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Sulfur-utilizing cytoprotection and energy metabolism

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Sulfur, one of the most abundant chemical elements on earth, has had important roles in biosphere evolution. Unique roles of sulfur in living organisms are mainly related to the redox reaction, whose functions include cytoprotection and energy metabolism. KEAP1–NRF2 system is a sulfur-utilizing cytoprotection mechanism, and its role in metabolic regulation has been attracting increasing attention. Persulfides, which have rapidly emerged as common biomolecules, also play important roles in cytoprotection and mitochondrial energy metabolism. Chemical regulation by persulfides and transcriptional regulation by the KEAP1–NRF2 system act in concert and make up a sulfur-utilizing dual system for cytoprotection and energy metabolism. Clarification of this system is expected to promote understanding of the aging process in organisms and aging-related diseases.

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Introduction

Many organisms possess sulfur-utilizing cytoprotection mechanisms that depend on cysteine residues and sulfur–metal complexes, including the iron–sulfur complex or [Fe–S] cluster, formed in specific regulatory proteins. An example is the KEAP1 (Kelch-like ECH-associated protein 1)[3] -NRF2 (NFE2L2; Nuclear factor erythroid 2-like 2)[4] system, which is highly conserved in animals. KEAP1 is a thiol-rich protein and negatively regulates NRF2, which is a potent transcription activator that coordinately induces many cytoprotective genes encoding antioxidant proteins and detoxification enzymes, thereby regulating redox reactions that involve sulfur. Recent advances in analytical chemistry have unexpectedly revealed appreciable amounts of persulfide produced *in vivo*, which implies a substantial contribution of persulfides to an additional layer of cytoprotection. Persulfides have important functions not only in cytoprotection but also in mitochondrial energy metabolism. Sulfur-dependent energy metabolism is utilized by microbes living in the deep-sea hydrothermal vent, and this type of metabolism appears to be a major energy-producing process for early ancestral organisms, as well as for modern higher animals including humans. This review introduces recent progresses on persulfide research and proposes functional interactions between chemical regulation by persulfides and transcriptional regulation by the KEAP1–NRF2 to make up a sulfur-utilizing dual system.

