due to the low levels of H2S when compared to other thiols (such as glutathione or cysteine) and the very slow reaction rate. Zhang et al. (2014) did not observe any S-sulfhydration as a consequence of the reaction of H2S with disulfides of BSA, and a study by Wedmann et al. (2014) confirmed this observation on immunoglobulins purified from human blood. The use of very high, non-physiological concentrations of H2S or its donors in experimental setups could, nonetheless, overcome this limitation and lead to protein modification.
2.3 Nonenzymatic S-sulfhydration by Species Originating from $\text{H}_2\text{S}$

2.3.1 Reaction with Polysulfides

Polysulfides ($\text{HS}_\text{x}^-$) are the products of incomplete $\text{H}_2\text{S}$ oxidation on its way to total oxidation to elemental sulfur. Contrary to a widely spread opinion in a biological community that $\text{H}_2\text{S}$ easily oxidizes, $\text{H}_2\text{S}$ is known as a very persistent and difficult-to-get-rid-off pollutant in industrial processes. The oxidation that indeed does occur on air is most probably caused by the impurities present in the solutions such as traces of metal ions, which are inevitable contaminants of all standard buffer solutions (Kotronarou and Hoffmann 1991).

The length of polysulfide chain can be from 2 to 7. It is worth noting that older chemical literature clearly points out that $\text{H}_2\text{S}_2$ is a molecule that can be prepared under extreme conditions (Parsons and Walton 1921). It is a substance of pungent smell that cannot even stand the humidity from air, and it immediately decomposes to give $\text{H}_2\text{S}$ and elemental sulfur (Parsons and Walton 1921). Therefore, assuming that $\text{H}_2\text{S}_2$ is an important player in the biological system would be wrong. If formed, $\text{H}_2\text{S}_2$ would immediately decompose.

Longer polysulfides are more stable, with $x = 4$ and/or 5 being the most abundant species. They are formed even in biological milieu and could be responsible for the part of the effects assigned to $\text{H}_2\text{S}$ (Fig. 3e). Indeed, Kimura et al. (2013) demonstrated that polysulfides have a 300 times higher potency in activating TRP channels. In addition, Greiner et al. (2013) linked polysulfides to protein thiol oxidation suggesting that all sources of $\text{H}_2\text{S}$ (salts and $\text{H}_2\text{S}$ donors) inevitably contain polysulfides, which in turn oxidize cysteine residues leading to persulfide formation. All inorganic polysulfides with more than two sulfur atoms contain sulfane sulfur atoms, which could undergo nucleophilic attack by free protein thiols leading to the formation of protein persulfides. However, polysulfides could also serve as reducing agents. Francoleon et al. (2011) suggested that persulfides are much more potent reducing agents than $\text{H}_2\text{S}$, while Wedmann et al. (2014) recently showed that polysulfides are capable of completely cleaving intramolecular disulfides present in immunoglobulins. $\text{H}_2\text{S}$ solutions prepared with care, however, could not do the same. Only when mixed with traces of metal ions, they achieved the same effect (Wedmann et al. 2014).

Although there is very little doubt that polysulfides are formed in the cells, it is still unclear how they could serve as signalling molecules. Signalling molecules should be able to achieve the effect at low doses, but at the same time, their production should be tightly controlled, and their reactivity should be specific, just like in the case of NO or $\text{H}_2\text{O}_2$. Formation of polysulfides cannot be controlled; it is guided by stochasticity as the reactions that polysulfide undergo are highly unpredictable and will largely depend on the availability of oxygen and metal centres on the one hand and the protein thiols/disulfides on the other hand. In addition, polysulfides are charged, and it is almost impossible to achieve their fully protonated form under physiological condition, making the diffusion through the membrane impossible without a facilitator.
2.3.2 Reaction with Metal Centres and Generation of HS•

Although not studied into too much detail, metal centres could play an important role in catalysing formation of protein persulfides (Fig. 3f). Namely, some iron haem centres are able to oxidize hydrogen sulfide forming HS• (Miljkovic et al. 2013), which could in turn react with free thiols to finally generate protein persulfides (Zhang et al. 2014). Cytochrome c, for example, readily reacts with H₂S (Wedmann et al. 2014). Iron porphyrins in general could be a good source of HS•. HS• reacts further with H₂S, in a diffusion-controlled reaction, to give H₂S₂•⁻ (Das et al. 1999) which should be a powerful persulfidation agent when formed intracellularly, something that is yet to be confirmed. As demonstrated by Zhang et al. (2014), incubation of GAPDH with iron porphyrin and H₂S leads to the strongest generation of protein persulfides. This complemented with their observation that a large portion of intracellular S-sulfhydration co-localizes with mitochondria, the organelle richest in metallo-proteins.

