



The mechanism of action of N-acetylcysteine (NAC): The emerging role of H₂S and sulfane sulfur species

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ARTICLE INFO

Available online 23 June 2021

Keywords:

N-acetylcysteine
Cysteine
Hydrogen sulfide
Sulfane sulfur species
Persulfides
Glutathione
Disulfide reduction
Cytoprotection
Oxidative stress
ROS scavenging
Antioxidants

ABSTRACT

Initially adopted as a mucolytic about 60 years ago, the cysteine prodrug N-acetylcysteine (NAC) is the standard of care to treat paracetamol intoxication, and is included on the World Health Organization's list of essential medicines. Additionally, NAC increasingly became the epitome of an "antioxidant". Arguably, it is the most widely used "antioxidant" in experimental cell and animal biology, as well as clinical studies. Most investigators use and test NAC with the idea that it prevents or attenuates oxidative stress. Conventionally, it is assumed that NAC acts as (i) a reductant of disulfide bonds, (ii) a scavenger of reactive oxygen species and/or (iii) a precursor for glutathione biosynthesis. While these mechanisms may apply under specific circumstances, they cannot be generalized to explain the effects of NAC in a majority of settings and situations. In most cases the mechanism of action has remained unclear and untested. In this review, we discuss the validity of conventional assumptions and the scope of a newly discovered mechanism of action, namely the conversion of NAC into hydrogen sulfide and sulfane sulfur species. The antioxidative and cytoprotective activities of per- and polysulfides may explain many of the effects that have previously been ascribed to NAC or NAC-derived glutathione.

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Abbreviations: BSO, buthionine sulfoximine; CARS, cysteinyl-tRNA synthetase; CAT, cysteine aminotransferase; CBS, cystathionine-β-synthase; CDO, cysteine dioxygenase; CSE, cystathionine-γ-lyase; Cys, cysteine; ETC, electron transport chain; GSH, glutathione; H₂S, hydrogen sulfide; i.v., intravenous; MPST, 3-mercaptopyruvate sulfurtransferase; NAC, N-acetylcysteine; NACA, N-acetylcysteine amide; NACET, N-acetylcysteine ethyl ester; pK_a, logarithmic acid dissociation constant; PLP, pyridoxal 5'-phosphate; ROS, reactive oxygen species; SQR, sulfide:quinone oxidoreductase.

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1. Introduction: the popularity of N-acetylcysteine

It would be an overwhelming, if not impossible, task to comprehensively review the whole body of publications associated with the use of N-acetylcysteine (NAC). As of early 2021 PubMed listed a total of 17027 papers mentioning N-acetylcysteine (or its synonyms N-acetyl-L-cysteine and acetylcysteine) in the title and/or abstract. Even this number is an underestimate, as there are many papers that mention NAC only in the results or methods part, and hence are invisible in the PubMed search.

The popularity of NAC has continuously increased over the last 60 years. Since 2009, the yearly number of NAC-related publications has been well above 900, and since 2013 the number has plateaued at around 1000 papers per year (Fig. 1A). Even if the increasing number of NAC-related publications is to be normalized against the overall increase in PubMed publications, it is still apparent that interest in NAC has remained at a high level over the last 20 years (Fig. 1B).

Studies involving NAC are extremely diverse, ranging from cell-free systems to living organisms. On the one hand, there is the idea that administration of NAC can be beneficial in the context of human disease. Many studies explore the pharmacological use of NAC in animal disease models and clinical studies. Beyond the original uses of NAC as a mucolytic agent and an antidote against paracetamol overdose (to be discussed below), more recent work investigates its potential use in many other pathophysiological scenarios, e.g. psychiatric and neurological disorders (Deepmala et al., 2015; Fernandes, Dean, Dodd, Malhi, & Berk, 2016), or even coronavirus disease 2019 (COVID-19) (Bourgonje et al., 2021; Ibrahim et al., 2020). On the other hand, there is the idea of using NAC as a tool for the removal (scavenging) of oxidants in cell culture or animal experiments. NAC is often considered a generic “antioxidant”, and many investigators using or testing NAC adhere to the notion that NAC lowers reactive oxygen species (ROS) levels and relieves “oxidative stress”, whatever these umbrella terms may mean in any specific context. In a typical scenario, when researchers are asking if some observed effect has been caused by ROS, they apply NAC to their system (cell culture or animals), making the assumption that it will remove the oxidant. If the phenomenon of interest (e.g. cell death) is abolished or diminished by NAC, it is then concluded that ROS have been causative.

All these studies differ in many ways, e.g. in how the NAC solution has been prepared, and in the route and duration of application or administration. What most of these studies lack is an understanding of the connection between the experimental or clinical outcome and the

molecular mechanism of action. Matters are further complicated by the fact that improper use of NAC can lead to artefacts and misinterpretations, especially in cell culture experiments. An often overlooked complication is the acidity of unbuffered or weakly buffered NAC solutions. NAC is acidic due to its carboxyl group ($pK_a = 3.14$) (Fazary et al., 2020) and the absence of a free amino group. A final concentration of ~10 mM, as often used in cell culture experiments, can bring down the pH of phosphate buffer (PBS) and cell culture medium (RPMI) to 3.8 (Kundukad et al., 2020) and 5.8 (Amaral et al., 2016), respectively. When using non-pH-adjusted NAC (stock) solutions, it is difficult to know whether the experimental outcome has been influenced by acidification of the medium. Unfortunately, the preparation of NAC solutions is rarely described in the methods section of papers.

To rationalize the observed (or anticipated) effects of NAC, the literature has propagated three major explanatory narratives, which have been retold for decades. These are the narratives of NAC as a disulfide reductant, as a direct scavenger of oxidants and as a driver of glutathione (GSH) synthesis (Fig. 2). These narratives have an origin and some justification, yet all too often they extrapolate findings made under very specific circumstances to a more general context, typically without further questioning or testing. Accordingly, authors reporting on the effects of NAC have expressed very different viewpoints about our understanding of NAC's mechanism of action. Many authors state that NAC has “a well-defined mechanism of action” (Millea, 2009) or even is “one of the best understood drugs” available. Others say the exact opposite, namely that a mechanism of action for NAC is still elusive and remains to be established. Below we discuss the history and assumptions behind the three major narratives, as well as their limitations, and then consider alternative mechanisms by which NAC may exert its effects.

2. Three classical narratives

2.1. The disulfide reductant narrative

The NAC story started in earnest in the early 1960s when scientists at Mead Johnson recognized that NAC is an effective agent for the liquefaction of mucoid secretions. The first clinical study confirmed it to clear the tracheobronchial airway in patients suffering from a range of acute and chronic obstructive lung diseases (Webb, 1962). On May 28, 1963, the US Patent Office granted patent 3.091.569 for “Mucolytic N-Acylated Sulfhydryl Compositions and Process for Treating Animal Mucus” to Mead Johnson & Company, Evansville, Indiana. Further

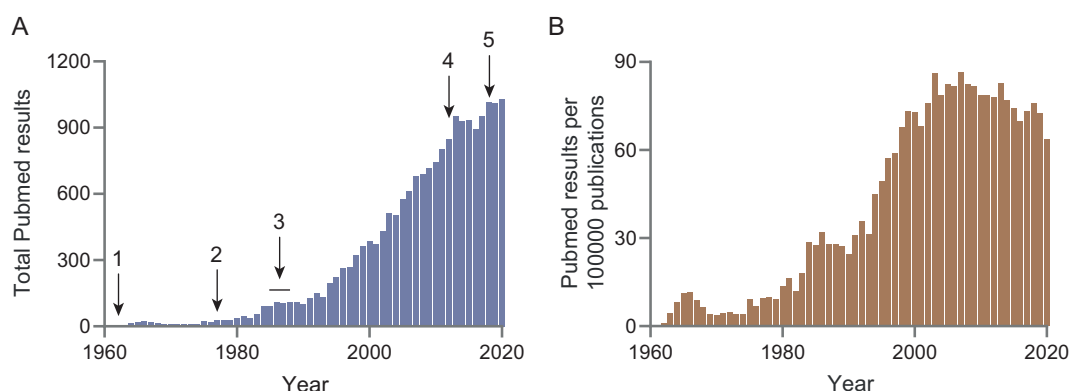


Fig. 1. Number of NAC-related publications per year.

(A) Yearly total number of PubMed-listed publications with “N-acetylcysteine” or “N-acetyl-L-cysteine” or “acetylcysteine” in the title and/or abstract. Key discoveries are indicated by numbers: 1, NAC liquefies lung mucus by disulfide bond reduction (Webb, 1962); 2, NAC is an antidote against paracetamol poisoning (Prescott et al., 1977); 3, NAC protects cells against oxidative insults (several authors, 1985–1988); 4, NAC is a source of hydrogen sulfide in bacteria (Kartha et al., 2012); 5, NAC is a source of hydrogen sulfide and sulfane sulfur in mammalian cells (Ezerina et al., 2018).

(B) The results shown in (A) were normalized against the total number of PubMed publications in each year (NAC-related publications per 100,000 publications). Normalization was performed with the “Pubmed by Year” tool (Sperr, 2016).

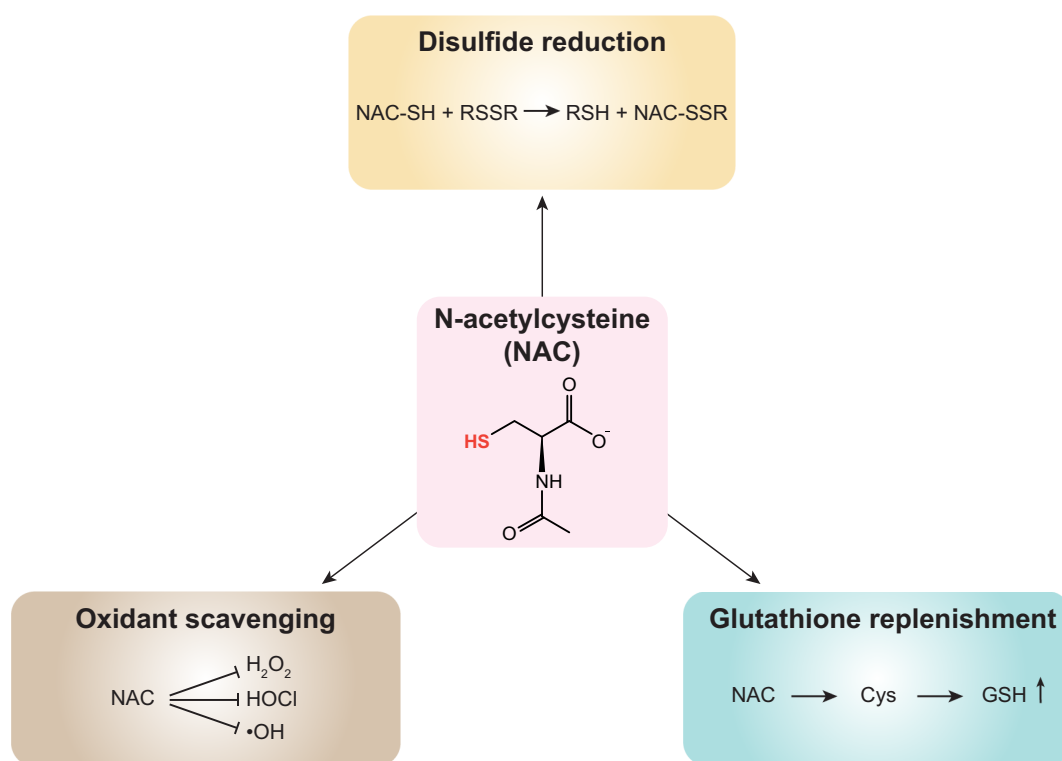


Fig. 2. The three classical narratives of how NAC exerts its biological effects.

Three narratives have been cultivated in the literature to explain the observed biological effects of NAC. The disulfide reductant narrative postulates that the beneficial effects of NAC are due to its capacity to reduce extra- and/or intracellular disulfide bonds. The oxidant scavenger narrative argues that the NAC sulfhydryl group (highlighted in red) is effective in removing one- and two-electron oxidants, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) or hydroxyl radicals ($\bullet\text{OH}$). The glutathione replenishment narrative proposes that NAC delivers Cys for GSH synthesis, hence boosting GSH levels.

research by Mead Johnson scientists concluded, based on *in vitro* experiments with patient samples, that NAC liquefies mucus by reducing disulfide bonds in mucus proteins (Sheffner, 1963a). This mechanism of action is indeed highly plausible in the context of the original clinical studies which directly supplied a 20% (w/v) NAC solution (1.22 M) into the lung, either as an aerosol mist or by intratracheal instillations (e.g. by catheter) (Sheffner, 1963b), hence directly exposing the mucus to very substantial amounts of NAC. Indeed, the major mucus proteins, the mucins, are rich in cysteine (Cys) and known to form massive aggregates which are stabilized by intra- and intermolecular disulfide bridges (Janssen, Stefanski, Bochner, & Evans, 2016), as recently detailed by structural analysis (Javitt et al., 2020). Additionally, intrabronchially applied NAC may achieve some of its benefits by reducing disulfide bonds in proteins other than mucins. For example, disulfide bonds are recognized to stabilize the hydrophobic surface of bacterial biofilms (Arnaouteli et al., 2017) and NAC has been proposed to disrupt bacterial biofilms, potentially also in the lung (Blasi et al., 2016). However, we could not find publications that provide direct evidence for protein disulfide reduction in the original clinical context, i.e. experiments that biochemically demonstrate the breaking of mucin disulfide bonds (and/or the formation of mixed mucin NAC disulfide conjugates) in patient samples obtained before and after *in situ* NAC inhalation/instillation. Instead, we encountered reports of recent clinical studies that have struggled to confirm the efficacy of inhaled NAC with respect to improvement of lung function or reduction in pulmonary exacerbations (Nash, Stephenson, Ratjen, & Tullis, 2009; Rogers, 2002; Tam, Nash, Ratjen, Tullis, & Stephenson, 2013). This includes a study that found that inhaled NAC did not alter sputum biophysical properties in cystic fibrosis patients and concluded that NAC is largely ineffective due to its low reducing activity (Ehre et al., 2019). It is interesting to note that some studies even suggested that a modest

improvement of lung function after NAC inhalation is caused by the osmotic activity of highly concentrated NAC solutions and can be mimicked with equi-osmotic NaCl solutions (Clarke, Thomson, & Pavia, 1980; Pavia, Sutton, Lopez-Vidriero, Agnew, & Clarke, 1983), thus being unrelated to reducing activity.