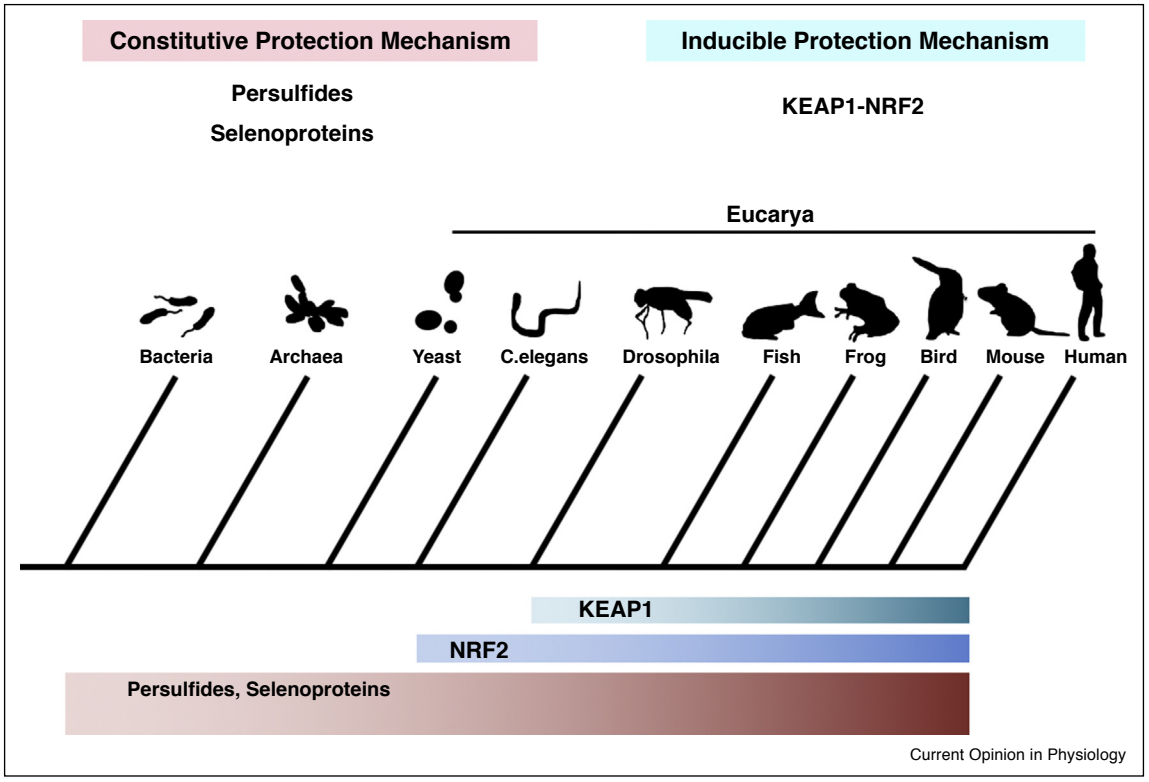
Sulfur utilization by living organisms

A review of the long history of the earth can reveal that sulfur, together with iron and oxygen, made a substantial contribution to biogeological evolution [1]. Sulfur is indeed one of the most abundant chemical elements on this planet and has had important roles in the biosphere. Sulfur present as cysteine thiols in proteins is often involved in many fundamental redox reactions in living organisms. For example, sulfide residues in low-molecular-weight (LMW) thiols, such as glutathione in eukaryotes and Gram-negative bacteria and bacillithiol in Gram-positive bacteria [2], play valuable roles in cytoprotection and detoxification. Inorganic sulfur, such as elemental sulfur and sulfide, is a major energy source for a group of chemolithotrophic bacteria. They obtain reducing equivalents from inorganic sulfur compounds for biosynthesis. Among the diverse functions of sulfur-containing proteins and metabolites, energy metabolism and redox regulation are two of the most fundamental activities for the survival of organisms.

Sulfur is unique compared with other major elements in the biosphere in that it undergoes the largest valence change, from −2 to +6, which is consistent with its substantial roles in biological redox reactions. Particularly for organisms living under aerobic conditions, an appropriate control of levels of reactive oxygen species (ROS) is essential, and disturbance of the balance between production and elimination of ROS often leads to pathological states. Cysteine thiol residues and sulfide-related

[3] KEAP1 (Kelch-like ECH-associated protein 1); Gene ID 9817 (human), Gene ID 50868 (mouse).
[4] NRF2 (NFE2L2; Nuclear factor erythroid 2-like 2); Gene ID 4780 (human), Gene ID 18024 (mouse).

Figure 1



Cross-species conservation of constitutive and inducible protection mechanisms against electrophilic and oxidative stress. Persulfides and selenoproteins are believed to be constitutive protection mechanisms, whereas the KEAP1–NRF2 system is an inducible protection mechanism. Persulfides and selenoproteins are present in all three kingdoms of life. KEAP1 is a unique sensor molecule for electrophilic and oxidative stress and occurs in flies and higher animals but not in worms. NRF2 is a master transcription factor of antioxidant genes as well as other cytoprotective genes. Worms have a homologue of NRF2, named Skn-1. *C. elegans*, *Caenorhabditis elegans*.

compounds such as persulfides[5] and sulfide-metal complexes such as iron–sulfur [Fe–S] clusters of specific regulatory proteins are thought to have critical roles in avoiding such pathological conditions. Bacterial SoxR contains an [Fe–S] cluster, whereas bacterial OxyR, yeast Yap1, plant NPR1, and animal KEAP1–NRF2 utilize the thiols of cysteine residues in proteins [3]. In addition, various LMW persulfides, that is, cysteine persulfide (CysSSH) and glutathione persulfide (GSSH), demonstrate potent antioxidant effects [4••].

