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Original Contribution

# Inactivation of thiol-dependent enzymes by hypothiocyanous acid: role of sulfenyl thiocyanate and sulfenic acid intermediates

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ABSTRACT

Myeloperoxidase (MPO) forms reactive oxidants including hypochlorous and hypothiocyanous acids (HOCl and HOSCN) under inflammatory conditions. HOCl causes extensive tissue damage and plays a role in the progression of many inflammatory-based diseases. Although HOSCN is a major MPO oxidant, particularly in smokers, who have elevated plasma thiocyanate, the role of this oxidant in disease is poorly characterized. HOSCN induces cellular damage by targeting thiols. However, the specific targets and mechanisms involved in this process are not well defined. We show that exposure of macrophages to HOSCN results in the inactivation of intracellular enzymes, including creatine kinase (CK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In each case, the active-site thiol residue is particularly sensitive to oxidation, with evidence for reversible inactivation and the formation of sulfenyl thiocyanate and sulfenic acid intermediates, on treatment with HOSCN (less than fivefold molar excess). Experiments with DAz-2, a cell-permeable chemical trap for sulfenic acids, demonstrate that these intermediates are formed on many cellular proteins, including GAPDH and CK, in macrophages exposed to HOSCN. This is the first direct evidence for the formation of protein sulfenic acids in HOSCN-treated cells and highlights the potential of this oxidant to perturb redox signaling processes.



Myeloperoxidase (MPO)[2] catalyzes the reaction of hydrogen peroxide ($H_2O_2$) with halide and pseudohalide ions ($Cl^-$, $Br^-$, $I^-$, and thiocyanate, $SCN^-$) to form hypohalous acids (hypochlorous acid, HOCl, from $Cl^-$; hypobromous acid, HOBr, from $Br^-$; and hypothiocyanous acid, HOSCN, from $SCN^-$), which are potent oxidants (reviewed in [1,2]). $SCN^-$ is the favored substrate of MPO and many other peroxidase enzymes (inclusive of eosinophil peroxidase and lactoperoxidase), which results in the formation of HOSCN in a variety of physiological settings [3–7]. It is postulated that HOSCN production is particularly important in smokers, owing to the higher levels of $SCN^-$ in their plasma from detoxification of cyanide in cigarette smoke [8]. The hypohalous acids are powerful antibacterial agents and, as such, play an important role in the human immune system [9]. However, recent evidence suggests that these oxidants can also induce host cell damage, particularly under inflammatory conditions, which may contribute to the development of a number of diseases, including atherosclerosis, neurodegenerative disorders, arthritis, and some cancers (reviewed in [1,2]).

HOCl and HOBr have been implicated in a number of pathologies owing to the detection of elevated levels of their biomarkers 3-chloro-Tyr and 3-bromo-Tyr, respectively, in diseased tissues and inflammatory fluids [2,10–12]. In contrast, the role of HOSCN in disease has not been widely studied [13]. Serum $SCN^-$ levels correlate with the extent of fatty streak formation and macrophage foam cell populations, consistent with a role for HOSCN in atherosclerosis [14,15]. Moreover, cyanate ($OCN^-$) formation from the decomposition of HOSCN has been implicated as the major pathway for inducing elevated homocitrulline formation in atherosclerosis in humans [16]. However, HOSCN production has also been proposed to be beneficial, by preventing disease through the detoxification of other, more potent, oxidants such as HOCl (e.g., [17,18]). Similarly, there is some controversy regarding the role of HOSCN in the induction of mammalian cell damage [19,20].

It is well established that exposure of bacteria, mammalian cells, and plasma to HOSCN results in specific and selective damage to thiols (reviewed in [13,21]). This targeting of thiols results in the

*Abbreviations:* CK, creatine kinase; DAz-2, 4-(3-azidopropyl)cyclohexane-1,3-dione; dimedone, 5,5-dimethylcyclohexane-1,3-dione; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC, liquid chromatography; MPO, myeloperoxidase; MS/MS, tandem mass spectrometry; RS–OH, sulfenic acid; R–$SO_2$H, sulfinic acid; R–$SO_3$H, sulfonic acid; RS–SCN, sulfenyl thiocyanate; $SCN^-$, thiocyanate ion; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TNB, 5-thio-2-nitrobenzoic acid.

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inhibition of bacterial glycolysis via inactivation of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hexokinase, glucose-6-phosphate dehydrogenase, and aldolase [22–24]. Similarly, HOSCN depletes thiols and inactivates thiol-dependent enzymes, including GAPDH, caspases, glutathione transferases, membrane ATPases, and protein tyrosine phosphatases in mammalian cells [5,20,25]. HOSCN can also perturb various cell signaling pathways, with evidence for alterations in the phosphorylation of mitogen-activated protein kinase proteins and the release of inflammatory mediators in macrophages and endothelial cells [25–27]. In macrophages, HOSCN is a potent inducer of cellular apoptosis [20], though in human umbilical vein endothelial cells, this oxidant induces cell damage via alternative pathways [19]. In each case, these cellular effects are attributed to the selectivity of HOSCN for thiol-containing proteins [19,20,25–27].