The recent failure of confirmation of the original results reported by Sheffner et al. may be explained by differences in concentrations and mode of application. While there is little doubt that NAC can directly reduce disulfide bonds in extracellular environments to which it is applied at extremely high concentration (1.22 M), as in the original experiments of Sheffner, for concentrations in the low-to-mid mM range this seems rather unlikely. Indeed, the direct exposure of pulmonary sputum to 10 mM NAC hardly reduced any mucin disulfide bonds, in contrast to 10 mM dithiothreitol which effectively reduced mucin multimers to monomers (Ehre et al., 2019). As will be discussed below, NAC is an intrinsically inefficient reducer of disulfide bonds, no more efficient than other small monothiols, including Cys. The rate constant for NAC-mediated disulfide reduction is very low ($<1 \text{ M}^{-1} \text{ s}^{-1}$) (Whillier, Raftos, Chapman, & Kuchel, 2009), meaning that high NAC concentrations are needed to achieve any noticeable reduction within relevant periods of time (Burgunder, Varriale, & Lauterburg, 1989; Chen et al., 2011; Harada et al., 2004; Tersteeg et al., 2017; Whillier et al., 2009).

How do these considerations relate to applications beyond lungs and pulmonary tissue? Direct reduction of protein disulfides on the cell surface seems to be plausible for *in vitro* cell culture experiments, because these often apply NAC at final concentrations in the mM range (5–20 mM) and over long incubation times. Such treatments have been found to lead to detectable protein disulfide reduction on the cell surface, although the efficiency seems to be low and the proportion of reduced disulfides is difficult to quantitate (Garant, Kole, Maksimova, & Bernier, 1999; Gelderman, Hultqvist, Holmberg, Olofsson, & Holmdahl,

2006; Kim, Rhim, Choi, & Kim, 2001; Laragione et al., 2003, 2006). It has remained unclear whether any of the NAC effects observed in cell culture are causally connected to protein disulfide reduction on the cell surface.

Concerning animal experiments, it has been reported that intravenous (i.v.) injections of NAC achieve serum concentrations between 100 and 1500 μM (Medved, Brown, Bjorksten, & McKenna, 2004; Medved et al., 2003; Olsson, Johansson, Gabrielson, & Bolme, 1988). Intravenous NAC was observed to cause measurable reduction of cystine (Cys-S-S-Cys) (Harada et al., 2001), Cys-protein mixed disulfides (Harada et al., 2004) and von Willebrand Factor intermolecular disulfides (Chen et al., 2011; Tersteeg et al., 2017) in the bloodstream. As will be discussed below, it is possible that the reduction of cystine or Cys-protein mixed disulfides is a mechanism by which NAC increases the availability of reduced Cys in the circulation (Harada et al., 2001; Zhou et al., 2015).

In contrast, oral administration is reported to lead to NAC concentrations below 15 μM in the circulation (Holdiness, 1991). Thus, oral administration is the route least likely to support direct disulfide reduction in the circulation, or elsewhere in the body. It is therefore surprising that oral NAC, typically provided as effervescent tablets, is marketed in Europe as an expectorant, although it seems very unlikely that orally supplied NAC can reach the lung in sufficient amounts to actually cause disulfide reduction in the mucus. Apparently, the benefit and likely mechanism established for one particular treatment (liquefaction of bronchial mucus by intrabronchially applied NAC) has been extrapolated to a very different situation (oral NAC administration), contrary to expectations and seemingly without evidence.

Our discussion above relates to disulfide bond reduction by NAC itself, i.e. direct thiol-disulfide exchange, as this is the likely mechanism of mucin reduction in the original tracheobronchial treatment. It may still be asked if cell surface disulfide reduction observed in cell culture or in the serum after intravenous injection is indeed the consequence of direct thiol-disulfide exchange. Although this seems possible in principle, none of the above-mentioned studies provides direct evidence for this mechanism, which would require the demonstration of intermediary NAC-protein disulfide conjugates. It is therefore an open question whether NAC may feed, directly or indirectly (e.g. after being transformed into some other species), into an enzymatic pathway of disulfide reduction that affects cell surface or extracellular proteins.

In summary, like other small monothiols, NAC clearly has the ability to directly reduce disulfide bonds in proteins or other molecules. However, the low reactivity and correspondingly slow kinetics argue against a widespread and general role as an extracellular disulfide reductant, limiting this mechanism to situations where the NAC concentration is at least reaching into the mM range. Also, the related idea that NAC taken up by cells (to be addressed below), is directly contributing to the reduction of intracellular disulfide bonds appears very unlikely. As will be discussed later, uptake of NAC into cells is rather slow and the disulfide reduction rate afforded by NAC is negligible when compared to the highly efficient enzymatic reducing systems (Nagy, 2013).

2.2. The oxidant scavenger narrative

It is often claimed or assumed that NAC can protect cells against oxidative damage by directly scavenging ROS, in particular superoxide and peroxides. This notion seems to be the least supported and most problematic of the three classical narratives. It got off the ground in the 1970s and 1980s when protective effects of NAC in rats and isolated cells against cigarette smoke (assumed to contain or induce ROS) were noted (Moldéus, Berggren, & Grafström, 1985; Sprince, Parker, Smith, & Gonzales, 1975). Furthermore, cell culture experiments showed that NAC protected against exogenously applied oxidants, as generated from either natural (phagocyte co-culture) (Kharazmi, Nielsen, & Schjøtt, 1988; Simon & Suttrop, 1985) or artificial (bolus addition) sources (Junod, Jornot, & Grichting, 1987; Moldéus, Cotgreave, &

Berggren, 1986). Although direct evidence was lacking, many studies attributed the observed cytoprotective effects to direct (i.e. uncatalyzed) oxidant scavenging by NAC.

This notion was widely accepted without direct evidence, probably because it has long been known that thiol groups are susceptible to oxidation *in vitro* and that their reaction with oxidants is highly favorable from the thermodynamic point of view. Not surprisingly, *in vitro* experiments can be designed to show that NAC consumes H_2O_2 (Cotgreave, Sandy, Berggren, Moldéus, & Smith, 1987). However, the critical parameter is the reaction kinetics. The rates for the reaction between NAC and various oxidants were first reported in 1989 (Aruoma, Halliwell, Hoey, & Butler, 1989). The reaction with H_2O_2 was very slow ($\sim 0.85 \text{ M}^{-1} \text{ s}^{-1}$) and the reaction with superoxide undetectable. The only fast reaction rate was with hydroxyl radicals ($\sim 10^{10} \text{ M}^{-1} \text{ s}^{-1}$), but those react with almost any biomolecule at such a rate, making a potential contribution of NAC irrelevant. Until today, the paper reporting the NAC rate constants has been cited more than 1400 times, in most cases, paradoxically, as evidence supporting the direct oxidant scavenger function of NAC in biological systems. Over the years it has become clearer and more widely known that thiol oxidation in general is subject to substantial kinetic barriers (Winterbourn, 2013) and that specific catalytic mechanisms are needed for efficient thiol-based oxidant scavenging in the cellular context (Hall, Parsonage, Poole, & Karplus, 2010; Pedre et al., 2018; Peralta et al., 2015). For instance, the reduction of H_2O_2 by NAC is 10^4 – 10^8 fold slower than the reduction of H_2O_2 by thiol peroxidases (Trujillo, Alvarez, & Radi, 2016; Winterbourn & Metodiewa, 1999).

Hypochlorite, an oxidant produced by activated phagocytes, is intrinsically more thiol-reactive than peroxides or superoxide, but the reactivity of NAC towards HOCl is not higher than the reactivity of endogenous low-molecular-weight thiols (Cys, GSH) or methionine. In fact, NAC is 12-fold slower than Cys, 4-fold slower than GSH, and about as effective as methionine (Storkey, Davies, & Pattison, 2014). Under most conditions intracellular NAC concentrations are unlikely to approach or surpass the endogenous concentration of GSH, Cys and/or methionine. In human red blood cells, the highest concentration of intracellular NAC reported after i.v. administration in humans was 200 μM (Medved et al., 2004), which is well above the intracellular concentration of Cys ($\sim 15 \mu\text{M}$) (Medved et al., 2003) and methionine ($\sim 25 \mu\text{M}$) (Canepa et al., 2002), but well below the intracellular concentration of GSH ($\sim 1500 \mu\text{M}$) (van't Erve, Wagner, Ryckman, Raife, & Buettner, 2013). In animal experiments, relatively high NAC concentrations were observed in the lung ($\sim 320 \mu\text{M}$), kidney ($\sim 250 \mu\text{M}$), heart ($\sim 170 \mu\text{M}$) and liver ($\sim 100 \mu\text{M}$), 5 minutes after i.v. injection (Giustarini, Milzani, Dalle-Donne, Tsikas, & Rossi, 2012). However, GSH concentrations in these tissues were still 6 to 75 times higher (Giustarini et al., 2012), and endogenous Cys and Met concentrations were in a similar range (Baydoun, Emery, Pearson, & Mann, 1990; Giustarini et al., 2012; Ueki, Roman, Hirschberger, Junior, & Stipanuk, 2012). Therefore, it seems unlikely that NAC would be able to contribute significantly to the direct scavenging of HOCl.

In summary, it seems highly unlikely that the observed cytoprotective effects of NAC are due to the direct (i.e. uncatalyzed) reaction between NAC and ROS. The narrative of NAC acting as a direct oxidant scavenger of ROS is contradicted by chemical kinetics and indeed very poorly supported by the literature, if at all. Given that NAC indeed does exert antioxidative (reductive) effects inside cells, it must do so indirectly, either by being converted into more reactive species, and/or by fueling enzymatic scavenging systems, as will be discussed in later chapters.

Although it may be difficult to give a generally binding definition for the term “antioxidant”, it is usually understood to mean a compound that directly and efficiently intercepts oxidants, thereby preventing the oxidation of other molecules. Under this definition, given the lack of evidence, NAC does not seem to qualify as an antioxidant in any relevant biological context. It may be argued that NAC still qualifies to be

called an antioxidant when it acts indirectly, e.g. by being metabolized into a compound that is a bona fine antioxidant. While this position is debatable, we think that NAC should not be described as an antioxidant, as this propagates misunderstandings about the chemical nature of NAC.

2.3. The glutathione replenishment narrative

Another major chapter of the NAC story unfolded in the 1970s when intravenous NAC was established to protect against severe liver damage caused by paracetamol (acetaminophen) poisoning. Following the demonstration of its protective effects in animals (Piperno & Berssenbruegge, 1976), clinical studies confirmed the efficacy of NAC in the treatment of paracetamol poisoning. Intravenous NAC, supplied within 10 hours of paracetamol ingestion, at a dose of 300 mg/kg (given over 20 hours) prevented liver damage, renal failure, and death in patients with severe paracetamol poisoning (Prescott, Park, Ballantyne, Adriaenssens, & Proudfoot, 1977). Later studies demonstrated that orally administered NAC is also effective (Burgunder et al., 1989; Green, Heard, Reynolds, & Albert, 2013), with similar efficacy (Hodgman & Garrard, 2012; Prescott, 2005). To this day, NAC remains the standard of care for the treatment of paracetamol poisoning, and therefore is included in the World Health Organization's list of essential medicines (World Health Organization, 2019).

In the context of paracetamol poisoning, NAC acts as a pro-drug of Cys to replenish GSH that is consumed by the electrophilic paracetamol metabolite N-acetyl-p-benzoquinone imine (Hinson, Monks, Hong, Highet, & Pohl, 1982; van de Straat, de Vries, Debets, & Vermeulen, 1987). This mechanism is highly plausible in the specific setting: high amounts of electrophile are formed and conjugated to a corresponding number of GSH molecules. The GSH-electrophile conjugates are exported from liver cells through ABC transporters and excreted from the organism (Koenderink et al., 2020), leading to a substantial loss of GSH from liver cells. Intracellular GSH re-synthesis is needed and Cys is the limiting substrate for *de novo* GSH synthesis (Lu, 2009), meaning that external Cys supply is critical (Hazelton, Hjelle, & Klaassen, 1986; Miners, Drew, & Birkett, 1984; Williamson, Boettcher, & Meister, 1982). Hence, NAC provides a source of Cys to cope with acute GSH depletion.

Following the paracetamol case, NAC has been shown to protect against other GSH-depleting hepato- and/or nephrotoxic xenobiotics, by the same mechanism, i.e. GSH replenishment. For example, NAC protected animals exposed to pesticides like fipronil, glyphosate, or paraquat (Abdel-Daim, Dessouki, Abdel-Rahman, Eltaysh, & Alkahtani, 2019; Turkmen et al., 2019; Yeh et al., 2006) or antibiotics like isoniazid, rifampicin, sulfasalazine or tetracycline (Attri et al., 2000; Farombi, Ugwuezunmba, Ezenwadu, Oyeyemi, & Ekor, 2008; Heidari et al., 2016). Overall, the idea of using NAC as a pro-drug of Cys to replenish GSH levels and exert a protective effect against GSH-depleting xenobiotics is well supported by the literature, in stark contrast to the disulfide reducing and oxidant scavenger narratives.