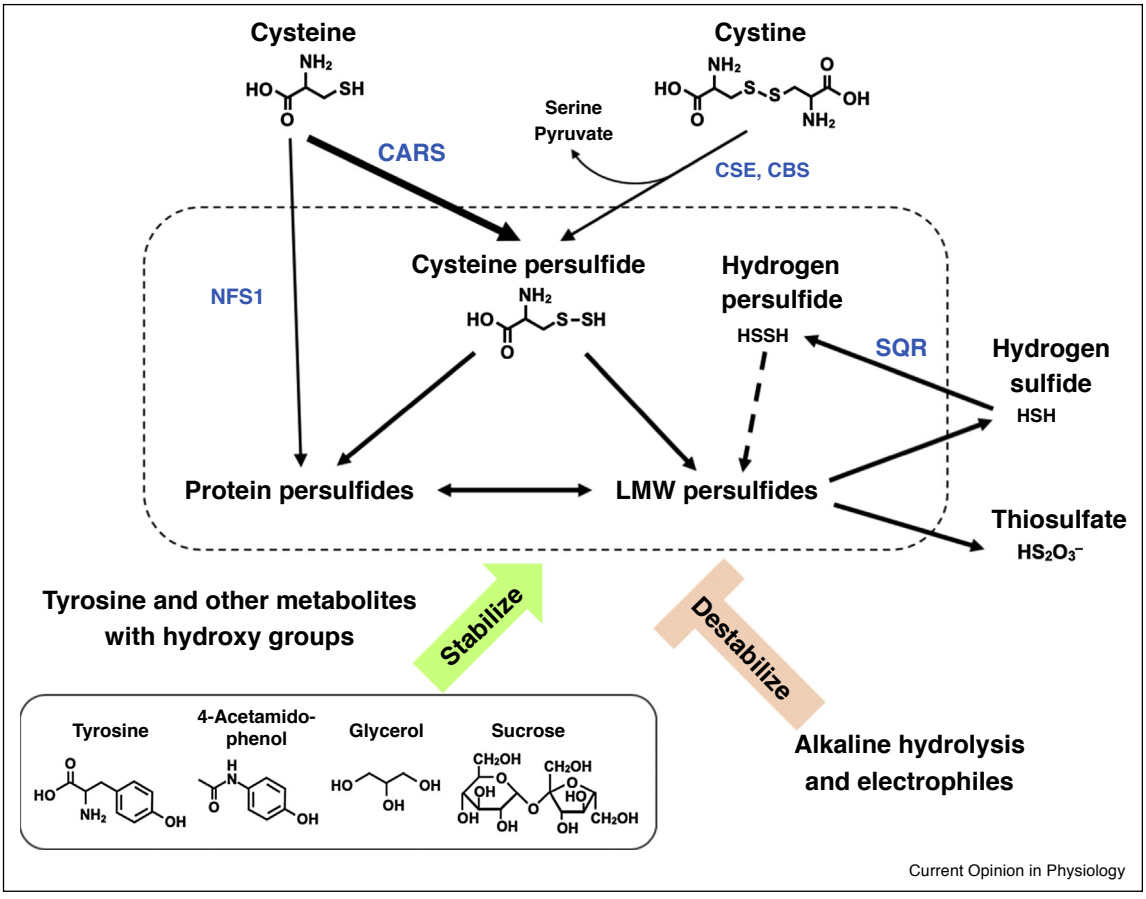
Inducible and constitutive protection systems

The KEAP1–NRF2 system is a sulfur-utilizing defense mechanism that is highly conserved in animals (Figure 1) [5•]. KEAP1 is a thiol-rich protein serving as a sensor for electrophiles and ROS, and NRF2 is a potent transcription activator acting as an effector that regulates a battery of genes encoding enzymes in sulfur-related redox regulation. KEAP1 is a substrate-recognizing subunit of Cullin3-based ubiquitin E3 ligase and mediates ubiquitination of NRF2 for proteasomal degradation under steady state. Alkylation and/or oxidation of KEAP1 thiols stop NRF2 ubiquitination, which results in the stabilization of NRF2 and the NRF2-mediated induction of antioxidant proteins and detoxifying enzymes in cytoprotection, including enzymes that function in glutathione metabolism (synthesis, reduction, conjugation, and degradation), thioredoxin, and thioredoxin reductase.

