

Recent Developments in Research of Reactive Sulfur Species Guest Editor: Tomohiro Sawa

Antioxidative and anti-inflammatory actions of reactive cysteine persulfides

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Cysteine persulfide (CysSSH) and polysulfides (CysS[S]_nH, *n*>1) are cysteine derivatives having sulfane sulfur atoms bound to cysteine thiol. Recent advances in the development of analytical methods for detection and quantification of persulfides and polysulfides have revealed the biological presence, in both prokaryotes and eukaryotes, of persulfide/polysulfide in diverse forms such as CysSSH, glutathione persulfide and protein persulfides. Accumulating evidence has suggested that persulfide/polysulfide species may involve in a variety of biological events such as biosyntheses of sulfur-containing molecules, tRNA modification, regulation of redox-dependent signal transduction, mitochondrial energy metabolism via sulfur respiration, cytoprotection from oxidative stress via their antioxidant activities, and anti-inflammation against Toll-like receptor-mediated inflammatory responses. Development of chemical sulfur donors may facilitate further understanding of physiological and pathophysiological roles of persulfide/polysulfide species, including regulatory roles of these species in immune responses.

Key Words: persulfide, reactive sulfur species, antioxidant, anti-inflammatory effect, electrophile

Cysteine persulfide (CysSSH) and polysulfides (CysS[S]_nH, *n*>1) are cysteine derivatives having sulfane sulfur atoms bound to cysteine thiol.⁽¹⁻⁴⁾ Various forms of persulfide/polysulfide exist in both prokaryotes and eukaryotes as cysteine-containing low molecular weight compounds such as glutathione persulfide (GSSH) and CysSSHs in proteins (Fig. 1).⁽⁵⁻¹¹⁾ CysSSH and related reactive sulfur species have been suggested to involve in a variety of biological processes. Hidese *et al.*⁽¹²⁾ and Zhang *et al.*⁽¹³⁾ reported that CysSSH serves as an important intermediate by donating its sulfane sulfur atoms during biosynthesis of sulfur-containing biofactors such as iron-sulfur clusters, biotin, and lipoic acid. Takahashi *et al.*⁽¹⁴⁾ demonstrated that CysSSH is involved in the regulation of tRNA methylthiolation and insulin secretion. Recent study has revealed that CysSSH can participate in energy metabolism through sulfur respiration in mitochondria.^(3,4,6) CysSSH can also act as a strong nucleophile and an antioxidant and may play an important role in regulating oxidative stress and redox signaling in cells.^(1,5-7,15) We recently identified that CysSSH and related molecules possess strong anti-inflammatory properties.⁽¹¹⁾ In this review article, we discuss the antioxidative and anti-inflammatory actions of CysSSH and related persulfide/polysulfide. Readers are also referred to useful review articles on roles of CysSSH in sulfur respiration.^(3,4)

Antioxidative and Nucleophilic Properties of Persulfide/Polysulfides

Persulfide and polysulfide species have been reported to be more nucleophilic and superior reducing agents compared with their corresponding parental thiols.^(2,15,16) We demonstrated that GSSH derived from glutathione reductase-mediated reduction of oxidized glutathione trisulfide (GSSG) very efficiently reduced hydrogen peroxide (H₂O₂).⁽⁵⁾ Under the same conditions, parental glutathione (GSH) or hydrogen sulfide (H₂S) failed to reduce H₂O₂.⁽⁵⁾ Li *et al.*⁽¹⁷⁾ reported that GSSH was 50-fold more reactive than H₂S towards H₂O₂ at physiological pH. In cell systems, overexpression of cystathione γ -lyase (CSE) enhanced GSSH levels, and more importantly, protected the cells from H₂O₂-induced cell death.⁽⁵⁾ Kunikata *et al.*⁽⁷⁾ also reported that exogenous addition of GSSG significantly suppressed cultured cell death induced by H₂O₂ exposure. These data suggest that persulfide/polysulfide species may act as important antioxidants inside cells and protect cells from oxidative stress.

Everett and Wardman⁽¹⁸⁾ reported that persulfides can efficiently scavenge free radical species. Persulfides are stronger acids than thiols and at physiological pH (at which many thiols are predominantly in the protonated form) a significant proportion of alkyl persulfide (RSSH) exists as the deprotonated alkyl persulfide anion (RSS⁻).⁽¹⁵⁾ In fact, Li *et al.*⁽¹⁷⁾ recently reported that pK_a of GSSH was determined to be 6.9, that is two orders of magnitude smaller than GSH (pK_a = 8.9). RSS⁻ thus formed readily reacts with radical species to form alkyl persulfide radical (RSS•).⁽¹⁸⁾ RSS• are significantly less reactive than their thiyl radical counterparts and are less likely to pose a threat if generated within the cells.