While NAC is effective in replenishing GSH under conditions of GSH deficiency, it is typically ineffective in elevating GSH levels under normal conditions (Bernhard, Junker, Hettinger, & Lauterburg, 1998; Cotgreave, Schuppe, & Moldéus, 1991; Giustarini et al., 2012). For example, neither short (5 minutes) nor long term (2 weeks) NAC administration elevated organ GSH levels in the rat (Giustarini et al., 2012). The maintenance of normal GSH levels in spite of NAC delivery (i.e. extra Cys availability) can be explained by the well-characterized negative feedback regulation (product inhibition) of the GSH biosynthesis pathway (Richman & Meister, 1975) (Fig. 3). Indeed, oral NAC administration to humans only increased blood cell GSH levels in individuals which had unusually low GSH levels before treatment (Paschalis, Theodorou, Margaritelis, Kyparos, & Nikolaidis, 2018; Treweeke et al., 2012).

Apart from acute intoxication with xenobiotics, there are also chronic pathological conditions for which GSH depletion has been observed. Several studies claim that NAC elevated GSH levels and at the same time caused positive health effects. For example, NAC was reported to attenuate liver fibrosis in rats (Vendemiale et al., 2001; Y.-Y. Yang et al., 2008) and cystic fibrosis in humans (Tirouvanziam et al., 2006). Also, NAC was reported to protect rats against diabetic complications, decreasing damage of the sciatic nerve (Kamboj, Vasishta, & Sandhir, 2010) and of the liver (de Rosa et al., 2018). A clinical study reported that oral administration of NAC to HIV-infected subjects elevated whole blood and T cell GSH levels, and increased the two-year survival rate (De Rosa et al., 2000). In principle, it is plausible that the restoration of pathologically low GSH levels to normal levels can have positive effects beyond the protection against electrophiles and xenobiotics. GSH is a cofactor for glutaredoxins, hence involved in the catalytic reduction of disulfides (Deponte, 2013), and for glutathione peroxidases, hence involved in the catalytic reduction of peroxides (Brigelius-Flohé & Maiorino, 2013), including lipid peroxides (Brigelius-Flohé & Maiorino, 2013; Maiorino, Conrad, & Ursini, 2018). Indeed, several studies report that GSH depletion is associated with increased levels of the lipid peroxidation end product malondialdehyde, and that NAC can decrease these levels (Abdel-Daim et al., 2019; Attri et al., 2000; Farombi et al., 2008; Kamboj et al., 2010; Turkmen et al., 2019; Vendemiale et al., 2001; Y.-Y. Yang et al., 2008; Yeh et al., 2006). Moreover, the GSH redox potential is influenced by the total concentration of GSH (Meyer & Dick, 2010). Hence restoration of normal GSH levels may facilitate enzymatic disulfide reducing and oxidant scavenging activities that have been attributed to NAC itself.

It is interesting to note that almost all studies that show a correlation between GSH replenishment and some health benefit jump to the conclusion that the elevation (restoration) of GSH levels is the cause of the beneficial outcome. It is rarely questioned or investigated whether there is indeed a causal connection between the two. If there is one, the beneficial effect of NAC should disappear when the GSH biosynthesis pathway is blocked with buthionine sulfoximine (BSO), an irreversible inhibitor of γ -glutamylcysteine ligase, which catalyzes the first step in GSH biosynthesis (Griffith, 1982) (Fig. 3). Under BSO, NAC-derived Cys should not be able to restore GSH levels and should therefore not be beneficial. Depending on the study context, it is not always possible to use BSO, but it has been used in animal experiments to positively confirm the role of GSH replenishment in NAC-mediated obviation of paracetamol toxicity (Miners et al., 1984). However, for other conditions associated with low GSH levels, we could not find reports directly demonstrating that NAC's positive effects depend on increased GSH biosynthesis.

On the contrary, several reports have found that NAC exerts a beneficial effect even when the observed GSH replenishment is blocked by BSO (Gleixner et al., 2017; Konarkowska, Aitken, Kistler, Zhang, & Cooper, 2005; Nazıroğlu, Cığ, & Özgül, 2013; Steenvoorden & Beijersburgen van Henegouwen, 1998; Yan, Ferrari, & Greene, 1995), suggesting that NAC protects cells through mechanisms independent of GSH biosynthesis. None of these studies identified the mechanism by which NAC is cytoprotective. Some authors speculated that NAC acts as an oxidant scavenger (Nazıroğlu et al., 2013; Steenvoorden & Beijersburgen van Henegouwen, 1998), whereas others rejected this notion (Konarkowska et al., 2005). As discussed above, it is very unlikely that NAC directly scavenges oxidants, suggesting that a previously unexplored mechanism is behind the GSH-independent protective effect. Similar to the disulfide reductant narrative, it seems that a mechanism (GSH replenishment) established for one particular situation (acute xenobiotic intoxication) has been assumed to apply to very different situations, usually without further questioning or testing.

In summary, it is clear that NAC provides a source of Cys for *de novo* GSH biosynthesis under conditions in which GSH is severely depleted, and that restoration of GSH can be cytoprotective. This is typically the case when substantial amounts of xenobiotics need to be detoxified

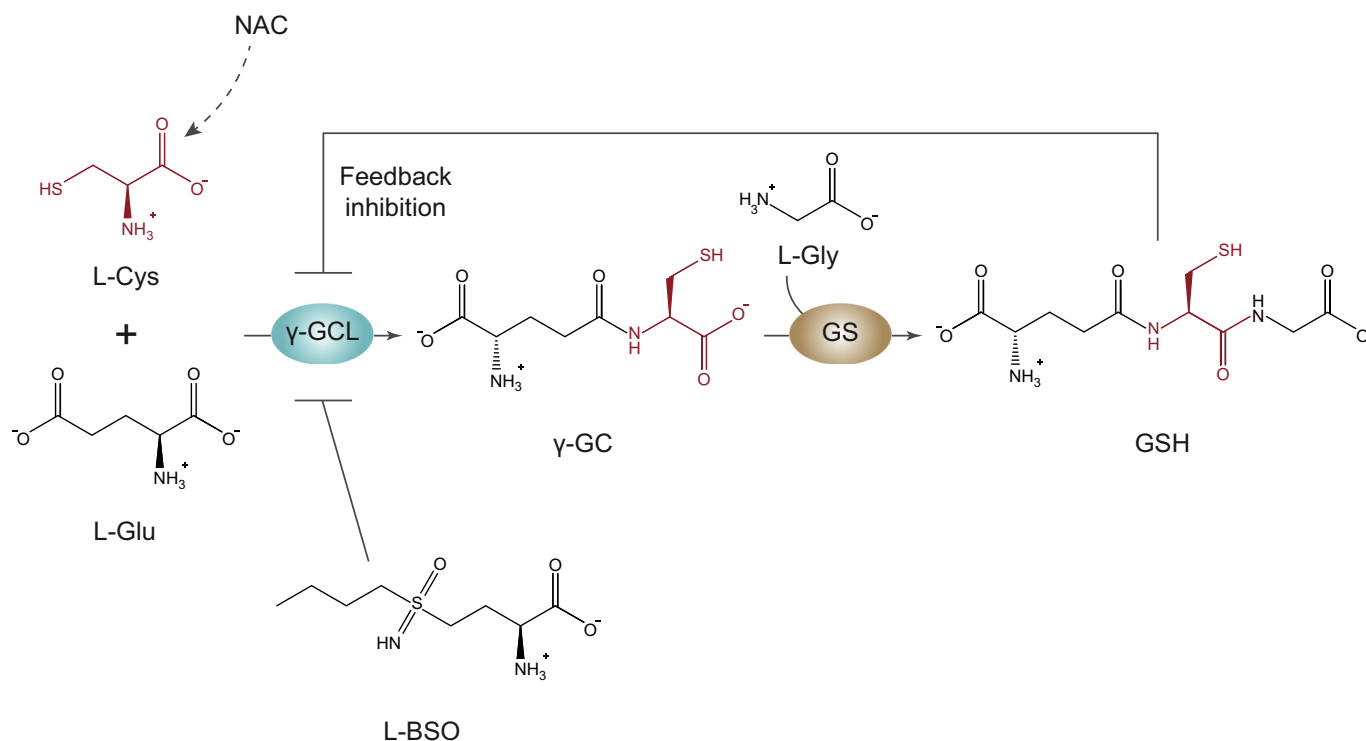


Fig. 3. The GSH biosynthesis pathway and its regulation

Two enzymatic steps facilitate the synthesis of GSH. The first step is catalyzed by γ -glutamylcysteine ligase (γ -GCL) which conjugates L-Cys and L-glutamate (L-Glu) into γ -glutamylcystine (γ -GC). The second step is catalyzed by GSH synthase (GS), which incorporates L-glycine (L-Gly) to form the final GSH tripeptide. As L-Cys typically is the limiting substrate, supplementation with L-Cys (directly or through NAC) can restore GSH levels when depleted. However, in the absence of GSH depletion, feedback inhibition of γ -GCL activity will prevent GSH biosynthesis, even when extra L-Cys is available. At high concentrations, GSH competitively binds to the γ -GCL active site, preventing further GSH formation. A pharmacological inhibitor of GSH biosynthesis is L-buthionine sulfoximine (BSO), which irreversibly binds to the γ -GCL active site.

and removed from the organism. However, NAC has also been observed to exert cytoprotective effects that cannot be ascribed to GSH replenishment. These observations imply an alternative mechanism beyond the classic narratives.

Before we consider alternative mechanisms of action we would like to discuss in more detail the chemical differences between Cys and NAC, as such differences will illuminate potential mechanisms by which NAC can be cytoprotective.

3. Why use NAC rather than cysteine?

It may be asked why all the treatments and experiments were (and still are) done with NAC and not with Cys. Given the fact that NAC shares the thiol group with Cys and is a prodrug for Cys, why not directly use Cys? Arguably, Cys would look like the more obvious choice. Is this just a historical accident, i.e. somebody having started with NAC and everybody else following? Does NAC have positive properties that Cys does not have? Or does Cys have negative properties not shared by NAC?

As Sheffner, the originator of NAC-based mucus liquefaction, explained in his initial publications, Cys was not considered ideal for therapeutic use, because in solution it oxidized rather rapidly, the oxidized form (cystine, Cys-S-S-Cys) being mostly insoluble and precipitating from the solution (Sheffner, 1963a). The crystalline cystine precipitates were feared to cause irritation in the respiratory tract. Moreover, Cys solutions were judged to have an obnoxious odor and a nauseating taste. Sheffner found NAC solutions to be more stable and to have a more agreeable taste and odor. Prescott, who first used NAC for the treatment of paracetamol poisoning in humans, stated to have used NAC (rather than Cys or other thiol compounds) because it was already

commercially available as a sterile 20% solution, as produced for the previously established intrabronchial application (Prescott et al., 1977).

Sheffner's argument about the greater stability of NAC in solution is borne out by numerous experiments. Freshly prepared NAC solutions are more resistant to air oxidation than corresponding Cys solutions. In one experiment, NAC was 50% oxidized (to NAC-S-S-NAC) after 13.7 h, while Cys was already 50% oxidized (to Cys-S-S-Cys) after 5.2 h (Held & Biaglow, 1994). Similar observations apply to copper-catalyzed air oxidation and to specific oxidant species, including superoxide, hydrogen peroxide and hypochlorite: NAC is always less rapidly oxidized than Cys (Table 1) (Held & Biaglow, 1994; Newton et al., 1995; Winterbourn & Metodiewa, 1999; Winterbourn, Peskin, & Parsons-Mair, 2002).

The higher resistance of NAC to oxidation, relative to Cys, is readily explained by the acetylation of the amino group. In general, oxidation of a thiol group requires its deprotonation to the thiolate anion (van Bergen, Roos, & De Proft, 2014). Cys is more prone to form the thiolate than NAC. This is because the amino group of Cys is protonated at physiological pH. The positive charge of the amino group supports the deprotonation of the thiol group, because the resulting thiolate anion is stabilized by electrostatic interactions. By contrast, the acetylated amino group of NAC cannot form a positive charge that would support thiol deprotonation. Hence, the thiol dissociation constant (pK_a) of the NAC thiol should be higher than the thiol pK_a of Cys. Indeed, the microscopic thiol pK_a for Cys (with a protonated amino group) has been reported as 8.53 (Benesch & Benesch, 1955), 8.29 (Portillo-Ledesma et al., 2014), or 8.45 (Noszál, Visky, & Kraszni, 2000) (Table 1), while that of the NAC thiol group has been determined as 9.55 (Connett & Wetterhahn, 1986), 9.85 (Noszál et al., 2000), or 9.74 (Portillo-Ledesma et al., 2014) (Table 1). Hence, there is a difference between Cys and NAC of at least one pK_a unit. Of note, the thiol pK_a of Cys with

Table 1
Chemical properties of Cys, NAC and their oxidation products.

	L-cysteine	N-acetyl-L-cysteine	L-cystine	N, N'-diacetyl-L-cystine
Abbreviation	Cys	NAC	Cys-S-S-Cys	NAC-S-S-NAC
MW	121.16	163.19	240.30	324.37
CAS number	52-90-4	616-91-1	56-89-3	5545-17-5
Solubility [g/L]	277	100	0.112	1000 ^a
Thiol pK _a	8.29–8.53	9.55–9.85	N/A	N/A
t _{1/2} air oxidation (h) ^b	5.2	13.7	N/A	N/A
k ₂ H ₂ O ₂ (M ⁻¹ s ⁻¹) (pH 7.4) ^c	2.9	0.16	N/A	N/A
k ₂ HOCl (M ⁻¹ s ⁻¹) (pH 7.4) ^d	3.6 × 10 ⁸	0.28 × 10 ⁸	N/A	N/A
Thiol autooxidation rate (μM/min) ^e	11.7	0.1	N/A	N/A

^a Experimentally determined by us.

^b Held and Biaglow (1994).

^c Winterbourn and Metodiewa (1999).

^d Storkey et al. (2014).