In addition to these inducible protection systems, selenoproteins constitute the first-line defense mechanism against electrophilic stress and oxidative stress, and the selenoprotein-mediated protection is present in all three kingdoms of life (Figure 1) [6]. Although sulfur and selenium have very similar physical and chemical properties and share all of the same oxidation states and functional groups, selenium-containing compounds and proteins are more reactive and possess higher catalytic

[5] In this article, the term 'persulfide' is defined as all molecular species containing more than one sulfur atom in each LMW and protein/peptidyl thiol moiety. The term persulfide, therefore, is used to represent various hydropolysulfide species of the general form RS_nH ($n > 1$) and dialkyl polysulfides generally referred to as RSS_nR ($n > 1$). When a specific polysulfide is named, the number of its sulfur atoms is indicated, for example, RSSSH and RSSSR are alkyl hydrotrisulfide and dialkyl trisulfide, respectively.

Figure 2



Synthesis and metabolism of persulfides. Persulfides exist as LMW persulfides and protein cysteinyl persulfides. CARS is a major enzyme for the synthesis of cysteine persulfide. LMW persulfides are metabolized in mitochondria to hydrogen sulfide and thiosulfate, which contributes to the maintenance of mitochondrial membrane potential. Several compounds are known to stabilize and destabilize persulfides. SQR, sulfide quinone oxidoreductase; NFS1, cysteine desulfurase.

activity than sulfur-containing counterparts [7]. This situation exists because selenium, by virtue of its heavier atomic weight compared with sulfur, is more polarizable and possesses both electrophilic and nucleophilic properties and oxidation-reduction reversibility. In the context of redox reactivity, nucleophilicity of sulfur is markedly enhanced when thiols are polysulfidated to become persulfides. For example, cysteine persulfide is much more nucleophilic than is the parental cysteine [4••,8••].

Functional defects of selenoproteins caused by selenocysteine-tRNA disruption increase cellular oxidative stress, which is sensed by KEAP1 and results in activation of the NRF2-mediated stress response pathway. This response suggests that selenoproteins and the KEAP1–NRF2 system serve as the primary and secondary defense mechanisms against oxidative stress, respectively [9,10]. Persulfides, because of their potent antioxidant effects, are likely to serve as another first-line defense mechanism before KEAP1 responds to the redox disturbance for NRF2 activation (see below).

Detection and quantification of biological persulfides

Although chemists had already described persulfides by the early 1920s [11], biological persulfides were not actively studied until the beginning of this century, 2000. Persulfides occur as LMW persulfides and protein cysteinyl persulfides (i.e. protein-bound persulfides) (Figure 2). Similar to selenium, persulfides possess both electrophilic and nucleophilic properties [12–14]. Because the nucleophilicity of persulfides is generally greater than that of parental thiols, as stated above, persulfides readily react with even weak electrophiles and thus efficiently quench all electrophiles including ROS, which allows persulfides to contribute to the

protection of cells against redox disturbances with much greater potency than simple thiols do (Figure 1) [4••].