2.3.3 “Trans-persulfidation” by Polythiolated Cysteine or Glutathione

A recent study by Ida et al. (2014) suggested very high levels of circulatory LMW persulfides. Francoleon et al. (2011) noticed that treatment of papain with GSSH leads to the enzyme inhibition in the same manner as it does the persulfidation of this enzyme. The authors recently went a step further suggesting that cysteine persulfide and/or glutathione persulfide could be the main persulfidating agents in the cells (Ono et al. 2014). Cysteine persulfides could even be transported through the membrane. S-nitrosocysteine does the same and is considered as one of the main carriers of “NO⁺⁻” moiety in trans-nitrosation reactions (Broniowska and Hogg 2012). Making a parallel with S-nitrosothiol chemistry, the term “trans-persulfidation” could be coined to describe this process (Fig. 3g).

The mechanism of this process is still unclear. As mentioned above, the persulfides would react with electrophiles rather than with nucleophiles (Artaud and Galardon 2014; Bailey et al. 2014), and when they do react with nucleophiles (such as other free thiols), this reaction leads to the release of H₂S (which represents the basis of one of the methods for persulfide detection: reduction with DTT). The reaction with nucleophiles could only go as an attack to sulfane sulfur, but that leads to the formation of a mixed disulfide and elimination of H₂S. Some literature data exist suggesting that persulfides could exist in the tautomeric thiosulfoxide form (Scheme 1), which would then act as a perfect donor of sulfane sulfur (Kutney and Turnbull 1982; Steudel et al. 1997). However, it is worth noting that although this may be true for the extreme case of F₂S₂, neither experimental nor computational data support the existence of this tautomeric form (Steudel et al. 1997). Therefore, the elucidation of this mechanism is to be done in some future studies.
Detection of Protein Persulfides

Detection of protein S-sulfhydration represents a certain challenge as the persulfide group exhibits the reactivity similar to other, free thiols (Flavin 1962; Heimer 1981; Mueller 2006; Pan and Carroll 2013; Zhang et al. 2014). Four distinctive approaches have been proposed (Fig. 5) for the persulfide detection, all of which are summarized here with a particular emphasis on the potential experimental problems that might occur.

The original method for protein persulfide detection was suggested by Mustafa et al. (2009b), and it was based on a premise that protein persulfides would not react with electrophilic thiol-blocking reagent S-methyl methanethiosulfonate (MMTS). In the subsequent step, persulfides were labelled with N-[6-(biotinamido)hexyl]-3′-(2′-pyridylthio)propionamide (biotin-HPDP) (Fig. 5a). This method allowed the simultaneous labelling of S-sulfhydration and S-nitrosation. Using this method, Mustafa and colleagues suggested that a large number of proteins were a target for H₂S signalling and that basal protein persulfidation is as high as 25 % (Mustafa et al. 2009b).