^e Catalyzed by Cu,Zn-superoxide dismutase (Winterbourn et al., 2002).

a neutral (non-protonated) amino group (as it would exist at very high pH), has been reported as 10.03 (Benesch & Benesch, 1955) or 9.67 (Portillo-Ledesma et al., 2014), i.e. values very close to the thiol pK_a of NAC.

N-acetylation of the amino group also increases the solubility of the disulfide form of NAC (i.e. N,N'-diacetylcysteine or NAC-S-S-NAC). As only the carboxyl groups of NAC-S-S-NAC can ionize, it is dianionic at pH 7.4 and hence well soluble in water (Table 1). In contrast, in cystine both the amino and carboxyl groups can ionize, forming a zwitterion at neutral pH. Without gross charge, the cystine zwitterion tends to form aggregates, and therefore is barely soluble in water (Table 1). Taken together, acetylation of the amino group renders NAC resistant to oxidation, and even when it does get oxidized, ensures solubility of the oxidation product. This explains why NAC solutions do not precipitate over time, even after prolonged storage.

Some authors have argued that NAC is a superior disulfide reductant in comparison to Cys (Aldini et al., 2018; Samuni, Goldstein, Dean, & Berk, 2013). This suggestion is based on the following logic: the higher the thiol pK_a, the more basic is the corresponding thiolate. For similar thiolates, the more basic ones (possessing higher electron density) will be better nucleophiles, that is, they will be faster in donating an electron pair to an electrophilic center, such as a sulfur atom in a disulfide bond. In other words, the NAC thiolate, having a higher pK_a, should be a better nucleophile and therefore a better disulfide reductant than the Cys thiolate. While this notion is correct in principle, one also needs to take into account that the concentration of the thiolate at physiological pH (~7.4) will mostly determine the rate of disulfide bond reduction, with more acidic ones (i.e. with a lower pK_a) having a greater proportion of the thiol in the thiolate form. At pH 7.4, only 1% of NAC molecules exist as a thiolate, in contrast to the 10% of Cys molecules. In accordance with this, it has been shown experimentally that Cys is approximately 10-fold faster than NAC at reducing disulfide bonds at physiological pH (Giustarini et al., 2012). Therefore, NAC's higher thiolate nucleophilicity is not brought to bear, making it an inferior disulfide reductant under physiological conditions.

In summary, there is little doubt that practical aspects, namely the better resistance of NAC solutions against oxidation and precipitation, have played a role in establishing the use of NAC and the disuse of Cys. However, NAC is not a better disulfide reducing agent than Cys.

Beyond the basic chemistry, there is another, far more critical argument in favor of NAC, namely its well-documented safety. NAC turned out to be safe in adults and children, even at very high doses, and studies in mice and rats show that toxic effects are only observed at very high dosages, above 6 g/kg when given orally and above 2 g/kg when injected intravenously (Bonanomi & Gazzaniga, 1980). For Cys, the situation looks very different. It has long been known that Cys can be toxic when administered at supraphysiological levels. Cys overdosing (oral LD₅₀ ~6 g/kg in rats (European Chemicals Agency, 2021) can have severe

pathophysiological consequences. For instance, intravenous or subcutaneous injections of Cys in rodents cause weight loss, brain damage, severe hypoglycemia and muscle spasms, with neonatal rodents being especially susceptible to these effects (Gazit, Ben-Abraham, Coleman, Weizman, & Katz, 2004; Gazit, Ben-Abraham, Pick, Ben-Shlomo, & Katz, 2003; Karlson, Grofova, Malthe-Sørensen, & Fonnum, 1981; Lehmann, Hagberg, Orwar, & Sandberg, 1993; Sawamoto, Hagiwara, & Kurisu, 2004; Wong et al., 2006). Oral Cys supplementation at very high doses is also toxic and leads to weight loss and lethality in rodents and poultry (Baker, 2006; Birnbaum, Winitz, & Greenstein, 1957; Dilger & Baker, 2008; Dilger, Toue, Kimura, Sakai, & Baker, 2007). In one of these studies, Cys was the only non-essential amino acid whose supplementation caused the death of all tested rodents (Birnbaum et al., 1957), indicating the particular pernicious nature of excess Cys. We could not find studies dedicated to testing high dose Cys treatment on human subjects, yet it has been noted that a single oral dose of 5 g Cys produced nausea, light-headedness and dissociation in normal human subjects (Anderson & Raiten, 1992), suggesting that humans are also susceptible to the toxic effects of Cys overdose. A summary of studies relating to Cys toxicity can be found in the report "Safety of amino acids used as dietary supplements" prepared by the Federation of American Societies for Experimental Biology (FASEB) for the FDA (Anderson & Raiten, 1992).

These and other observations have led to the notion that high concentrations of Cys are potentially toxic. It is interesting to note that normal intracellular Cys concentrations are lower than those of other amino acids (Bergström, Fürst, Norée, & Vinnars, 1974; Piez & Eagle, 1958; Soley & Alemany, 1980) and at the same time appear to be tightly regulated. For instance, Cys levels in rodent livers are kept between 50 and 240 μM, even under variations of protein intake (Giustarini et al., 2012; Lee, Londono, Hirschberger, & Stipanuk, 2004; Terrill, Grounds, & Arthur, 2015; Ueki et al., 2011; Vitvitsky et al., 2004). These observations suggest that cells need to keep Cys levels low and within a certain range to avoid toxicity.

The toxicity of Cys and the safety of NAC leads to an interesting question: Why is Cys toxic at supraphysiological levels, and how is toxicity prevented by N-acetylation? Surprisingly, little is known about the actual molecular mechanisms of Cys toxicity.

Most frequently, it has been suggested that Cys-derived toxicity is due to metal-catalyzed autooxidation of Cys, generating ROS (Saez, Thornalley, Hill, Hems, & Bannister, 1982; Wang & Cynader, 2001). Free copper and iron, or hemin-bound iron are potential catalysts of Cys autooxidation. Other transition metals may cause similar effects, though at much higher concentrations (Wang & Cynader, 2001). Contrary to Cys, NAC is more resistant to copper-catalyzed autooxidation and the associated ROS production occurs at a slower rate, potentially explaining the lower toxicity of NAC (Kachur, Koch, & Biaglow, 1999; Newton et al., 1995; Wang & Cynader, 2001; Winterbourn et al., 2002). In the case of iron, however, the situation is less clear, as no

experiments have directly compared iron-mediated ROS production between the NAC and Cys. Incubation of NAC or Cys (at a final concentration of 1 mM) in cell culture medium (containing trace iron) led to similar H_2O_2 production rates, suggesting that there is little difference between NAC and Cys with regard to iron-catalyzed ROS production (Hua Long & Halliwell, 2001).

Damage caused by metal-catalyzed Cys autooxidation has been documented in bacteria (Park & Imlay, 2003). A recent paper suggests that this mechanism may also play out in eukaryotes, with dysregulation of Cys storage in lysosomes potentially perturbing the cytosolic iron pool (Hughes et al., 2020). However, it has not been shown that disturbance of the cytosolic iron pool is actually related to Cys autooxidation. In any case, the low concentrations of free transition metals in mammalian cells combined with low intracellular Cys concentrations speak against widespread damage caused by this mechanism. While in erythrocytes the concentration of free copper and hemin is within the range that may potentially cause Cys-catalyzed autooxidation ($\sim 0.8 \mu\text{M}$ and $\sim 15 \mu\text{M}$, respectively) (McMillin, Travis, & Hunt, 2009; Oh et al., 2016), in other cells the former is nearly undetectable (Rae, Schmidt, Pufahl, Culotta, & O'Halloran, 1999) and of the latter is in the submicromolar range (Yuan et al., 2016). This suggests that damage caused by Cys-catalyzed autooxidation would be limited to the bloodstream, and even this would be questionable, as most of the free Cys in blood is oxidized and therefore unable to generate radicals (Turell, Radi, & Alvarez, 2013).

However, there is also the possibility that Cys autooxidation is catalyzed by enzymatic metal centers. The Cu center of Cu,Zn superoxide dismutase (SOD) has also been found to catalyze O_2 -dependent Cys oxidation, generating H_2O_2 (Winterbourn et al., 2002). Interestingly, Cu,Zn-SOD was much less efficient in catalyzing NAC oxidation (Table 1), in accordance with the notion that amino thiols are generally more susceptible to Cu-catalyzed oxidation than thiols lacking a free amino group (Kachur et al., 1999). Hence if Cu centers contribute to Cys toxicity, the lower toxicity of NAC would be readily explained. Nonetheless, it seems unlikely that the rate of Cys-dependent H_2O_2 production afforded by Cu,Zn-SOD (and/or other Cu sites) is significant enough to cause toxicity, also considering that cells are highly efficient in removing H_2O_2 .

Another potential mechanism of cysteine toxicity, specific to the nervous system, is excitotoxicity, i.e. damage of nerve cells due to pathologically high concentrations of excitatory neurotransmitters. While the mechanism remains unclear, two hypotheses have been proposed: firstly, extracellular cystine may drive the cystine-glutamate antiporter (system X_c) to release large amounts of intracellular glutamate, hence causing neurotoxicity (Janáky, Varga, Hermann, Saransaari, & Oja, 2000). Secondly, oxidation products of Cys, such as cysteine sulfinic acid or S-nitrosocysteine, may cause excitotoxicity by structurally mimicking glutamate (Janáky et al., 2000). NAC is indeed less neurotoxic than Cys at the same concentration (Puka-Sundvall, Eriksson, Nilsson, Sandberg, & Lehmann, 1995). However, excitotoxic effects of Cys would be limited to the nervous system and would not explain the toxic effects observed in other organs.

It has also been suggested that Cys toxicity could be due to interference with enzymatic reactions that use pyridoxal 5'-phosphate (PLP) as a cofactor. About 1.5% of human protein-coding genes encode PLP-dependent enzymes (Percudani & Peracchi, 2003), and Cys can form a stable thiazolidine derivative with PLP that would block its activity (Buell & Hansen, 1960; Mackay, 1962). For NAC, the acetylation of the amino group would prevent this reaction. However, the rate constant for the reaction between Cys and PLP to form the thiazolidine derivative is very low ($\sim 2 \text{ M}^{-1} \text{ s}^{-1}$) (Schonbeck, Skalski, & Shafer, 1975). Indeed, only extremely high concentrations of Cys (10 mM), i.e. 100-fold higher than typically found in cells, led to noticeable inhibition of PLP-dependent enzymatic activity *in vitro* (Lowther, Beattie, Langridge-Smith, Clarke, & Campopiano, 2012). In conclusion, Cys toxicity due to interference with PLP-dependent enzymes seems rather unlikely.

Another possible cause of Cys toxicity may be the limited solubility of oxidized Cys, cystine, in contrast to oxidized NAC, NAC-S-S-NAC,

which is highly soluble. Cystine can precipitate and crystallize into renal calculi, as observed in cystinuria. However, these rare cases are associated with genetic mutations in cystine transporters (Dello Strologo et al., 2002). To our knowledge, there have been no reports connecting Cys toxicity to cystine precipitations.

Overall, the mechanisms behind Cys toxicity (and lack thereof in NAC) remain largely unclear. The above proposed mechanisms remain speculative and it seems unlikely that they fully explain Cys toxicity.

4. Hydrogen sulfide: the missing link in cysteine toxicity?

While the above suggested mechanisms may explain some of the toxic effects created by supraphysiological Cys administration, experimental evidence for them is either weak (e.g. interference with PLP-dependent enzymes), or appears to be limited to specific conditions (e.g. high metal concentrations) or tissues (neuronal excitotoxicity). It is therefore interesting to note that one of the known products of Cys catabolism is hydrogen sulfide (H_2S), long recognized as a highly toxic molecule (Szabo, 2018). This raises the question whether Cys toxicity is partly or mainly due to H_2S toxicity. Surprisingly, it seems that this question has not been explicitly addressed in the literature.

The concentration of H_2S in blood and tissues is normally well below 100 nM (Furne, Saeed, & Levitt, 2008; Levitt, Abdel-Rehim, & Furne, 2011; Olson, 2012; Vitvitsky, Kabil, & Banerjee, 2012). Toxicity caused by an excess of H_2S is commonly attributed to inhibition of cytochrome c oxidase ($K_i = 0.2 \mu\text{M}$ *in vitro*), which is part of complex IV in the mitochondrial respiratory chain (Petersen, 1977). Notably, the inhibitory potency of H_2S is equivalent to that of cyanide (Petersen, 1977). Complex IV poisoning by H_2S uncouples oxidative phosphorylation and causes metabolic acidosis (Derwall et al., 2011; Khan et al., 1990). Chicken fed with diets containing supraphysiological amounts of Cys were found to succumb to acidosis (Dilger & Baker, 2008), a finding potentially in line with the notion that Cys-derived H_2S is the toxic agent. H_2S not only inhibits cytochrome c oxidase but also interferes with other protein metal centers. For example, it interferes with O_2 binding to hemoglobin and myoglobin, and hence impairs O_2 transport to tissues (Ríos-González, Román-Morales, Pietri, & López-Garriga, 2014).

In mammals, Cys is the exclusive source of endogenously produced H_2S . Three kinds of experiments have established the notion that an increased supply of Cys enhances H_2S production:

Firstly, feeding of Cys enhances H_2S production in organisms ranging from bacteria to plants and animals (Else, Fowkes, & Baxter, 2010; Kartha, Zhou, Hovde, Cheung, & Schröder, 2012; Sekiya, Schmidt, Wilson, & Filner, 1982; Stipanuk & Beck, 1982; Vitvitsky et al., 2012). Similar observations have been made with mammalian cell cultures (Ezerina, Takano, Hanaoka, Urano, & Dick, 2018). In general, the amount of H_2S formed depends on the amount of Cys administered (Kartha et al., 2012; Sekiya et al., 1982; Vitvitsky et al., 2012).