Although the high reactivity of persulfides is advantageous for protection, it is an inherent critical feature that makes precise quantification of persulfides difficult. However, our application of a derivative of iodoacetamide (IAM)—β-(4-hydroxyphonyl)ethyl iodoacetamide (HPE–IAM)—has great benefits for the successful establishment of a persulfide quantification system [8••,15••]. HPE–IAM has mild electrophilicity and establishes stable adducts with persulfides without degrading their polysulfide structures. In addition, to our surprise, HPE–IAM produced greater stabilization of persulfides compared with IAM, possibly because of an inhibitory effect of the hydroxyphenyl moiety of HPE–IAM on alkaline hydrolysis of persulfides [16] (see below). This result indicates that HPE–IAM is the ideal and most preferable reagent to minimize persulfide degradation, which is caused mostly by alkaline hydrolysis and is accelerated by various electrophiles. Another benefit of the use of HPE–IAM is increased hydrophilicity and thus longer retention of various HPE–IAM adducts on a reverse column, which can greatly improve resolution of their elution profiles in liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis. Thus, LMW persulfides, but only after conjugation with HPE–IAM, can be measured in a rigorously quantitative manner via LC–MS/MS. For quantification of protein-bound persulfides, the protein of interest is labeled with HPE–IAM, digested with pronase or trypsin, and analyzed by means of LC–MS. We also developed a quick, convenient alternative method: the biotin-polyethylene glycol-conjugated maleimide (biotin-PEG-MAL) labeling gel shift assay [15••]. These newly developed methods of detection and quantification have revealed unexpectedly high concentrations of persulfides in cells. For example, mouse liver has a concentration of cysteine persulfide that is close to 20% of that of cysteine, and almost 10–20% of protein thiols are in the form of persulfides [8••].

Cysteinyl-tRNA synthetase as a major cysteine persulfide synthase (CPERS)

A debate has continued for some time about which enzyme is responsible for production of persulfides [8••,17]. Cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) have been proposed as persulfide-synthesizing enzymes [4••,18]. Indeed, CSE and CBS generate cysteine persulfide from cystine by cleaving a C–S bond and leaving serine or pyruvate *in vitro* (Figure 2). However, endogenous persulfide production is not directly regulated by these enzymes [8••], which strongly suggests the presence of alternative enzymes responsible for persulfide production *in vivo*.

We recently identified cysteinyl-tRNA synthetase (CARS) as a major cysteine persulfide-synthesizing enzyme (CPERS) [8••]. Prokaryotes possess a single CARS, whereas eukaryotes possess two isoforms of CARS, cytoplasmic CARS1 and mitochondrial CARS2. CARS1 and CARS2 likely contribute to the generation of protein-bound persulfides and LMW persulfides, respectively. Of four highly conserved motifs of CARS, two are specifically required for cysteinyl-tRNA-synthesizing activity, and the other two are specifically required for CPERS activity. The latter two motifs contain lysine residues for pyridoxal phosphate (PLP) binding, which is essential for CPERS activity. Mutation of the PLP-binding motifs led to successful generation of CPERS activity-deficient CARS, which is a powerful tool for proving the biological significance of persulfides *in vivo*.