To date, this is the most used methodological approach in reporting protein S-sulfhydration of different proteins. However, certain methodological limitations arise. Although MMTS has been widely used to study S-nitrosation (Forrester et al. 2009) and has been an efficient tool in trapping mixed disulfides in vivo (Peaper et al. 2005), Karala and Ruddock (2007) were able to show that in vitro MMTS treatment of both peptides and proteins resulted in the artificial formation of intramolecular and intermolecular protein disulfide bonds which could lead to
Fig. 5 An overview of the methods currently employed for the detection of protein persulfides. (a) The first method was based on a chemically wrong premise that protein persulfides would not react with electrophilic thiol-blocking reagent S-methyl methanethiosulfonate (MMTS). In the subsequent step, persulfides were labelled with N-[6-(biotinamido)hexyl]-3′-(2′-pyridylidithio) propionamide (biotin-HPDP). (b) In the second method, iodoacetic acid (IAA) is used to initially block both free thiols and protein persulfides. In the subsequent steps, alkylated persulfide is cleaved with DTT and then labelled with iodoacetamide-linked biotin (IAP). Although DTT would indeed cleave this adduct, it is unclear how this method distinguishes the persulfides from intramolecular disulfides and S-nitrosothiols, which would also be reduced by DTT. (c) In this method, both persulfide and free thiol would be blocked by the thiol fluorescently labelled N-ethyl maleimide (Cy5-conjugated maleimide). The adduct of persulfide and Cy5-maleimide is a disulfide that will be then cleaved by the DTT leading to a decrease of the in-gel fluorescence signal in the samples containing persulfides. (d) Finally, the use of the methylsulfonyl benzothiazole (MSBT) to block thiols and persulfides in the first step, followed by the tag switch with cyanoacetate derivatives in the second step, leads to the efficient labelling of persulfides, by the method called tag-switch technique.
general data misinterpretation. In addition, Pan and Carroll (2013) studied how persulfides react with both electrophilic and nucleophilic species, reaffirming the nucleophilic properties of the persulfide sulfane sulfur. They unambiguously showed that persulfides react with MMTS (and its analogue S-4-bromobenzyl methanethiosulfonate) as readily as free thiols, questioning the interpretation of the data obtained by modified biotin-switch technique (MBST) approach. Two possible models were proposed to explain the data generated by MBST: (1) free thiols may be incompletely blocked in the first MMTS alkylation step and subsequently react with the pyridyldisulfide biotin reagent; (2) alternatively or in addition, labelling may be achieved via stepwise thiol-disulfide exchange in a reaction catalysed by trace free thiols (RSH).

In their attempt to identify persulfidation of protein tyrosine phosphatase 1B (PTP1B), Krishnan et al. (2011) proposed an approach for persulfide detection based on a completely opposite chemical premise. Namely, they proposed that thiol-blocking reagent, iodoacetic acid (IAA), will react with both free thiols and protein persulfides (Fig. 5b), which is in agreement with the persulfides’ reactivity similar to that of free thiols. In the subsequent steps, however, they proposed the cleavage of the alkylated persulfide with DTT and then labelling of that particular cysteine with iodoacetamide-linked biotin (IAP). Although DTT would indeed cleave this adduct, it is unclear how this method distinguishes the persulfides from intra- and intermolecular disulfides and S-nitrosothiols, which would also be reduced by DTT.

Sen et al. (2012) proposed an alternative method for the persulfide detection. This method is based on the fact that both persulfide and free thiol would be blocked by the thiol-blocking reagent N-ethyl maleimide. The authors used Cy5-conjugated maleimide in the first step followed by the used of DTT in the second (Fig. 5c). The adduct of persulfide and Cy5-maleimide is a disulfide that will be cleaved by the DTT leading to a decrease of the in-gel fluorescence signal in the samples containing persulfides. This method has its advantage of being relatively simple and available to every researcher (because the reagents are commercially available). The only limitation of the method is that it does not offer actual persulfide labelling which would allow wide proteomic analysis. Particular care should be taken to ensure that all thiols and persulfides are indeed blocked in the initial step, as that is the crucial step for the subsequent data interpretation.

Most recently, Zhang et al. (2014) proposed a different approach for protein persulfide detection, named tag-switch assay (Fig. 5d). This method was based on an idea that thiol-blocking reagent should be introduced in the first step, which would tag both free thiols and persulfides. If an appropriate tag is employed, the disulfide bond in persulfide adducts might show much enhanced reactivity to certain nucleophiles than common disulfides in proteins. Therefore, a tag-switching reagent (containing both the nucleophile and a reporting molecule such as biotin) could be introduced to label only the persulfide adducts. It should be noted that thiol adducts from the first step are thioethers, which are not expected to
react with the nucleophile. A major challenge in this technology was for the newly generated disulfide linkage from persulfide moieties to display a unique reactivity for a suitable nucleophile to an extent that distinguishes them from common disulfides. Using the methylsulfonyl benzothiazole (MSBT) to block thiols in the first step in combination with the tagged-cyanoacetate derivatives in the second step, persulfides could be efficiently labelled. Neither free thiols, intramolecular disulfides, S-glutathionylated or sulfenylated proteins were tagged by this approach (using BSA as a model protein). This method was further adapted for visualization of intracellular S-sulfhydration by fluorescence microscopy. Despite the lack of reactivity of BSA sulfenic acid derivative with the method, it is expected that sulfenic acids should react with cyanoacetate derivatives. Protein sulfenic acids are, in general, very unstable and prone to further oxidation, so it is improbable that any of them will remain in the solution after 30–45 min of initial incubation with the MSBT. Nonetheless, it is better to treat the cell lysates with dimedone, prior to the incubation with the first component of tag-switch assay, in order to eliminate any doubts of potential cross-reactivity (Park et al. 2015).