Secondly, the physiological effects of Cys administration can often be mimicked by directly providing an H_2S donor from outside, suggesting that endogenously produced H_2S is indeed the link between elevated Cys uptake and physiological (or pathological) Cys effects (Ezerina et al., 2018; Leffler et al., 2011; Sidhu, Singh, Samir, & Carson, 2001; Xue et al., 2013).

Thirdly, pharmacological inhibition or genetic depletion of enzymes facilitating H_2S production diminishes Cys-induced H_2S elevation and the related physiological effects (Else et al., 2010; Ezerina et al., 2018; Huang et al., 2016; Kartha et al., 2012; Leffler et al., 2011; Mani, Yang, & Wang, 2011; Stipanuk & Beck, 1982).

Three enzymes are known to facilitate Cys desulfuration in order to release H_2S : cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE), and the combination of cysteine aminotransferase (CAT, also known as GOT or AST) and 3-mercaptopyruvate sulfurtransferase (MPST) (Fig. 4). CBS and CSE belong to the transsulfuration pathway,

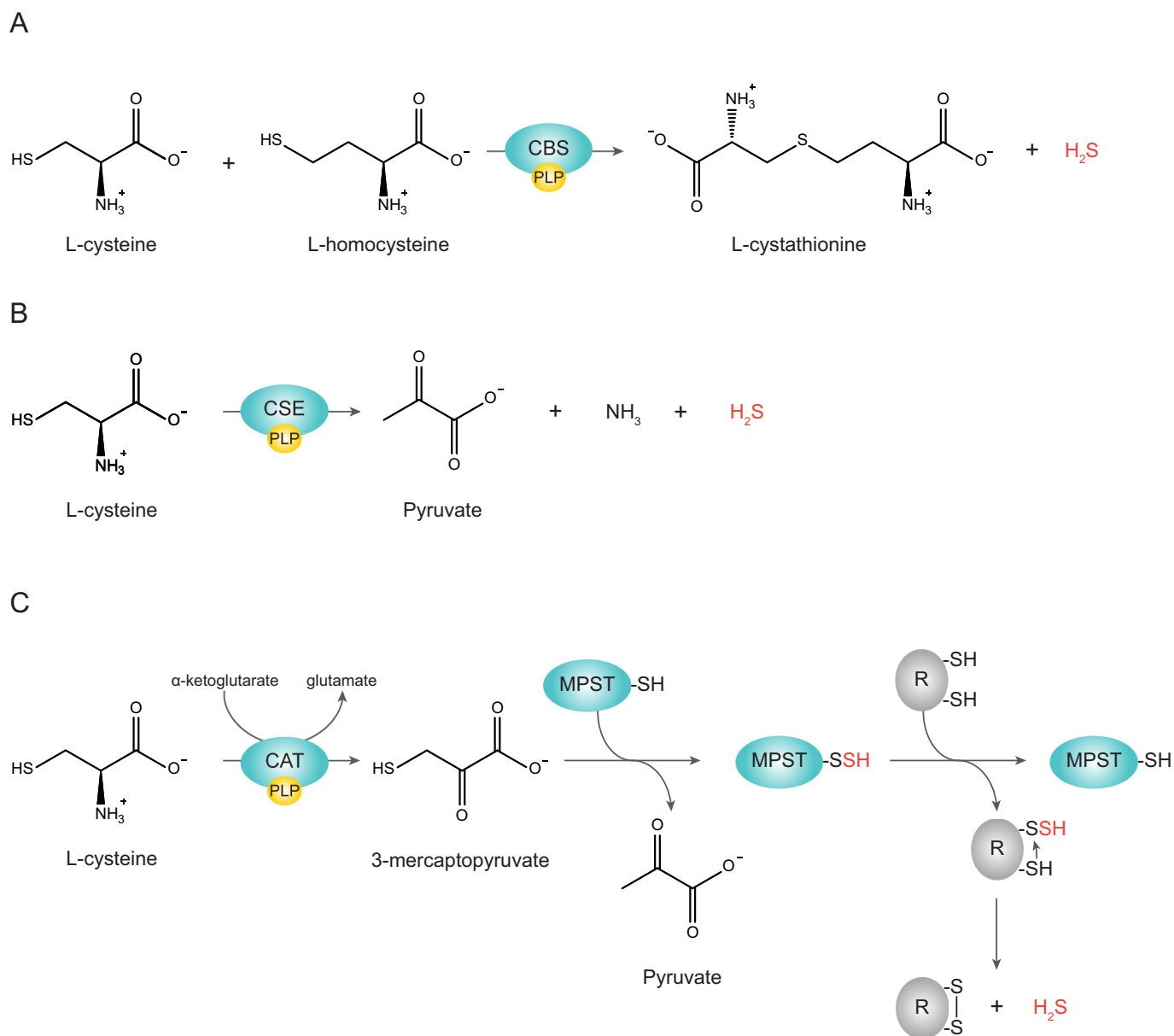


Fig. 4. Three enzymatic systems generate H₂S from Cys

(A) Cystathionine-β-synthase (CBS) condenses Cys and homocysteine to form cystathionine and H₂S.

(B) Cystathionine-γ-lyase (CSE) eliminates H₂S from Cys, forming pyruvate and NH₃.

(C) Cysteine aminotransferase (CAT) deaminates Cys by transamination to generate 3-mercaptopyruvate. 3-mercaptopyruvate sulfurtransferase (MPST) desulfurates 3-mercaptopyruvate to generate pyruvate and an enzyme-bound persulfide. MPST then transfers the outer sulfur of the persulfide to a dithiol acceptor molecule (R), in particular thioredoxin, which forms an intramolecular disulfide bond to release H₂S.

which normally generates Cys from methionine via the intermediate homocysteine. CBS catalyzes the condensation of serine and homocysteine to form cystathionine, and CSE catalyzes the α,γ-elimination of cystathionine to produce Cys, α-ketobutyrate and NH₃. However, both enzymes also possess kinetically relevant auxiliary activities that produce H₂S from Cys. CBS catalyzes the condensation of Cys and homocysteine to form H₂S and cystathionine, while CSE catalyzes the α,β-elimination of Cys to generate pyruvate, NH₃ and H₂S (Fig. 4A-B) (Chiku et al., 2009; Singh, Padovani, Leslie, Chiku, & Banerjee, 2009). Unlike CBS and CSE, the CAT/MPST enzymatic tandem does not directly produce H₂S. Cys transamination catalyzed by CAT first generates 3-mercaptopyruvate (3MP), which is subsequently desulfurated by MPST, generating pyruvate and an MPST-bound persulfide (RSSH) (Pedre & Dick, 2020). The outer sulfur atom of the MPST-bound

persulfide is then transferred to thiol-containing acceptors from which H₂S can be released (Fig. 4C). Acceptors with vicinal dithiols such as thioredoxin and dihydrolipoic acid are especially efficient H₂S releasers (Mikami et al., 2011; Westrop, Georg, & Coombs, 2009; Yadav, Yamada, Chiku, Koutmos, & Banerjee, 2013).

It is important to mention that tissue H₂S concentrations reported in the literature vary dramatically, from low nanomolar to high micromolar. One of the main issues is that some methods cause the release of H₂S from endogenous sulfur compounds, leading to substantial overestimations (Olson, 2012). In particular, reports on medium-to-high micromolar H₂S concentrations in tissues are highly implausible, considering that the smell of a 50 μM H₂S solution is experienced as obnoxious (Furne et al., 2008) and causes eye and lung irritation (Olson, 2012). The methods least prone to artifacts determine blood and tissue H₂S levels

consistently below 100 nM (Furne et al., 2008; Levitt et al., 2011; Olson, 2012; Vitvitsky et al., 2012).

Under physiological conditions, enzymatic H₂S production from Cys is highly regulated and context-dependent. Factors influencing H₂S production not only include Cys availability and the expression levels of the necessary enzymes, but also the competing activity of other Cys-utilizing enzymes (e.g. cysteinyl-tRNA synthetase, γ -glutamyl-cysteinyl ligase, cysteine dioxygenase (Lu, 2009; Stipanuk, 2004)), and the presence and amount of various enzymatic co-substrates, activators, co-factors and allosteric inhibitors (Filipovic, Zivanovic, Alvarez, & Banerjee, 2018). The roles and interplay of all these factors in the regulation of H₂S production is not yet understood, but some aspects have emerged.

For example, in mammals, the liver seems to be the main organ of endogenous H₂S production, while the brain and kidney also produce notable amounts (Furne et al., 2008; Levitt et al., 2011; Olson, 2012; Vitvitsky et al., 2012). However, the enzymatic source of H₂S differs between tissues. In the liver, H₂S production is thought to be mainly facilitated by CSE, while H₂S generated in kidney, brain and vascular endothelium is CSE-independent and thought to originate from CBS and/or CAT/MPST (Abe & Kimura, 1996; Kabil, Vitvitsky, Xie, & Banerjee, 2011; Shibuya et al., 2009; Shibuya, Mikami, Kimura, Nagahara, & Kimura, 2009). In addition, tissues contain different baseline Cys concentrations, with lower concentrations (~30–90 μ M) in the brain and the lung, intermediate concentrations (~50–240 μ M) in the liver, and higher concentrations (~300–1000 μ M) in the kidney (Giustarini et al., 2012; Lee et al., 2004; Stipanuk, Londono, Lee, Hu, & Yu, 2002; Terrill et al., 2015; Ueki et al., 2011; Vitvitsky et al., 2004). All H₂S-generating pathways require PLP as a cofactor (MPST is a PLP-independent enzyme, but CAT requires PLP, Fig. 4C). Various metabolites allosterically or competitively modulate H₂S production by targeting particular enzymes: for instance, S-adenosyl methionine is an allosteric activator of CBS, and aspartate is a competitive inhibitor of CAT (Filipovic et al., 2018; Miyamoto, Otsuguro, Yamaguchi, & Ito, 2014).

While there is a significant amount of evidence linking the uptake of Cys to increased H₂S production, and some understanding on how H₂S is produced, the potential connection between H₂S and Cys toxicity has not received much consideration. Only a few studies have experimentally established a causal link between the increase of Cys levels and H₂S-mediated toxicity (Roman et al., 2013; Ueki et al., 2011; Wong et al., 2006). The main evidence in favour of this connection comes from the analysis of mice deficient in cysteine dioxygenase (CDO). CDO lowers Cys levels by catalyzing its oxidation to cysteine sulfinate, which is then further degraded to taurine and sulfate (Ueki et al., 2011). Mice lacking CDO not only exhibit increased plasma and organ Cys levels but also higher H₂S levels, concomitantly causing a decrease in cytochrome c oxidase activity, along with pathological abnormalities (Roman et al., 2013; Ueki et al., 2011). Although the authors used methylene blue to detect H₂S (Ueki et al., 2011), a method prone to overestimate H₂S levels, enhanced generation of H₂S was also suggested by increased urinary thiosulfate, one of the products of H₂S catabolism (Roman et al., 2013). This implies that elevated Cys causes pathology through elevated H₂S, and suggests that previous observations of Cys-mediated toxicity are also related to Cys-dependent H₂S production. Indeed, the phenotypes caused by chronic supraphysiological Cys feeding of wild type mice, obesity and hypoglycemia, are similar to those of CDO-deficient mice (Dilger et al., 2007; Gazit et al., 2004; Sawamoto et al., 2004; Ueki et al., 2011). In addition, acute exposure of animals to supraphysiological Cys produces phenotypes similar to those produced by acute H₂S exposure, such as breathing difficulties (Reiffenstein, Hulbert, & Roth, 1992; Sprince, Parker, & Josephs, 1969) and somnolent behavior/lethargy (Khan et al., 1990; Reiffenstein et al., 1992; Sprince et al., 1969).

Taken together, in many systems and situations H₂S appears to be a previously overlooked contribution to Cys toxicity.

5. How does NAC circumvent the problem of cysteine toxicity?

As discussed above, NAC is well tolerated and effectively non-toxic at concentrations that would be considered unsafe (i.e. potentially toxic) in the case of Cys. Given the fact that the organism converts NAC to Cys, why is NAC so much safer than Cys? Most likely, the answer is connected to the pharmacokinetic bottlenecks that are imposed by the N-acetyl group. It appears that NAC releases Cys slowly, thus preventing acute exposure of tissues to high (i.e. supraphysiological) concentrations of Cys, and thus of H₂S. Although we could not find studies directly comparing the pharmacokinetics of Cys and NAC, the idea of NAC being a 'slow donor' of Cys is well supported by cell-based studies which show a much slower catabolism of NAC relative to Cys (Banks & Stipanuk, 1994; Raftos, Whillier, Chapman, & Kuchel, 2007).

In principle, NAC may deliver Cys to cells by two different mechanisms (Raftos et al., 2007). The first one is the direct uptake of NAC into the cytosol, followed by its enzymatic deacetylation to Cys (Fig. 5). The second one is the reduction of extracellular Cys disulfide conjugates (e.g. Cys-S-S-Cys or Cys-S-S-Protein) by NAC, which would release reduced Cys to be taken up by the cell (Fig. 5). Extracellular deacetylation of NAC is also conceivable, but to our knowledge has not been reported.

In comparison to Cys, the direct cellular uptake of NAC is slow (Raftos et al., 2007). Apparently, the N-acetyl group hampers both passive and active transport across the plasma membrane. Passive transport is expected to be disfavoured, as NAC is negatively charged at physiological pH (unlike Cys, which is a zwitterion lacking a net charge), thus lowering its membrane permeability (Faria et al., 2019). Indeed, charge-neutralizing modifications of the NAC carboxyl group (amidation or esterification) greatly improve cellular uptake (Giustarini et al., 2012; Grinberg, Fibach, Amer, & Atlas, 2005). Evidence suggesting active membrane transport of NAC does not seem to exist. The canonical importer of reduced Cys is ASCT1 (also known as neutral amino acid transporter SLC1A4) (Franchi-Gazzola, Gazzola, Dall'Asta, & Guidotti, 1982; Scopelliti, Heinzelmann, Kuyucak, Ryan, & Vandenberg, 2014; Scopelliti, Ryan, & Vandenberg, 2013). Since ASCT1 does not transport negatively charged amino acids, as demonstrated for cystate (Scopelliti et al., 2013), it appears unlikely that NAC is a substrate for this transporter. No other potential NAC transporters have been reported, although one study implicated anion exchanger 1 (AE1) as a facilitator of NAC uptake into erythrocytes (Raftos et al., 2007) (Fig. 5).