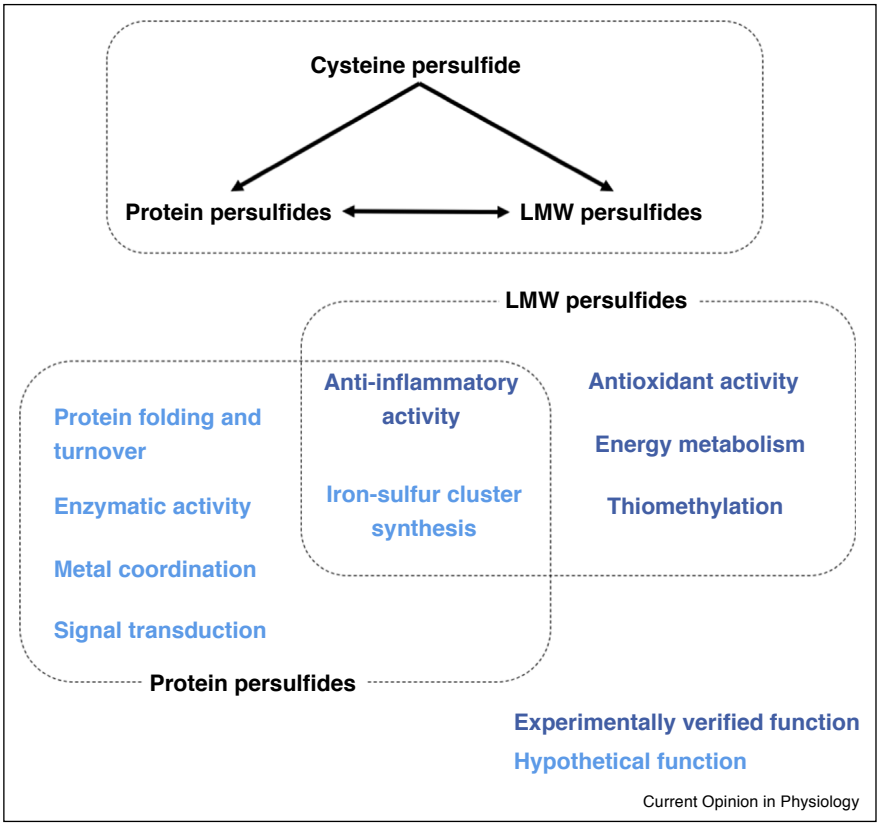
Biological roles of persulfides (Figure 3)

As mentioned earlier, LMW persulfides possess antioxidant functions and highly nucleophilic activity [4••]. Even though intracellular concentrations of cysteine persulfide and glutathione persulfide are lower than those of cysteine and glutathione, respectively, the increased reactivities of LMW persulfides are likely to overcome this quantity difference and result in a significant overall contribution to antioxidant capacity [4••,19].

Another role of LMW persulfides produced by CARS2 in mitochondria is the maintenance of mitochondrial membrane potential [8••]. Cysteine persulfide synthesized by CARS2 is reduced to hydrogen sulfide in the presence of mitochondrial electron transfer chain activity [8••], which is likely followed by hydrogen sulfide oxidation coupled with membrane potential generation [20]. Sulfide is an energy source for microbes living in the deep-sea hydrothermal vent; this mechanism appears to be a major energy metabolic pathway for early ancestral organisms [21]. Although many organisms came to utilize oxygen for energy production after the Great Oxygenation Event three billion years ago, when molecular oxygen started to accumulate in the atmosphere, an interesting fact is that higher animals including humans are still equipped with the ability to utilize sulfur for respiration that was originally used by ancient organisms. An important question is whether any specific conditions for this sulfur respiration are especially critical.

LMW persulfides also provide sulfur atoms for thiomethylation of tRNA [22] and possibly for iron–sulfur cluster synthesis. Administration of *N*-acetylcysteine tetrasulfide, one of the LMW persulfides, results in a potent anti-inflammatory activity [23], which may be due to the transfer of sulfur from the persulfide to the protein thiols of redox-sensitive components in the inflammasome machinery complex that are critically involved in inflammatory responses.

Figure 3



Biological significance of persulfides. Experimentally verified functions and hypothetical functions are indicated in dark blue and light blue, respectively.