3.1 Detection of Sulfane Sulfurs

Although the methods for sulfane sulfur detection detect more than just protein persulfides, they could serve as a useful tool for the fast and easy proof-of-concept type of experiments. With recent advances in making these methods quantitative, it is also possible to assume that the results suggesting an increase or decrease of the sulfane sulfur levels do suggest the same trends for protein persulfides.

The traditional method for sulfane sulfur detection is cyanolysis (Wood 1987). The method is based on the reaction between sulfane sulfurs and cyanide under alkaline conditions (pH > 8.5). In this reaction, thiocyanate (SCN\(^-\)) is formed, which in the reaction with Fe\(^{3+}\) generates the Fe(SCN)\(^{2+}\) complex with characteristic absorbance maximum at 460 nm.

Recently, two new methods have been proposed. The first one is designed to be used for the detection of sulfane sulfur levels in the cells. Chen et al. (2013) based their discovery on an assumption that sulfane sulfurs are likely to react with the nucleophile components of a fluorescent probe, which could then undergo spontaneous and fast cyclization to release fluorophore. They successfully produced two probes, named SSP1 and SSP2, which could be easily used for intracellular detection of sulfane sulfur. The same group improved the probes designing a new DSP-3 fluorescence sensor for hydrogen polysulfides (Liu et al. 2014a).

Most recently, the group led by Ming Xian established isotope dilution mass spectrometric method for the quantification of sulfane sulfurs (Liu et al. 2014b). Accurate and reliable measurements of sulfane sulfurs in biological samples are required in order to understand the impact of H\(_2\)S signalling. The method that Liu et al. (2014b) proposed employs a triphenylphosphine derivative to capture sulfane
sulfurs as a stable phosphine sulfide product. The concentration of this product can be determined by isotope dilution mass spectrometry using a $^{13}$C$_3$-labelled phosphine sulfide as an internal standard. Using this method, the authors found that average concentrations of sulfane sulfur were 57.0 (liver), 150.9 (kidney), 46.0 (brain), 61.8 (heart), 56.1 (spleen) and 20.8 nmol/g (lungs).

4 Persulfidation in Action

As already mentioned, H$_2$S regulates the plethora of biological functions from neurotransmission and blood pressure to cardioprotection. To date, several key protein targets have been identified which do undergo oxPTM of the cysteine residue, suggesting that this modification can be responsible for some of the H$_2$S effects such as vasodilation, prevention of cell death and senescence, cell differentiation, etc.

4.1 S-sulfhydration of K$_{ATP}$ Regulates Vasodilation

Seminal work by Yang et al. (2008) showed that CSE knockout mice develop hypertension, confirming previous assumptions about H$_2$S being the endogenous regulator of blood pressure. Several other studies have been published recently showing that majority of H$_2$S-induced vasodilation goes via its interaction with NO. This effect can be dual, via direct reaction with NO that leads to the nitroxy (HNO) formation and subsequent activation of HNO–TRPA1–CGRP signalling cascade (Eberhardt et al. 2014) or by inhibiting the phosphodiesterase 5 and increasing the cGMP levels (Colleta et al. 2012). Nonetheless, the part of H$_2$S-induced vasodilation could be assigned to the activation of K$_{ATP}$ channels. Persulfidation of C34 on the Kir6.1 subunit of K$_{ATP}$ channel on smooth muscle cells prevents its association with ATP and promotes its binding to phosphatidylinositol-4,5-bisphosphate (PIP2). This leads to the channel opening and K$^+$ influx and subsequently to the smooth muscle cells’ relaxation. Several other Ca$^{2+}$ channels have been also implicated in the H$_2$S-induced vasodilation.