Following its uptake, NAC must be deacetylated to yield Cys. Aminoacylase 1 is thought to be the main NAC-deacetylating enzyme ($K_M = 0.5\text{--}1.4$ mM, $k_{cat}/K_M = 2\text{--}6 \times 10^4$ M⁻¹s⁻¹) (Lindner, Täfler-Naumann, & Röhm, 2008; Stocker et al., 2012). The combination of slow uptake and a deacetylation step that is not extremely efficient is expected to limit the intracellular delivery of Cys. Assuming a maximal NAC serum concentration of 500 μ M (Rushworth & Megson, 2014), an elevation of intracellular Cys concentration in the low micromolar range can be expected. Most likely, uptake rather than deacetylation is the rate-limiting step, as a more lipophilic NAC derivative, NAC ethyl ester, shows enhanced intracellular Cys delivery relative to NAC (Giustarini et al., 2012). Both i.v. and oral administrations are presumed to deliver Cys into cells via NAC import and deacetylation. For oral administration, this seems to be the predominant mechanism, with deacetylation taking place in intestinal enterocytes and Cys being forwarded through the portal vein (Cotgreave, Berggren, Jones, Dawson, & Moldéus, 1987), leading to a bioavailability of 9 and 4% for total and reduced NAC, respectively (Olsson et al., 1988). The slow cellular uptake and deacetylation of NAC explains the time needed to reach peak concentration (≥ 0.5 h) (Holdiness, 1991) and the mean residence time in the bloodstream (≥ 2 h) (Borgström, Kågedal, & Paulsen, 1986; Olsson et al., 1988) following oral administration.

The second mechanism of NAC-to-Cys conversion is indirect and involves disulfide exchange between NAC and disulfide-linked Cys in the extracellular space (mainly Cys-S-S-Cys and Cys-S-S-Protein),

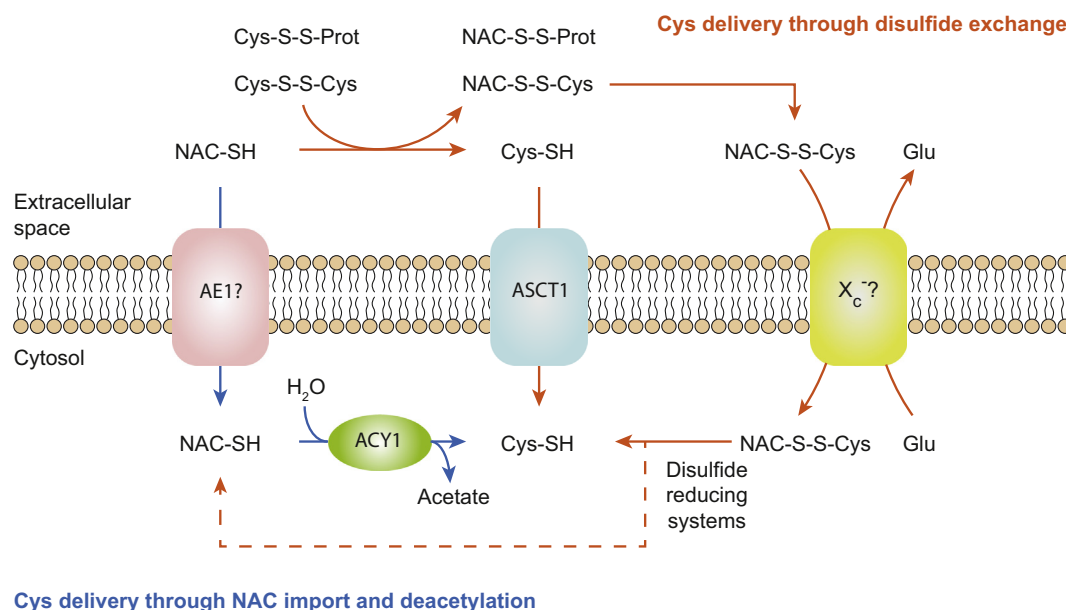


Fig. 5. Two mechanisms by which NAC can increase intracellular Cys levels.

In principle, NAC can deliver Cys in direct (grey) and indirect (orange) ways. How NAC enters cells is not fully understood. Anion exchanger 1 (AE1) may play a role in erythrocytes. Following import, NAC is deacetylated by aminocysteine 1 (ACY1), releasing Cys in the process. Alternatively, NAC can deliver Cys indirectly through disulfide exchange with oxidized Cys in the plasma (Cys-S-S-R), to release reduced Cys (Cys-SH), which is then rapidly imported by the neutral amino acid transporter ASCT1. Although direct evidence is lacking, it seems likely that NAC-S-S-Cys can be imported into cells by the system X_c^- anion exchanger. Inside the cell, NAC-S-S-Cys is reduced by intracellular disulfide reducing systems, releasing Cys and NAC, the latter being deacetylated into Cys by ACY1.

generating corresponding NAC mixed disulfides (NAC-S-S-Cys and NAC-S-S-Protein) and releasing reduced Cys that can be taken up by surrounding cells, presumably by the ASCT1 transporter mentioned above (Radtke et al., 2012; Raftos et al., 2007) (Fig. 5). Extracellular disulfide exchange between NAC and oxidized Cys is likely to occur in the plasma, known to contain relatively high concentrations of cystine (~40–60 μ M) and Cys-protein disulfide conjugates (~150–180 μ M) (Turell et al., 2013). While definite evidence seems to be missing, it has been suggested that extracellular disulfide exchange is the main mechanism by which NAC generates Cys following an i.v. injection (Whillier et al., 2009). This notion is supported by three observations: (i) administration of isotopically labeled NAC to mice increased erythrocyte GSH content by 20%, of which only 1% was labeled (Zhou et al., 2015); (ii) NAC was unable to supply Cys to cultured endothelial cells unless traces of oxidized Cys were present in the cell culture medium (Giustarini et al., 2018); and (iii) addition of the D-stereoisomer of NAC (D-NAC), which cannot be deacetylated (Sjödin, Nilsson, Hallberg, & Tunek, 1989; Wong, Chan, & Corcoran, 1986), to cystine-containing cell culture media, increases intracellular GSH levels to the same extent as L-NAC (Ferrari, Yan, & Greene, 1995). These results suggest that the slow process of extracellular thiol-disulfide exchange can be more efficient in delivering Cys to cells than NAC uptake and deacetylation, at least under some conditions.

Disulfide exchange between reduced NAC and oxidized cysteine generates the mixed disulfide conjugate NAC-S-S-Cys. We could not find studies investigating whether NAC-S-S-Cys can be taken up by cells. In principle, given the similarity between NAC-S-S-Cys and cystine, NAC-S-S-Cys may be a substrate for the cystine/glutamate transporter (system X_c^-). System X_c^- is an antiporter coupling the import of cystine to the export of glutamate (Bannai & Kitamura, 1981). Since NAC-S-S-Cys is an anion and system X_c^- has been shown to transport various anionic compounds of similar size (e.g. DL- α -amino adipate and L-homocysteate (Bannai, 1986)), it seems possible that system X_c^- also accepts NAC-S-S-Cys as a substrate for import (Fig. 5). In this case, imported NAC-S-S-Cys would constitute another source of intracellular Cys, upon reduction by cytosolic disulfide-reducing systems (Fig. 5).

Since NAC is subject to considerable pharmacokinetic bottlenecks, researchers have looked for modifications that improve its bioavailability and rate of cellular uptake. These modifications aimed at removing the negatively charged carboxyl group, to increase lipophilicity and improve passive transport across the plasma membrane. Two of these variants have been studied in some detail: N-acetylcysteine amide (NACA) and N-acetylcysteine ethyl ester (NACET). In NACA, the carboxyl group has been converted to the corresponding amide. NACA is more efficient than NAC in terms of promoting GSH replenishment and protecting intracellular thiols and metal centers (e.g. hemoglobin) against the effect of externally applied oxidants (Grinberg et al., 2005; Offen et al., 2004). Unlike NAC, NACA can cross the blood-brain barrier (Offen et al., 2004), making it a potential therapeutic for conditions affecting the brain (Bhatti et al., 2017). The improved intracellular delivery of NACA may also lead to enhanced H_2S production relative to NAC, but this remains to be tested. In the case of NACET, the carboxyl group has been converted into the corresponding ethyl ester. NACET is advantageous over NAC in terms of Cys delivery (Giustarini et al., 2012; Giustarini et al., 2018; Tosi et al., 2021), GSH replenishment (Giustarini et al., 2012; Tosi et al., 2021), and protection against oxidants (Giustarini et al., 2012; Kularatne et al., 2020; Tosi et al., 2021). The improved cellular uptake of NACET also led to stronger endogenous H_2S production, relative to NAC (Giustarini et al., 2012). Both NACA and NACET were more efficient than NAC in protecting against paracetamol-induced liver toxicity (Giustarini et al., 2012; Khayyat, Tobwala, Hart, & Ercal, 2016). One caveat is that both NACA and NACET are less stable than NAC, in that they are more susceptible to oxidation (Grinberg et al., 2005; Tosi et al., 2021). In addition, no toxicology studies have evaluated the safety of NACA or NACET, limiting options to use these compounds in clinical trials. It remains to be seen if NAC-derivatives with improved cellular uptake kinetics also exhibit elevated toxicity relative to NAC, as potentially suggested by the stronger endogenous H_2S production that is induced by NACET.

In summary, it seems that NAC delivers Cys at such a slow and (presumably) steady pace that it avoids the toxic effects that have been associated with corresponding dosages of unmodified Cys. In other words,

N-acetylation of Cys slows down the delivery of Cys, making NAC a Cys-prodrug that feeds cells with a trickle of Cys over a prolonged period of time.

6. Are the cytoprotective effects of NAC caused by low-level H₂S production?

As discussed above, the direct and acute administration of supraphysiological amounts of Cys can be toxic. However, the pharmacokinetic bottlenecks associated with NAC uptake and deacetylation decelerate and prolong Cys delivery, making it not just non-toxic, but actually cytoprotective. This leads to the question of why prolonged low-level-Cys delivery should provide protective effects. If high levels of Cys are cytotoxic because they trigger supraphysiological spikes in H₂S production, could it be that NAC-derived H₂S, produced more steadily at much lower concentrations, is behind the protective effects afforded by NAC?

The idea of NAC treatment leading to modestly increased endogenous H₂S production seems plausible. However, few studies actually investigated the connection between NAC and H₂S. This seems surprising, given the established role of Cys in H₂S production and NAC obviously being a Cys pro-drug. Only in recent years studies demonstrated that exposure of cells to NAC leads to enhanced endogenous H₂S production (Ezerina et al., 2018; Jurkowska & Wróbel, 2018; Kartha et al., 2012; Ono et al., 2017). This raises the question if modest elevations of H₂S production as caused by NAC treatment are indeed cytoprotective.

Elevations of H₂S levels within the physiological range (presumably up to ~20 nM) are indeed associated with a range of cytoprotective effects (Furne et al., 2008). At the organism level, H₂S is long recognized as a positive regulator of vasoactivity and a protective agent against ischemia-reperfusion injury (Li, Rose, & Moore, 2011; Nicholson & Calvert, 2010). At the cellular level, H₂S donors have been observed to inhibit apoptosis, enhance mitochondrial bioenergetics and protect against 'oxidative stress' (Filipovic et al., 2018; Predmore & Gojon, 2012; Szabo et al., 2014). There is also evidence in favor of the idea that endogenous fluctuations in H₂S production serve signaling functions that support cellular homeostasis (Wang, 2012).

It is not yet understood by which molecular mechanisms H₂S causes beneficial effects. In principle, these could be caused by H₂S per se, the electrons extracted from it, or its oxidation products:

Firstly, H₂S by itself has a well-known propensity to coordinate metal centers in enzymes, typically blocking the enzyme's normal activity (Ríos-González et al., 2014). This is also the basis of its main toxic effect (complex IV inhibition) (Petersen, 1977). In principle, it is possible that weak or transient attenuation of certain metal enzymes contributes to its protective effects. For instance, in a situation of chronic inflammation, inhibition of the heme-containing myeloperoxidase by H₂S (Pálincás et al., 2015) may limit tissue damage. However, it seems that specific connections between beneficial effects and metal enzyme binding have not been established so far (Ríos-González et al., 2014). Alternatively, H₂S may have a role in binding ('sequestering') free iron, to prevent its involvement in radical-generating Fenton reactions. This has been proposed to be the mechanism by which H₂S protects *E.coli* against oxidative stress (Mironov et al., 2017), yet to date there is no evidence for a similar role in eukaryotes.

Secondly, positive effects may be based on the utilization of H₂S as an alternative electron donor for oxidative phosphorylation. Low H₂S concentrations stimulate mitochondrial respiration and ATP production, because H₂S can be used as an alternative electron donor for the mitochondrial electron transport chain (ETC) (Lagoutte et al., 2010; Módis, Coletta, Erdélyi, Papapetropoulos, & Szabo, 2013; Szabo et al., 2014; Vitvitsky et al., 2018). The enzyme sulfide:quinone oxidoreductase (SQR) extracts the electrons from H₂S and feeds them into the quinone pool, thus permitting them to enter the ETC at the level of complex III (Lagoutte et al., 2010; Módis et al., 2013). Recent work suggests that H₂S can also feed electrons into the ETC at the level of complex IV, by reducing cytochrome c (Vitevitsky et al., 2018).