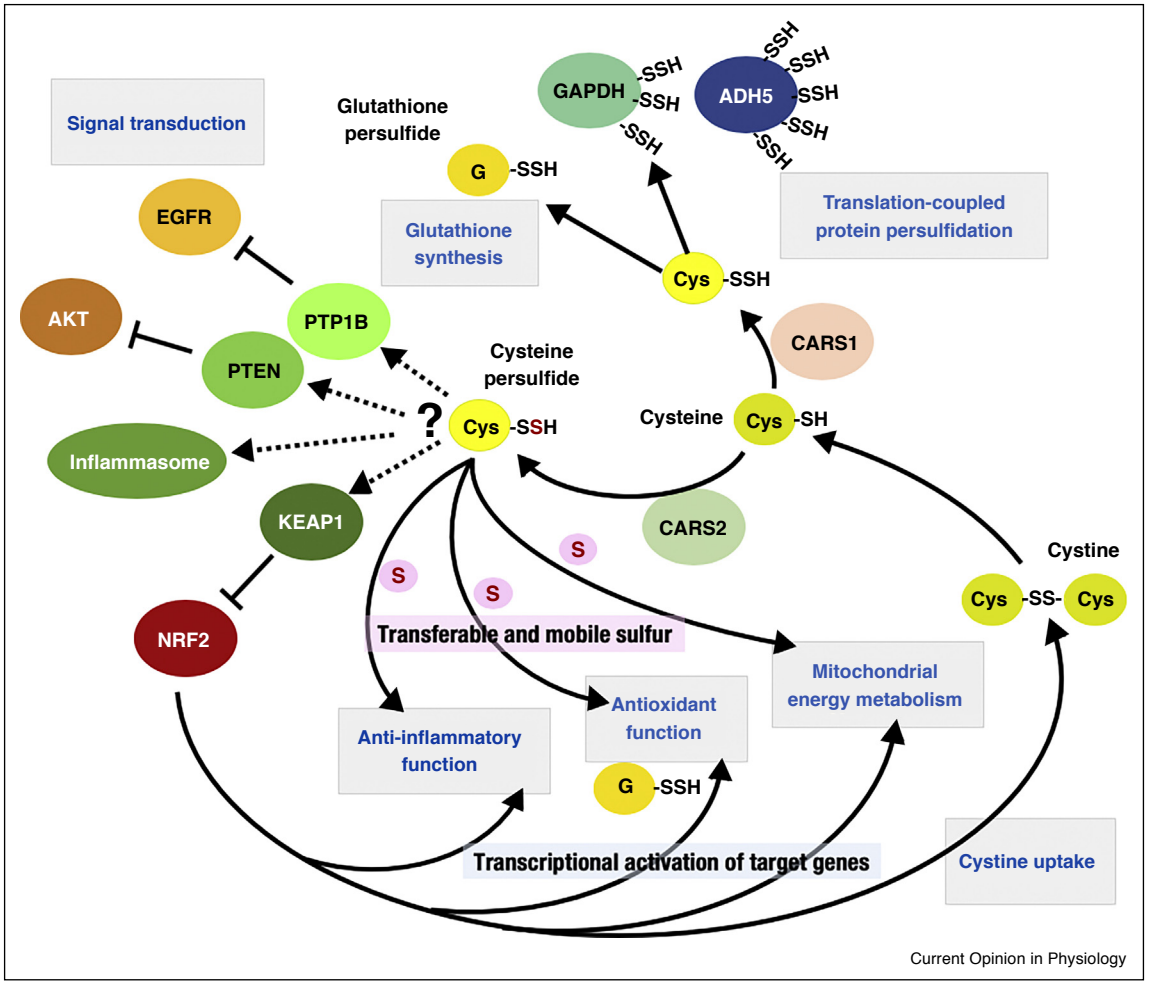
The biological roles of protein and even peptidyl persulfides are still under investigation. However, in the view of the substantially high ratios of persulfides versus those of simple thiols, which are both found in proteins, all the functions currently attributed to thiols should be re-evaluated. For instance, protein folding and turnover, enzymatic activity and protein function, and metal coordination for the structural and catalytic integrity of proteins would probably be better understood if protein-bound persulfides are considered. In particular, because of the high reactivity of persulfides, protein persulfides are likely to make important contributions to signal transduction in response to redox alterations. When thiols are chemically modified by alkylation or extensive oxidation such as sulfination ($-SO_2H$) and sulfonation ($-SO_3H$), regeneration of thiols basically requires *de novo* synthesis of the whole protein after proteolytic degradation via proteasome and/or autophagy machinery, because these modifications are all irreversible. In the case of persulfides, the distal sulfur atom rather than the proximal sulfur atom is modified because of to its higher nucleophilicity compared with that of the proximal sulfur atom, which enables the reversible regeneration or restoration and recycling of thiols simply by a cleaving off of the modified sulfur. This functional reversibility may allow protein cysteine persulfides to play a role in the finely tuned regulation of redox signaling.