Persulfide and polysulfide species can also efficiently react with various electrophiles.^(5,19) 8-Nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP) is an endogenously occurring weak electrophile and acts as a second messenger in redox signaling.⁽²⁰⁻³⁰⁾ We have found that persulfide/polysulfide species reacted with 8-nitro-cGMP to convert it to 8-mercapt-cGMP.^(5,25) These findings demonstrate the unique potency of persulfide/polysulfide species as strong reductants and nucleophiles, so that they possess greater reactivity than thiols and can scavenge oxidants and cellular electrophiles, thereby serving as important antioxidant molecules.

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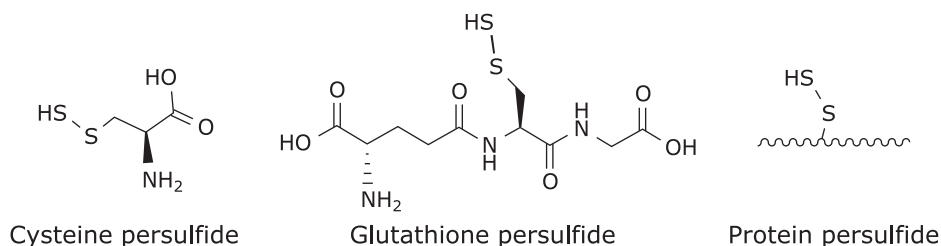


Fig. 1. Chemical structures of representative endogenous persulfide species.

NAC Polysulfides as Potent Sulfur Donors

Polysulfur donors that can increase intracellular persulfide/polysulfide levels by donating their sulfur atoms to the endogenous acceptor thiols become powerful chemical tools for understanding of the physiological and pathological roles of reactive sulfur species in biological systems. Powell *et al.*⁽³¹⁾ reported that *N*-acetyl-L-cysteine (NAC) conjugated to Bpin through disulfide bonding can act as a prodrug for persulfide donor (BDP-NAC). BDP-NAC can release free NAC hydropersulfide (NAC-SH) in response to H₂O₂ exposure.⁽³¹⁾ They demonstrated that BDP-NAC treatment protected cells from cell death caused by H₂O₂ exposure, possibly through producing antioxidative NAC-SH in cells.⁽³¹⁾ Zheng *et al.*⁽³²⁾ developed the persulfide prodrug that can generate a hydroxymethyl persulfide via esterase-dependent activation. They also reported that such a persulfide generating prodrug exhibited potent cardioprotective effects in a murine model of myocardial ischemia-reperfusion injury with a bell shape therapeutic profile.⁽³²⁾ Kang *et al.*⁽³³⁾ reported another type of persulfide precursors that produce hydropersulfides via pH- or F-mediated desilylation of *O*-silyl mercaptan based sulfur containing molecules.

One of the unique characteristics inherited to persulfide/polysulfides are that they are able to donate sulfane sulfur atoms to the acceptor thiols through sulfur transfer reactions.^(2,15,34) In this context, we demonstrated that *N*-acetyl-L-cysteine polysulfides (NAC polysulfides; Fig. 2) very efficiently increase intracellular levels of reactive sulfur species such as CysSSH and GSSH.⁽²⁾ Mass spectrometric analyses clearly indicated that NAC polysulfides were rapidly incorporated into cells and donated their sulfur atoms to the endogenous acceptor thiols such as cysteine and GSH.⁽¹¹⁾ NAC polysulfides may have some advantages as persulfide/polysulfide donors. First, NAC polysulfides are stable during storage even in aqueous media. This will save the researchers the trouble for preparing fresh solution just before each experiment. Second, one can easily prepare NAC polysulfides labeled with ³⁴S at sulfane sulfur moieties by using ³⁴S-sulfide as a starting material. Sulfur transfer from ³⁴S-labeled NAC polysulfides and cellular acceptor thiols can thus be determined

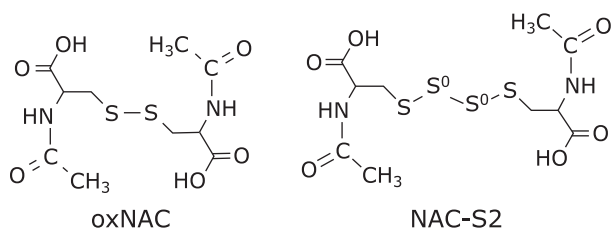


Fig. 2. Chemical structures of NAC polysulfide (NAC-S₂) and related compound. oxNAC is a control reagent having no polysulfide donating capability. NAC-S₂ is a polysulfide donor that transfers their sulfane sulfur (S⁰) to acceptor thiols.

by monitoring the isotope-targeted sulfur metabolomics in complex biological milieu. Furthermore, metabolisms of persulfide/polysulfides, particularly focusing on their sulfane sulfur atoms may be studied by detecting sulfur metabolites labeled with ³⁴S. Third, the decomposition metabolites derived from NAC polysulfides including NAC itself are expected to be biologically inert. Thus, one can expect that NAC polysulfides may be applicable for therapeutic purposes as prototype drugs if they have beneficial effects.