Thirdly, the beneficial effects of H₂S may be caused by its oxidation products, so-called sulfane sulfur species, in particular per-(RSSH) and poly-(RSS_nSR)sulfides (Paul & Snyder, 2015). These are mainly generated by SQR, which (after extracting electrons from H₂S) transfers the partially oxidized sulfur atom to GSH and perhaps other thiol-containing acceptors, thus generating persulfides (Landry et al., 2019; Libiad, Yadav, Vitvitsky, Martinov, & Banerjee, 2014) (Fig. 6, left side). In addition, oxidation of H₂S by cytochrome c can also lead to the formation of persulfides (Vitevitsky et al., 2018) (Fig. 6, left side). Finally, H₂S-derived persulfides can be the product of a non-enzymatic process, presumably much less efficient, namely

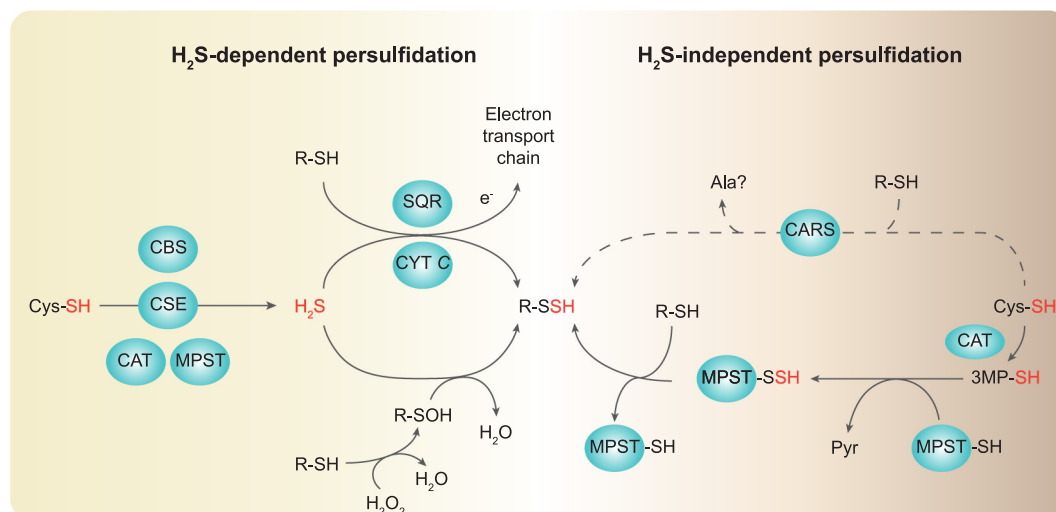


Fig. 6. Intracellular pathways leading from Cys to persulfides. Two main routes lead to the generation of persulfides. The first involves Cys-derived H₂S as an intermediate (left side), the second involves the direct transfer of sulfur (right side). H₂S-dependent persulfide generation is mainly catalyzed by sulfide:quinone oxidoreductase (SQR), with potential contributions from cytochrome c (CYT C), but may also to some extent result from the non-enzymatic reaction between H₂S and sulfenic acids (R-SOH). H₂S-independent persulfide generation is based on the transfer of sulfur from Cys-derived 3-mercaptopyruvate (3MP-SH) to thiols. Additionally, cysteinyl-tRNA synthetase (CARS) has recently been proposed to act as a cysteine persulfidase, although the exact mechanism remains unknown (the dashed line indicates uncertainty about the mechanism).

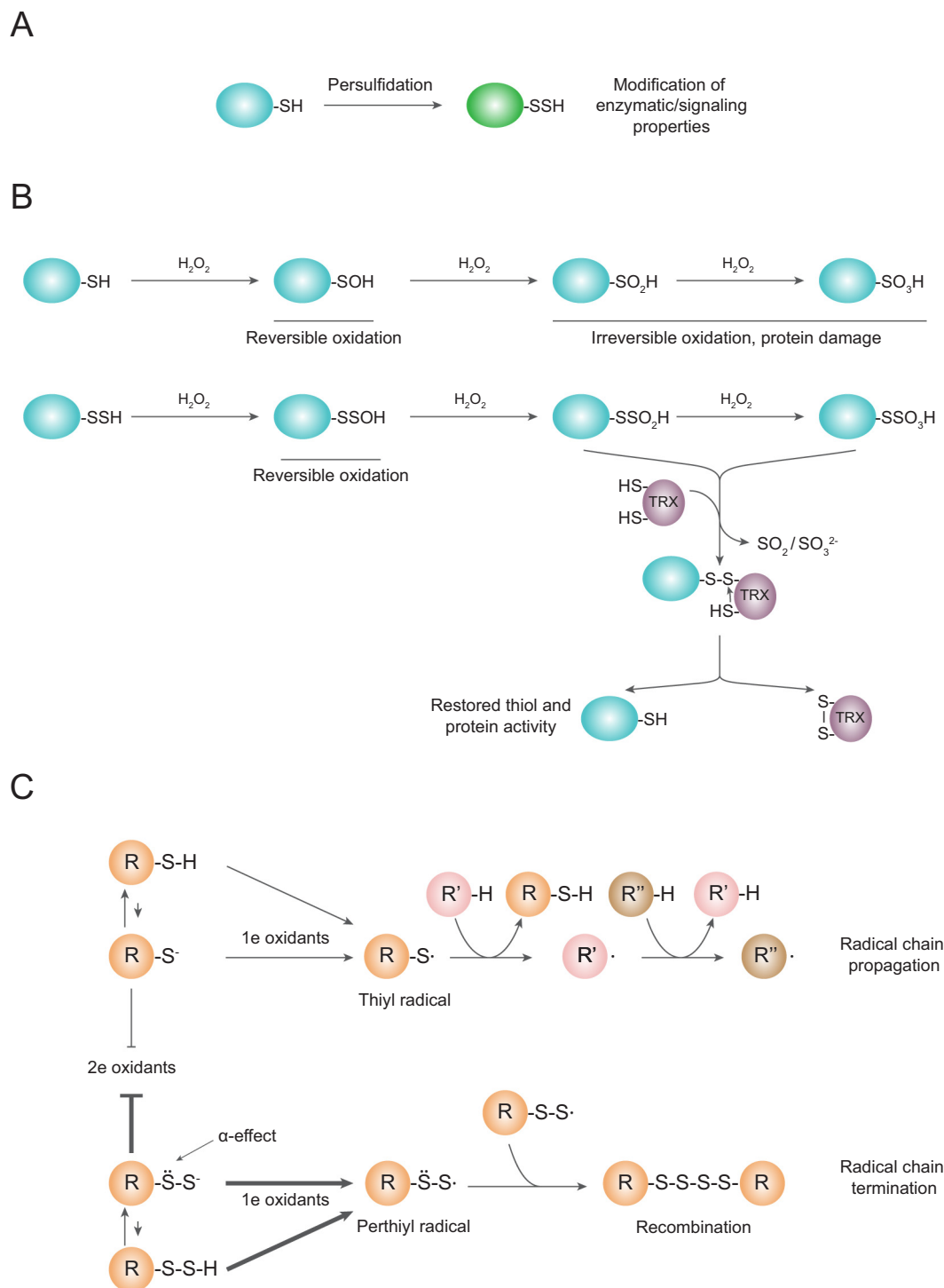


Fig. 7. Three hypotheses potentially explaining persulfide-mediated cytoprotection. (A) The first hypothesis argues that persulfidation of Cys residues on proteins changes their activity and/or signaling properties in an adaptive manner, e.g. to alter metabolism or gene expression.

(B) The second hypothesis argues that protein persulfidation protects thiol groups against irreversible oxidative damage. While sulfinic (SO_2H) and sulfonic (SO_3H) acid residues cannot be repaired, perthiosulfinic (SSO_2H) and perthiosulfonic (SSO_3H) acid residues are easily repaired by a disulfide reductase, such as thioredoxin (TRX). Reduction regenerates the original thiol and presumably releases the outer sulfur as sulfur dioxide (SO_2) or sulfite (SO_3^{2-}).

(C) The third hypothesis argues for a scavenging role of (low-molecular-weight) persulfides, as they are more reactive towards one- and two-electron oxidants than thiols. This is explained by (i) the α -effect, which increases the nucleophilicity of the persulfide's outer sulfur atom, and (ii) for two-electron oxidants, the higher availability of the reactive thiolate (i.e., lower pK_a). In addition, persulfides are proposed to act as highly efficient radical scavengers. They form stable perthiyl radicals, unable to propagate radical chain reactions, and capable of recombining into tetrasulfides, thereby eliminating radicals from the system.

the reaction of H_2S with sulfenic acids (which can be formed under oxidative stress conditions) (Cuevasanta et al., 2015) (Fig. 6, left side). Importantly, there is substantial evidence suggesting that

sulfane sulfur species have cytoprotective properties. In particular, it has been shown that sulfane sulfur species protect against the damaging effects of electrophiles (Abiko et al., 2017; Bianco et al.,

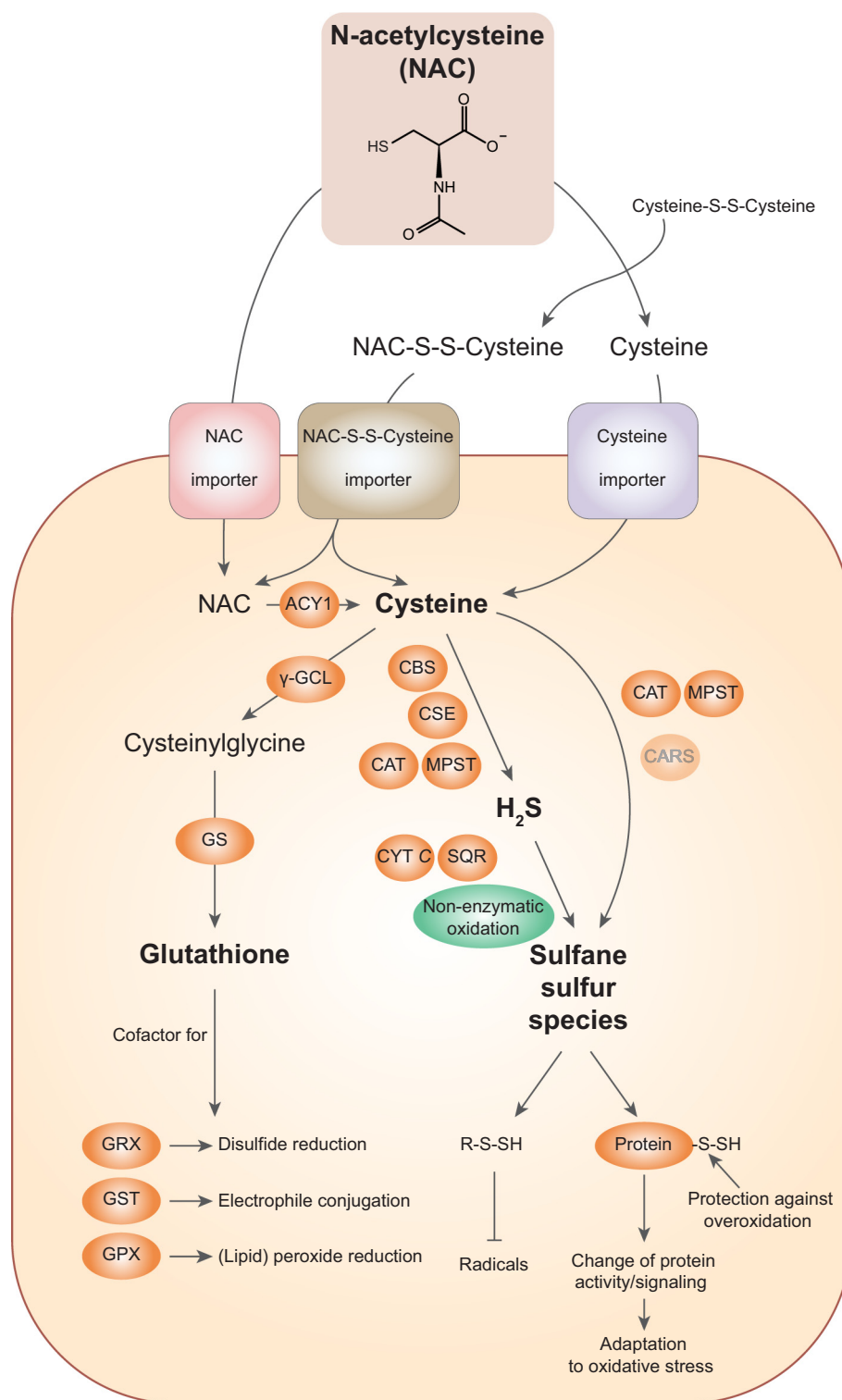


Fig. 8. NAC-derived metabolites facilitate disulfide-reducing and oxidant scavenging activities. The conventional narratives assume that disulfide-reducing and antioxidative activities are inherent to NAC. However, evidence suggests that these activities are facilitated by NAC-derived metabolites, GSH and H₂S/sulfane sulfur species. On the one hand, the replenishment of previously depleted GSH levels supports enzymatic disulfide reduction by glutaredoxins (GRX), electrophile detoxification by glutathione S-transferases (GST) and (lipid) peroxide scavenging by glutathione peroxidases (GPX). On the other hand, sulfane sulfur species can act as direct radical scavengers, and protect proteins against irreversible oxidative damage. Additionally, persulfide modifications on proteins may change their activity and signaling properties to trigger stress-adaptive cellular responses.

2019; Ihara et al., 2017), metals (Akiyama et al., 2017), oxidants (Ezerina et al., 2018), and cyanide (Baskin, Horowitz, & Nealley, 1992; Patterson et al., 2016).