We recently discovered that persulfides in the proximity of metabolites possessing hydroxy groups, such as tyrosine, glycerol, and sucrose, are stabilized (Figure 2) [16], which raises exciting possibilities about the functional contributions of persulfides *in vivo*. Nutrients and metabolites probably modulate the biological impacts of persulfides. Persulfidation of specific cysteine residues of certain proteins may be better understood by the effect of the presence of tyrosine near the persulfides on their chemical stability. Moreover, posttranslational modification of tyrosine may destabilize persulfides, which may regulate persulfide-mediated biological effects.

Functional relationship between the KEAP1–NRF2 system and persulfide-driven activities

Persulfides are likely to possess three major roles of great biological relevance, that is, strong antioxidant

Figure 4



Functional relationship of the KEAP1–NRF2 system and persulfides. Cysteine persulfude is synthesized in cytoplasm and in mitochondria by CARS1 and CARS2, respectively. CARS1 is thought to contribute mainly to co-translational persulfide synthesis in proteins, whereas CARS2 contributes to LMW persulfude synthesis. GAPDH and ADH5 are heavily persulfidated and are expected to be generated via translation-coupled protein persulfidation. An enhanced electrophile-quenching ability of LMW persulfides, such as cysteine persulfide and glutathione persulfide, is likely to suppress an electrophilic response including that of the KEAP1–NRF2 system. EGFR, epidermal growth factor receptor.

and anti-inflammatory functions and maintenance of mitochondrial energy metabolism. These functions are most likely shared by persulfides and NRF2 (Figure 4). In addition to the antioxidant function, NRF2 has potent anti-inflammatory activity, which alleviates chronic inflammation caused by autoimmunity as well as aging [24–26]. NRF2 activation is also beneficial for improving mitochondrial function, although the precise mechanisms of that process have not been clarified [27,28]. NRF2 can increase the expression of the cystine transporter xCT and increase cysteine availability [29]. With regard to this function, by contributing to the substrate supply, NRF2 is in the position to support persulfide generation and cellular activities driven by persulfides.

In turn, persulfides may suppress activation of the NRF2 pathway. Because persulfides quench electrophiles efficiently, the sensor thiols in KEAP1 would be spared, and the E3 ligase activity of KEAP1–Cullin3 complex would be retained. In fact, persulfides appear to attenuate the responses to electrophilic signaling in cells in general [4••,30,31]. Thus, persulfide abundance and NRF2 activity appear to be regulated in a negative feedback loop.

Given that NRF2 and persulfides may interact closely, the molecular mechanisms underlying the outcomes of NRF2 pathway activation and those of persulfide-driven signaling are distinct from each other. We propose that persulfide synthesis and metabolism are solely controlled through a unique process that depends on the transfer and

mobilization of sulfur among diverse biological molecules such as LMW and protein thiols and even metals such as iron and zinc ions. Therefore, such a transferable and mobile sulfur species primarily executes the biological actions of persulfides, apparently mimicking the chemical properties of selenium. In contrast, NRF2 may manage biological actions via transcriptional activation of its target genes, with this process being presumably independent of the one involving transferrable and mobile sulfur. The persulfides that function as transferable and mobile sulfur species and the KEAP1–NRF2 transcriptional regulation together make up a sulfur-utilizing dual system for cytoprotection and energy metabolism. The functional failure of this system may underlie various human pathological conditions, and clarification of changes in the function and activity of this system during the aging process may provide promising clues to understanding pathogenesis of aging-related diseases.

Conflict of interest statement

Nothing declared.

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