4.2 Persulfidation of Electrophilic Messengers and Cardioprotection

Recently, direct persulfidation of several electrophilic messengers (such as 8-nitro-cGMP) by sulfide has been reported offering additional mechanism for H$_2$S-mediated signalling (Nishida et al. 2012). Redox signalling by electrophilic by-products, such as nitrated cyclic nucleotides and nitro- or keto-derivatives of unsaturated fatty acids, all generated by the inflammation-related enzymes, ROS and/or NO, has attracted much interest lately (Nishida et al. 2014; Fujii and Akaike 2013). S-alkylation of cysteine residues by 8-nitroguanosine 3',5'-cyclic monophosphate mediates several redox signalling pathways. Nishida et al. (2012)
showed that HS\textsuperscript{−} could directly attack the above-mentioned electrophiles, forming derivatives that have biological effect of themselves. Namely, formation of 8-SH-cGMP blocks the S-guanylation of H-Ras, a modification which normally activates H-Ras to signal cell senescence as a response to stress (Fujii and Akaike 2013). The authors link the protective cardiovascular effects of H\textsubscript{2}S and its donors to increased formation of 8-SH-cGMP and inhibition of H-Ras signalling. It is worth mentioning, however, that Terzić et al. (2014) showed that nucleophilic attack of the hydrosulfide anion to 8-nitro-cGMP cannot take place, as previously proposed. Instead, the formation of reactive species containing sulfane sulfur, like persulfides, is required (Terzić et al. 2014).

4.3 S-sulfhydration of GAPDH and NF-κB Protects Against Apoptosis

GAPDH was the first protein characterized as S-sulfhydrated in the study that sparked the whole research field (Mustafa et al. 2009a). GAPDH has been known for a while as regulator of a cell death cascade (Hara et al. 2005). S-nitrosation of catalytic C152 abolishes its catalytic activity but makes it able to bind to Siah1, an E3 ubiquitin ligase. Siah1, which possesses a nuclear localization tag, helps the translocation of GAPDH to a nucleus where it enables Siah1 to degrade nuclear proteins, leading to cell death (Hara et al. 2005). Mustafa et al. showed that GAPDH is endogenously S-sulfhydrated at C150 which increases its enzymatic activity severalfold (Mustafa et al. 2009a). The authors also demonstrated that DTT treatment of GAPDH decreases its activity, suggesting that endogenous persulfidation regulates its function. CSE knockout mice showed ~35 % reduced activity of GAPDH when compared to control mice. Zhang and associates confirmed recently, using selective tag-switch assay, that GAPDH is indeed endogenously S-sulfhydrated (Zhang et al. 2014). The fact that S-sulfhydration of GAPDH increases its enzymatic activity means that it also prevents its interaction with Siah1 protecting the cells from apoptosis.

Nuclear factor-κB (NF-κB) is an anti-apoptotic transcription factor, which is under basal conditions kept in cytosol via interaction with the inhibitor-κBα (Napetschnig and Wu 2013). During the inflammation, cells produce tumour necrosis factor-α (TNF-α), which could lead to cell death (Aggarwal et al. 2012). H\textsubscript{2}S is known to have protective effects in inflammation but without distinctive mechanism that can explain it (Li et al. 2005; Whiteman and Winyard 2011). Sen and colleagues offered such mechanism in a study describing the persulfidation of NF-κB (Sen et al. 2012). Namely, they showed that C38 of p65 subunit of NF-κB is persulfidated which promotes its binding to the co-activator ribosomal protein S3, augmenting its binding to the promoters of anti-apoptotic genes (Fig. 6a). In addition, TNF-α stimulated transcription of CSE, increasing the total amount of S-sulfhydrated NF-κB. Conversely, Du et al. 2014 showed recently that hydrogen sulfide suppresses oxidized low-density lipoprotein-induced macrophage inflammation by inhibiting NF-κB. This study suggests that persulfidation of C38 on p65
in fact prevents the NF-κB from leaving the cytosol, therefore completely inhibiting its DNA binding activity. Further studies are obviously needed to give more conclusive answer about the role of H₂S in inflammation.

4.4 S-sulfhydration of Parkin Protects Against Parkinson’s Disease

Parkinson’s disease (PD) is a neurodegenerative disease caused by the death of dopamine-generating cells in the substantia nigra (Shulman et al. 2011). One of the proteins considered responsible for this cell death is parkin. Parkin is an E3 ubiquitin ligase that catalyses ubiquitination of diverse substrates. Mutations in parkin, which lead to the loss of its activity, are one of the causes of PD (Shulman et al. 2011; Moore et al. 2005). Parkin has reactive cysteine residues, which can be subjected to oxidative posttranslational modifications. S-nitrosation of parkin, for example, inhibits its E3 ubiquitin ligase activity contributing to the Parkinson’s disease (Chung et al. 2004).