Anti-inflammatory Functions of NAC Polysulfides

Studies using *in vitro* cell culture models as well as *in vivo* mice endotoxin shock models revealed that NAC polysulfides possess potent anti-inflammatory functions.⁽¹¹⁾ Macrophages are immunologically stimulated by lipopolysaccharide (LPS), a Gram-negative bacterial cell component, to activate Toll-like receptor 4 (TLR4) signaling for producing proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interferon- β (IFN- β). It was demonstrated that NAC polysulfide treatment remarkably inhibited the production of both TNF- α and IFN- β from mouse macrophage cell line Raw264.7 cells stimulated with LPS.⁽¹¹⁾ It has been known that several phosphorylation-transcription factor signals are activated in response to LPS during production of TNF- α , such as I κ B kinase (IKK)- and AKT-nuclear factor kappa B (NF- κ B), and mitogen-activated protein kinase (MAPK)-/c-Jun N-terminal kinase (JNK)/extracellular signal-regulated protein kinase (ERK)-AP-1 pathways.^(35,36) We observed that NAC polysulfides treatment significantly inhibited NF- κ B activation by suppressing phosphorylation of IKK. On the other hand, another phosphorylation signaling downstream to LPS-TLR4 such as AKT-NF- κ B, and MAPK-/JNK-/ERK-AP-1 pathways were not affected by NAC polysulfide treatment.⁽¹¹⁾ In innate immune responses, a variety of ligands such as zymosan A (by TLR2) and viral RNA duplex (by TLR3) are recognized by different TLRs.^(37,38) Our data suggested that NAC polysulfides can inhibit not only TLR4 but also TLR2 and TLR3 by suppressing IKK/NF- κ B signaling.⁽¹¹⁾ In addition to the inhibition of pro-inflammatory cytokine production, NAC polysulfides strongly suppressed cytokine-mediated inflammatory responses such as expression of inducible nitric oxide synthase (iNOS). IFN- β released extracellularly can activate signal transducer and activator of transcription 1 (STAT1) signaling, leading to the production of inflammatory mediator nitric oxide (NO) via expression of iNOS, in autocrine and/or paracrine manner. It was found that NAC polysulfides are capable of suppressing IFN- β dependent inflammatory responses by both inhibiting IFN- β production as well as STAT1 phosphorylation.⁽¹¹⁾

We observed the moderate but significant reduction of cell viability by NAC polysulfides treatment at high concentrations (>0.5 mM).⁽¹¹⁾ Under those conditions, cellular redox balance may become a more reducing state due to increased formation of strong antioxidants such as hydropersulfides and hydropolysulfides. Recent studies have suggested that such reducing cellular state

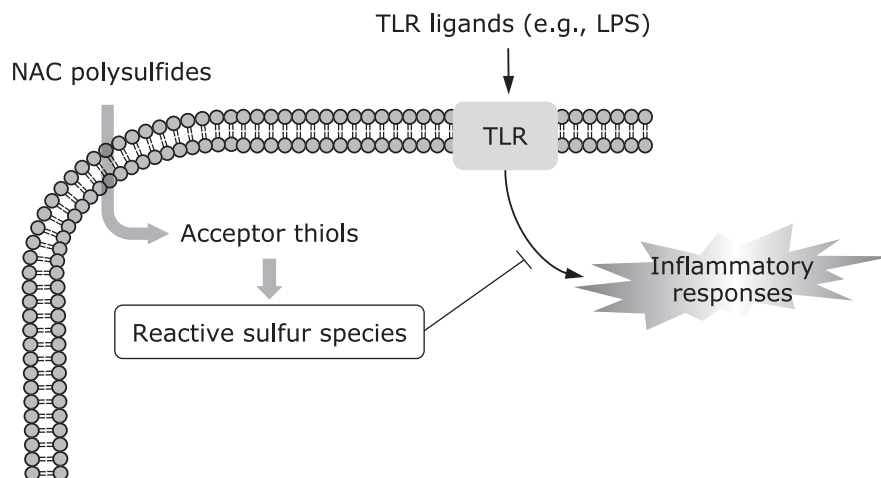


Fig. 3. Suppression of immune responses by NAC polysulfide. NAC polysulfide that penetrates plasma membrane increases intracellular reactive sulfur species by donating its sulfur to acceptor thiols. In inflammatory cells such as macrophages treated with NAC polysulfide, TLR ligand stimulation is remarkably suppressed.