On a final note, while endogenously produced sulfane sulfur species generally appear to be cytoprotective, it is definitely conceivable that excessive levels of sulfane sulfur species (as

potentially induced by supraphysiological Cys administration) can be disadvantageous or even toxic to cells. It is therefore possible that not only H₂S toxicity, but also sulfane sulfur toxicity contributes to Cys toxicity. Since H₂S and per/polysulfides are always co-occurring and interconverting, their relative contributions to toxicity are difficult to assess.

7. Are the cytoprotective effects of NAC caused by sulfane sulfur species?

Above considerations suggest that sulfane sulfur species, i.e. oxidation products of H_2S , may be more directly connected to the protective effects of NAC than H_2S itself. Most of the studies that report increases in cellular H_2S levels upon NAC administration also show increases in cellular sulfane sulfur content (Ezerina et al., 2018; Jurkowska & Wróbel, 2018; Ono et al., 2017), with an even earlier study demonstrating that NAC was a source of sulfane sulfur (Jurkowska & Wróbel, 2008). The methods used to detect an increase of sulfane sulfur content included the classical cold cyanolysis method (Jurkowska & Wróbel, 2008, 2018), and sulfane sulfur reactive fluorescent probes (Ezerina et al., 2018). Importantly, one study demonstrated that administration of ^{34}S -labeled NAC to cells led to the appearance of ^{34}S -labeled sulfane sulfur species, in particular persulfides of Cys and GSH (Ono et al., 2017).

How the sulfur atom of NAC finds its way into sulfane sulfur species may vary and has not been fully clarified. On the one hand, NAC-derived Cys may first be desulfurated to yield H_2S , which is subsequently oxidized to sulfane sulfur species, either enzymatically by SQR/cytochrome c or non-enzymatically (Fig. 6, left side). On the other hand, NAC-derived Cys may also generate persulfides independently of prior H_2S formation (Fig. 6, right side). A well established pathway is the deamination of Cys to 3MP by CAT. 3MP is in turn desulfurated by MPST to form an enzyme-bound persulfide whose outer sulfur can be transferred to the thiol group of other proteins or small molecules (Pedre & Dick, 2020; Yadav et al., 2013). Additionally, cysteinyl-tRNA synthetase (CARS) has been proposed to generate tRNA-bound cysteine persulfides directly from Cys (Akaike et al., 2017), although the mechanism remains unclear. How much each of the pathways contributes to sulfane sulfur production is not known, but there are indications that both H_2S -dependent and H_2S -independent routes contribute (Ezerina et al., 2018).

Overall, there is strong evidence that sulfane sulfur species have cytoprotective properties. However, the molecular mechanisms by which persulfides exert protective effects are not yet understood. There are three main hypotheses, not mutually exclusive, each being supported by some evidence (Fig. 7):

The first hypothesis (Fig. 7A) is based on the observation that many proteins in the cell are persulfidated on cysteine residues, especially under oxidative stress conditions. Although it is currently not understood how proteins are persulfidated, it is clear that sulfane sulfur generated by the above-mentioned pathways (Fig. 6) finds its way into proteins. It has been proposed that proteins are functionally modulated by persulfidation, in order to adapt metabolism and signaling transduction to oxidative stress situations. Several studies have connected persulfidation to enzymatic and/or physiological adaptations, such as potassium channel dependent vasorelaxation (Jiang, Tang, Cao, Wu, & Wang, 2010; Mustafa et al., 2011), the Keap1-Nrf2 antioxidant response (Xie et al., 2016; G. Yang et al., 2013) or the suppression of apoptosis by NF- κ B (Sen et al., 2012). Persulfidation of a Cys residue in aquaporin-8, a plasma membrane H_2O_2 channel, leads to its reversible closure, thereby restricting influx of extracellular H_2O_2 (Bestetti et al., 2018).

The second hypothesis (Fig. 7B) suggests that persulfidation protects protein thiols against irreversible oxidation (Heppner et al., 2018; Millikin et al., 2016; Ono et al., 2014; Zivanovic et al., 2020). Hyperoxidation of thiols, i.e. formation of either sulfinic or sulfonic acid, is (almost always) irreversible and often leads to protein malfunction. However, if hyperoxidation takes place on the outer sulfur of a persulfide (leading to perthiosulfinic -SSO $_2$ H or perthiosulfonic -SSO $_3$ H acid) the modification is reversible, because the oxidized outer sulfur atom can be removed by disulfide reduction, as catalyzed by thioredoxin or other disulfide-reducing oxidoreductases (Dóka et al., 2020; Millikin et al., 2016; Zivanovic et al., 2020). In other words, the outer sulfur of the persulfide is sacrificed to protect the inner sulfur against permanent oxidative damage. This mechanism has been

demonstrated with recombinant proteins *in vitro* (Dóka et al., 2020; Gao et al., 2015; Zivanovic et al., 2020), but remains to be demonstrated under physiologically relevant settings.

The third hypothesis (Fig. 7C) is that persulfides directly act as scavengers of one/two-electron oxidants and electrophiles, as they are more reactive than the corresponding thiols (Benchoam et al., 2020; Cuevasanta et al., 2015; Ida et al., 2014). The superior reactivity of persulfides is explained by the so-called α -effect, i.e. the lone pair electrons of the inner sulfur atom enhance the nucleophilicity of the adjacent outer sulfur atom (Edwards & Pearson, 1962; Jencks & Carriuolo, 1960). Likewise, persulfides have a lower pK_a than corresponding thiols, increasing the proportion of the reactive deprotonated form at physiological pH (Benchoam et al., 2020; Ono et al., 2014). Low-molecular-weight persulfides have been found at low micromolar concentrations inside cells (Ida et al., 2014), but it remains unclear if these concentrations are sufficient to allow them to make a significant contribution to oxidant scavenging. Relatively low concentrations of persulfide donors can provide protection against cytotoxic H_2O_2 concentrations (Bianco et al., 2019; Ezerina et al., 2018), but a direct H_2O_2 scavenging mechanism seems unlikely: persulfides in the low micromolar range should be easily outcompeted, not only by enzymatic H_2O_2 scavengers, but also by the highly abundant glutathione: while persulfidated glutathione is 22-fold faster in reducing H_2O_2 (Benchoam et al., 2020), the concentration of non-persulfidated glutathione is at least 25-fold higher (Ida et al., 2014).

However, it is possible that persulfides are highly effective as radical scavengers, even at relatively low concentrations. So far, there is only limited data on the interaction between persulfides and radicals (Bianco et al., 2016; Everett, Folkes, Wardman, & Asmus, 1994), but it has been suggested that persulfides may be highly efficient terminators of radical chain reactions (Fukuto, 2021). In one-electron reactions, persulfides form perthiyl radicals ($\text{RSS}\cdot$), which presumably are much less damaging than the corresponding thiyl radicals ($\text{RS}\cdot$) formed from thiols. Thiyl radicals are unstable and engage in further electron transfer, while perthiyl radicals are much more stable and have a strong tendency to recombine with each other to form tetrasulfides (RSSSSR),

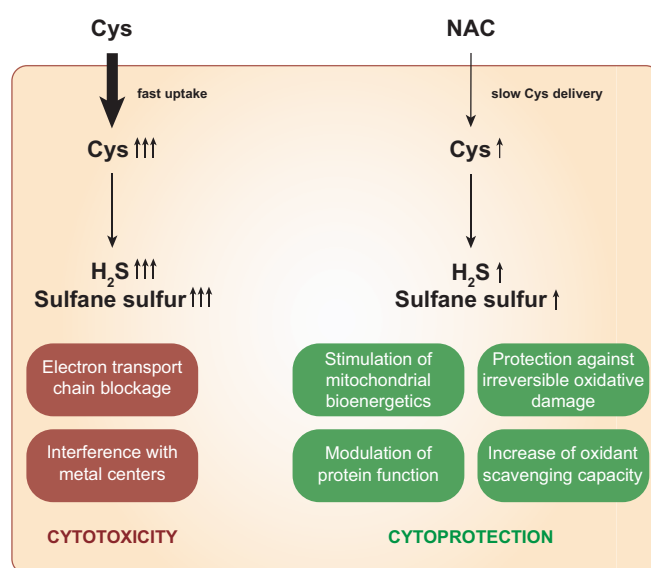


Fig. 9. The rate of Cys delivery may determine the difference between cytotoxic and cytoprotective effects. Direct Cys administration leads to a rapid and steep rise in intracellular Cys, which is metabolized into H_2S and sulfane sulfur species. The sudden increase in the concentration of these molecules leads to cytotoxicity, associated with the blockage of the mitochondrial ETC and potentially other enzymatic metal centers. In contrast, NAC elevates intracellular Cys at a much slower and more steady rate, allowing for a low-level H_2S and sulfane sulfur production, stimulating mitochondrial bioenergetics and protecting cells against oxidative damage.

hence eliminating radicals from the system (Fig. 7C) (Bianco et al., 2016; Everett et al., 1994; Nakabayashi & Tsurugi, 1963). The high stability of the perthiyl radicals is thought to be caused by the overlap between the unpaired electron orbital and the lone pair orbital of the adjacent sulfur atom (Everett et al., 1994; Ono et al., 2014).

These three explanations are not mutually exclusive and may all apply in combination. For instance, a persulfide on a protein may alter protein activity in a regulatory manner and also protect it against irreversible inactivation. At the same time, low molecular weight persulfides may engage in the direct scavenging of one-electron oxidants.

Taken together, multiple lines of evidence support the notion that NAC acts as a donor of sulfane sulfur when provided to cells. These species may form dependently or independently of H₂S, and are likely to exert a cytoprotective effect, through modulation of protein activity, protection of thiols against irreversible modifications and/or increased oxidant scavenging capacity.

8. The sulfane sulfur perspective: a new angle to look at the classical NAC narratives

In the initial chapters of this review we looked at the commonly told narratives of how NAC acts as a beneficial drug. We concluded that their validity is likely restricted to specific circumstances and that there is a paucity of evidence for any of these narratives to serve as a general explanation. In particular, it seems that the ability of NAC to directly scavenge oxidants and to directly reduce disulfide bonds has often been overestimated. The now emerging H₂S/sulfane sulfur narrative may in a sense reconcile the different narratives, as it proposes that NAC is metabolized into sulfur species that indeed can have a direct role in oxidant scavenging and in enhancing cellular reducing capacity.

To reiterate, the restoration of depleted GSH pools can in principle explain disulfide-reducing as well as electrophile- and oxidant-scavenging NAC effects (Fig. 8), as GSH is the required substrate for glutaredoxins, glutathione-S-transferases and glutathione peroxidases. However, this only applies to situations of severe GSH depletion, and even then the protective action of NAC can be independent of GSH biosynthesis. As discussed above, sulfane sulfur species derived from NAC may enhance oxidative stress resistance by protecting protein thiols, by triggering adaptive changes in protein activity, and by scavenging radicals (Fig. 8). If true, the conversion of NAC into sulfane sulfur species would explain the long-known antioxidative properties of NAC, first observed in cell culture and initially believed to represent the direct scavenging of oxidants by NAC itself (Junod et al., 1987; Kharazmi et al., 1988; Moldéus et al., 1986; Simon & Suttrop, 1985). Sulfane sulfur species may explain why NAC can protect against ROS and ROS-inducing treatments (e.g. UV light) in a GSH-independent manner (Konarkowska et al., 2005; Naziroğlu et al., 2013; Steenvoorden & Beijersbergen van Henegouwen, 1998) and reconcile the observed antioxidative properties of NAC with its intrinsic inefficiency as a direct antioxidant.

On a final note, it may be asked if NAC doses previously established for use in human subjects can be expected to support sustained H₂S/sulfane sulfur production and corresponding therapeutic benefits. Given the relatively long half-life of NAC in the circulation (mean residence time ≥ 0.8 h and ≥ 2 h in single i.v. and oral administrations, respectively (Borgström et al., 1986; Olsson et al., 1988)) this seems conceivable. However, given the discussed pharmacological bottlenecks, it is likely that NAC plasma concentrations need to be at least similar to those of circulating cystine and Cys-protein mixed disulfides to raise intracellular Cys levels and subsequently increase H₂S/sulfane sulfur production. From this perspective, it seems unlikely that ingestion of a single customary NAC tablet (200–600 mg; i.e. ~5–10 mg NAC/kg) is effective, as the maximum plasma NAC concentration (<15 μ M) (Holdiness, 1991) is low relative to circulating cystine (~40–60 μ M) and Cys-protein conjugates (~150–180 μ M) (Turell et al., 2013). On

the other hand, increased and sustained H₂S/sulfane sulfur production should be achievable with i.v. administrations (100–200 mg NAC/kg), which lead to NAC plasma concentrations above 500 μ M (Medved, Brown, Bjorksten, Murphy, et al., 2004; Medved et al., 2003; Pakravan et al., 2008). Oral doses used to treat paracetamol poisoning (140 mg NAC/kg) lead to peak plasma concentrations above 50 μ M (Ekins et al., 1987; North, Peterson, & Krenzelok, 1981), and were shown to increase total plasma Cys levels (Ferreira, Campbell, & Reid, 2011), suggesting that H₂S/sulfane sulfur will also be elevated. Additional work is needed to further define the conditions under which NAC produces sustained low-level H₂S/sulfane sulfur generation, and to which extent this translates into therapeutic benefits.

9. Conclusion

In summary, previously favoured explanations for the mechanism of action of NAC are either poorly supported (direct oxidant scavenging), restricted to very specific situations (disulfide reduction in lung mucins), or fail to explain all observations (GSH biosynthesis). A new conceptual framework for NAC's mechanism of action is emerging, namely as a Cys pro-drug that leads to modest elevations of H₂S and sulfane sulfur species inside cells. The slow release of Cys from NAC allows for sustained sulfane sulfur production, providing protective effects -independently of GSH replenishment (Fig. 9). The sulfane sulfur branch of NAC metabolism opens new perspectives on its therapeutic use.

Declaration of Competing Interest

All authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (SPP1710 to TPD).

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