Recently, Solomon Snyder and colleagues demonstrated that cysteines C59, C95 and C182 could undergo S-sulfhydration (Vandiver et al. 2013). This leads to the increase of parkin’s activity (Fig. 6b). Persulfidation of parkin is markedly decreased in PD brains, whereas S-nitrosation is increased. Development of H₂S donors opens up a possibility of their use in the early treatment of PD. Increase of parkin’s activity could salvage the neurons from the cell death by removing damaged proteins. Parkin is also an important regulator of mitophagy, leading to the removal of damaged mitochondria, particularly in ischemia–reperfusion injury. As H₂S is known to have great pharmacological potential in preventing ischemia–reperfusion injury, it is possible that part of this effect goes via persulfidation of parkin and increased removal of damaged mitochondria.

4.5 S-sulfhydration of PTP-1B Regulates ER Stress

Protein tyrosine phosphatases (PTPs) in conjunction with protein tyrosine kinases are important controllers of various biological functions. PTPs are particularly sensitive to oxidative posttranslational modifications of cysteine, as cysteine is present, is in the active site and is important for their function (van Montfort et al. 2003). H₂O₂ is known to modulate function of these enzymes by forming an inactive adduct which contains sulfenic acid in the active site (van Montfort et al. 2003). PTP-1B is one of the members of this class of enzymes, located in the cytoplasmic face of the endoplasmic reticulum, where it plays an important role in ER stress signalling (Paulsen et al. 2012).

Persulfidation of C215 leads to the loss of enzymatic activity, which increases the phosphorylation of Y619 and therefore the activation of PERK in response to ER stress (Krishnan et al. 2011). PERK activation leads to global inhibition of protein translation. The mechanism by which H₂S causes persulfidation of PTP-1B,
as in many other cases, is not clear. In the case of PTP-1B though, it is possible that ER stress in fact leads to PTP-1B inactivation by initial formation of sulfenic acid in the active site, which then reacts with $\text{H}_2\text{S}$ to give persulfide. Although both

Fig. 6 Biological role of protein persulfidation. (a) As a response to inflammation, tumour necrosis factor alpha (TNF-α) migrates to nucleus where it binds to the transcription factor specificity protein 1 (SP1) causing the transcription and overexpression of CSE. H$_2$S produced by CSE induces persulfidation of p65 subunit of NF-κB which translocates to nucleus where its interaction with co-activator ribosomal protein S3 (rps3) is enhanced, resulting in the transcription of anti-apoptotic genes. (b) S-nitrosation of parkin inhibits its E3 ubiquitin ligase activity which leads to accumulation of toxic proteins. This is found in Parkinson’s disease where H$_2$S production is reduced. Persulfidation of parkin, on the other hand, increases parkin’s enzymatic activity, which could be used as a therapeutic approach for Parkinson’s disease treatment. (c) Keap-1 keeps Nrf-2 in the cytoplasm where it can be ubiquitinated and proteolytically degraded. Persulfidation of critical cysteines on Keap1 disturbs its interaction with Nrf-2 leading to the release of Nrf-2 which can now move into the nucleus where it binds to antioxidant response element (ARE) causing the transcription of various antioxidant defence genes. (d) The hypothetical role of persulfidation in protection of protein function during oxidative stress. When exposed to hydrogen peroxide, proteins undergo oxidation to form sulfenic acids (P–SOH), sulfinic acids (P–SO$_2$H) and sulphonic acids (P–SO$_3$H), which cause the irreversible inactivation of the protein. Sulfenic acids could react with H$_2$S to form persulfides. In addition, protein persulfides could be formed by other mechanisms, and when exposed to ROS, they will readily react with it, forming P–S–SO$_3^-$, which can be enzymatically cleaved to restore free thiol on the protein
modifications are inhibitory, persulfide could be then removed easier and the enzyme’s activity restored. Therefore, H$_2$S could serve to protect the enzyme from a longer-lasting and potentially irreversible inhibition.