may be detrimental to cells named as “reductive stress” conditions.^(39,40) Thus, apparent adverse and/or toxic effects observed for NAC polysulfides may be associated with augmentation of reductive stress. Further study is warranted to clarify cell type specificity, reversibility, and mechanisms involved in occurrence of reductive stress caused by NAC polysulfides and related sulfur donors.

Macrophage activation in response to LPS is an important innate immune response that helps eradication of infected Gram-negative bacteria. However, when host individuals are suffered from LPS exposure continuously or in large quantity, macrophages are over-activated to produce excess amounts of pro-inflammatory cytokines (cytokine storm), and finally leading to lethal endotoxin shock.^(37,38) We investigated whether NAC polysulfides inhibit LPS-induced pro-inflammatory responses in mice, and hence, rescue mice from endotoxin shock.⁽¹¹⁾ The mice endotoxin shock model used showed that 80% of mice died within 96 h after receiving LPS intraperitoneally.⁽¹¹⁾ In the NAC polysulfide treatment group, mice survival rate was remarkably improved; 90% of mice were survived 96 h after LPS administration.⁽¹¹⁾ These data suggest that NAC polysulfides show anti-inflammatory functions *in vivo*, possibly through suppression of LPS-induced inflammatory responses (Fig. 3).

Endotoxin shock is an acute life-threatening organ dysfunction.^(41,42) Endotoxin shock with increased LPS levels in blood, overexpression of pro-inflammatory cytokines, activation of blood coagulation system, and accumulation of fibrinogen dysfunction products leads to a violation of local and general hemodynamics and endothelial dysfunction via TLRs signaling pathways.⁽⁴²⁾ Various compounds have been tested on animal models for their capacity to block TLR4-mediated cytokine production, and several have reached the clinical trials.⁽⁴²⁾ The known TLR4 antagonists belong to various classes of chemical compounds, mainly glycolipids that mimic the natural TLR4 ligand, lipid A, but also heterocycles, peptides, opioids, taxanes, steroids, etc, and have natural and synthetic origin.⁽⁴²⁾ In this study, we demonstrated that NAC polysulfide treatment protected mice from lethal endotoxin shock model. This result suggests that per/polysulfide donors may become a new class of TLR4 antagonist that can be applicable for treatment of endotoxin shock. Very recently, TLR4 has been suggested as a promising therapeutic target for drug abuse and major depressive disorders,^(43,44) as well as amyotrophic lateral sclerosis.⁽⁴⁵⁾ Possible application of TLR4 antagonists in treatment of peripheral neuropathic pain has

also been discussed.⁽⁴⁶⁾ Persulfide/polysulfide donors solely or in combination with other TLR4 antagonists warrants further investigation as potential therapeutic options.

In summary, reactive sulfur species are closely involved with immune function regulation, and that inflammatory pathology can be improved by artificially increasing reactive sulfur species. Excessive inflammatory reactions are greatly involved not only in endotoxin shock, but also in allergies and autoimmune diseases. Steroid hormones and immunosuppressants are the typical treatments for these disorders, but they come with various side effects. In the future, we expect to target intracellular active sulfur regulation to develop a new anti-inflammatory therapy.

Abbreviations

CysSSH	cysteine persulfide
ERK	extracellular signal-regulated protein kinase
GSH	glutathione
GSSH	glutathione persulfide
GSSSG	oxidized glutathione trisulfide
H ₂ O ₂	hydrogen peroxide
H ₂ S	hydrogen sulfide
IFN-β	interferon-β
IKK	Iκappa B kinase
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
NAC	<i>N</i> -acetyl-L-cysteine
NAC-SH	<i>N</i> -acetyl-L-cystine hydropersulfide
NAC polysulfide	<i>N</i> -acetyl-L-cystine polysulfide
NF-κB	nuclear factor kappa B
8-nitro-cGMP	8-nitroguanosine 3',5'-cyclic monophosphate
RSSH	alkyl persulfide
RSS ⁻	alkyl persulfide anion
RSS•	alkyl persulfide radical
STAT1	signal transducer and activator of transcription 1
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α

Conflict of Interest

No potential conflicts of interest were disclosed.

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