### 4.6 S-sulfhydration of Keap1 and p66Shc Increases Cellular Antioxidative Defence and Prevents Senescence

A major mechanism in which antioxidant enzymes are induced involves the activation of the antioxidant response element (ARE) by the oxidative-stress sensor protein Kelch-like ECH-associated protein 1 (Keap1) and the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) (Hybertson et al. 2011; Kaspar et al. 2009). Under basal conditions, Keap1 sequesters Nrf-2 in the cytoplasm by binding to its Neh2 domain (Hybertson et al. 2011; Kaspar et al. 2009; Wakabayashi et al. 2004). Chemical inducers, such as sulforaphane, are known to react with Keap1 cysteine residues, thereby promoting Nrf-2 nuclear accumulation and hence ARE activation (Wakabayashi et al. 2004). A widely accepted model for Nrf-2 nuclear accumulation describes that a modification of the Keap-1 cysteines leads directly to the dissociation of the Keap1–Nrf-2 complex and to the translocation of Nrf-2 into the nucleus.

H$_2$S cardioprotective effects in ischemia–reperfusion injury are partly caused by Nrf-2 nuclear translocation and activation of antioxidant defence enzymes (Calvert et al. 2009, 2010). Recently, two independent studies suggested that Keap-1 gets S-sulfhydrated when cells were exposed to H$_2$S. Modification of C151 on Keap1 stimulates dissociation of Nrf-2 enabling its translocation to nucleus where it regulates expression of cytoprotective genes and delays cellular senescence (Fig. 6c; Yang et al. 2013). In second study, Hourihan et al. (2013) found that H$_2$S stabilizes Nrf2 via covalent modification of amino acids C226 and C613 in the Keap1. The authors show that H$_2$S leads to the production of H$_2$O$_2$, which inhibits Keap1 by stimulating the formation of an intramolecular disulfide bond between C226 and C613. The Keap1 C226 and C613 residues are also persulfidated. This may be explained either by the ability of H$_2$S to reduce C226–C613 disulfides originally formed by H$_2$O$_2$ or by direct reaction of H$_2$S with sulfenylated residues formed by H$_2$O$_2$. More importantly, the authors observed that Nrf-2 controls CBS, CSE and sulfide–quinone reductase-like enzyme, suggesting that a feedback loop exists between Nrf-2 and H$_2$S.

P66Shc belongs to the ShcA family of proteins whose members share three common functionally identical domains: the C-terminal Src homology 2 domain (SH2), the central collagen homology domain (CH1) and the N-terminal phosphotyrosine-binding domain (PTB) (Giorgio et al. 2005). In response to oxidative stress such as UV exposure or H$_2$O$_2$, p66Shc gets activated by phosphorylation at Ser-36. The activated p66Shc is then dephosphorylated and translocates to mitochondria, where it binds to cytochrome c helping in electron transport process (Giorgio et al. 2005). P66Shc$^{-/-}$ mice show 30 % increase of the lifespan. It has been shown recently that sulfhydration of p66Shc impaired the association of PKC$\beta$I.
and p66Shc and attenuated H₂O₂-induced p66Shc phosphorylation, a critical step in p66Shc-mediated mitochondrial ROS generation (Xie et al. 2014). H₂S is known to have dramatic effects on inhibiting oxidative stress, something that cannot be simply explained by its direct redox chemistry. A study by Xie and associates suggests that H₂S may inhibit mitochondrial reactive oxygen species production via a p66Shc-dependent mechanism.

4.7 MEK1/PARP-1 Activation and DNA Damage Repair

The salvage of DNA damage is essential for normal cell function. DNA damage stimulates a complex and highly concerted DNA damage repair response, which includes poly(ADP-ribose)ation catalysed by poly(ADP-ribose)ation polymerases (PARPs). Upon DNA damage, PARPs bind to DNA strand breaks and catalyse poly(ADP-ribose)ation which attracts other DNA damage repair proteins (D’Amours et al. 1999). The activation of PARPs is regulated by several kinases, of which MEK/ERK signalling cascade plays an important role (Cohen-Armon et al. 2007). Zhao et al. (2014) reported recently that H₂S attenuates DNA damage in human endothelial cells by causing S-sulfhydration of cysteine 341 on MEK1. This facilitates the translocation of phosphorylated ERK1/ERK2 into nucleus where it activates PARP-1 and increases the DNA damage repair yield, protecting cells from senescence.

4.8 TRP Channel S-sulfhydration Regulates Osteogenic Differentiation

Bone marrow mesenchymal stem cells (BMMSCs) are nonhaematopoietic multipotent stem cells and play an important role in the maintenance of the bone marrow homeostasis (Prockop 1997; Pittenge et al. 1999). BMMSCs and BMMSC-derived osteoblasts are responsible for bone formation and balancing osteoclast-mediated bone resorption in order to maintain bone mineral density (BMD) (Pittenge et al. 1999). CBS-deficient patients exhibit a variety of phenotypes, including osteoporosis. Osteoporosis is characterized by low bone mass and deterioration of osseous microarchitecture, resulting in decreased bone strength and increased risk of fragility fractures. This phenotype is often observed in patients with hyperhomocysteinemia (Herrmann et al. 2005; Melton 2003).

It has been demonstrated recently that H₂S deficiency causes aberrant intracellular Ca²⁺ influx because of reduced persulfidation of cysteine residues on multiple TRP channels (Liu et al. 2014c). Decreased Ca²⁺ influx downregulates PKC-/Erk-mediated Wnt/beta-catenin signalling which controls osteogenic differentiation of BMMSCs. Therefore, the authors suggest that bone marrow mesenchymal stem cells produce H₂S in order to regulate their self-renewal and
osteogenic differentiation and that H\textsubscript{2}S deficiency results in defects in BMMSC differentiation. This study is in agreement with the previous observation that hydrogen sulfide protects MC3T3-E1 osteoblastic cells against H\textsubscript{2}O\textsubscript{2}-induced oxidative damage (Xu et al. 2011) suggesting that development of new H\textsubscript{2}S-releasing drugs could be a potential therapeutic route for the treatment of osteoporosis.

5 Placing Persulfidation in a Broader Biological Context and Future Directions

The biochemical properties of protein persulfides discussed here suggest a specific and enhanced reactivity, which can be used for the regulation of protein’s function. As mentioned above, persulfidation increases the nucleophilicity of protein thiols, and, as shown in several examples of proteins such as GAPDH and parkin, it increases their enzymatic activity. Further, the increased reducing power of persulfides suggests that they could act as efficient antioxidants. For example, GSSH reacts rapidly with H\textsubscript{2}O\textsubscript{2}, neutralizing its toxic effects. In fact, one of the possible roles of protein persulfidation could be the protection of a particular protein from irreversible damage induced by ROS and/or RNS (Fig. 6d). Thiol oxidation, which initially starts with the formation of sulfenic acids (still reversible modification), could proceed further with the formation of irreversible sulfonic acids. H\textsubscript{2}S could react with sulfenic acid preventing this oxidation. In addition, persulfidated protein, even when exposed to ROS/RNS, will form an adduct that could be cleaved by the action of certain enzymes restoring free thiol. Therefore, spatio-temporal distribution of sulfenylation vs. persulfidation could help us to understand the relative ratio and importance of these two oxPTMs of cysteine.

S-nitrosation of proteins is a very important posttranslational modification of proteins. S-nitrosation of parkin leads to its inactivation (Chung et al. 2004). Conversely, persulfidation of parkin stimulates the enzyme increasing its ligase activity, which prevents accumulation of toxic proteins (Vandiver et al. 2013). On the other hand, intracellular S-nitrosothiol levels were found to be lower in cells with lower H\textsubscript{2}S production, suggesting its role in trans-nitrosation reactions as well (Filipovic et al. 2012a). Therefore, the understanding of the NO and H\textsubscript{2}S crosstalk, with particular emphasis on S-nitrosation vs. persulfidation, should be one of the future tasks.

All these could be done with proper tools for persulfide detection. Further development of assays, which would allow easy labelling of protein persulfides and their subsequent proteomic analysis, would accelerate the progression of the field. It is worth mentioning that most of the protein persulfidation reports to date used the methodology, which has been shown to generate artefacts.

Very little is known about the mechanisms of protein persulfidation. Trans-persulfidation of proteins by low molecular weight persulfides is an exciting concept that warrants further exploring. The role of metal centres in facilitating
persulfidation is also of potential interest. Elucidation of all these mechanisms (the role of H$_2$S vs. polysulfides vs. LMW persulfide) could help us in getting a few steps closer to the understanding of actual contribution of different H$_2$S-producing enzymes in the regulation of the intracellular S-sulfhydration levels.

Finally, the question that warrants an equally important attention is the extent to which this modification is regulatory and to which it is just a consequence of stochastic events. Answering this could lead to the identification of specific targets that could later on be pharmacologically tempered with. Therefore, this field of research will remain one of the hot topics for many years to come.

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