Reaction of HOSCN with thiols results in the generation of sulfenyl thiocyanate derivatives (RS–SCN; Reaction (1)), which are postulated to hydrolyze to sulfenic acid intermediates (RS–OH; Reaction (2)) [22,28]:

$$\mathrm{RS{-}H + HOSCN \rightarrow RS{-}SCN} \quad (1)$$

$$\mathrm{RS{-}SCN + H_2O \rightarrow RS{-}OH + SCN^- + H^+} \quad (2)$$

Sulfenyl species have been reported in bacterial cells exposed to HOSCN, though direct evidence for protein sulfenic acid formation is lacking, owing to a lack of specificity in the methods used to assess these reactive species [22,29]. Similarly, although there are data on the targets of HOSCN in mammalian cells [5,20,25] there is little information available regarding the nature of the intermediates and mechanisms involved in thiol-dependent enzyme inactivation.

In this study, we examine the mechanism involved in HOSCN-mediated inactivation of GAPDH and creatine kinase (CK), both in isolation and in the cellular environment. We show that HOSCN induces enzyme inactivation in both a reversible and a nonreversible manner via the formation of sulfenyl thiocyanate/sulfenic acid intermediates and sulfinic/sulfonic acids, respectively. In addition, we provide the first direct experimental evidence for the formation of protein sulfenic acid intermediates in mammalian cells exposed to HOSCN. This suggests that HOSCN may have the potential to act as a second messenger in redox signaling processes, which has important implications for the development of inflammatory disease.

## Materials and methods

### *Reagents*

Aqueous solutions and buffers were prepared using Nanopure water filtered through a four-stage Milli-Q system (Millipore–Waters, Lane Cove, NSW, Australia). All proteins and 5-thio-2-nitrobenzoic acid (TNB) solutions were prepared in 0.1 M sodium phosphate buffer (pH 7.4) pretreated with Chelex resin (Bio-Rad, Hercules, CA, USA) to remove contaminating trace metal ions. Dimedone (505 mM; Sigma–Aldrich, Castle Hill, NSW, Australia) was prepared in 95% (v/v) ethanol. Lactoperoxidase (LPO; from bovine milk; Merck, Whitehouse Station, NJ, USA) was quantified by absorbance at 412 nm using ε 112,000 $M^{-1}$ $cm^{-1}$ [30]. $H_2O_2$ (30% v/v solution; Merck) was quantified at 240 nm using ε 39.4 $M^{-1}$ $cm^{-1}$ [31]. CK (from rabbit muscle) was obtained from Roche (Castle Hill, NSW, Australia), and GAPDH (from rabbit muscle) from Sigma–Aldrich.

### *Cell culture*

Murine macrophage-like J774A.1 cells (American Type Culture Collection; No. 915051511) were cultured under sterile conditions in Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with 10% (v/v) fetal bovine serum (Sigma–Aldrich) and 2 mM L-glutamine (Thermotrace, Melbourne, VIC, Australia). Cells were seeded at $1 \times 10^6$ cells $ml^{-1}$ in 12-well plates and allowed to adhere overnight. Before treatment, the medium was removed and cells were washed twice with phosphate-buffered saline (PBS; Amresco, Solon, OH, USA). Cells were washed twice with PBS after treatment and before lysis in 600 µl of $H_2O$ and centrifugation at 8000 *g* at 4 °C for 5 min. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) standards (Sigma–Aldrich).

### *Generation and quantification of HOSCN*

HOSCN was produced enzymatically from the reaction of $H_2O_2$ with $SCN^-$ in the presence of LPO as described previously [20,32]. The concentration of HOSCN was quantified immediately using the TNB assay [20,32,33] with absorbances recorded at 412 nm and ε 14,150 $M^{-1}$ $cm^{-1}$ [34]. Stock solutions of HOSCN were diluted into potassium phosphate buffer (10 mM, pH 6.6) for protein and cell lysate experiments or PBS for intact cell experiments.

### *$S^{14}CN^-$ incorporation studies with $HOS^{14}CN$*

$HOS^{14}CN$ was prepared as above except using $KS^{14}CN$ (3.75 mM) (American Radiolabeled Chemicals, St. Louis, MO, USA) and 1.875 mM $H_2O_2$, as previously described [32]. $HOS^{14}CN$ was added to CK or GAPDH (25 µM) and incubated for 5 or 30 min, respectively, before precipitation with 10% (w/v) trichloroacetic acid (TCA). Proteins were pelleted by centrifugation (10,000 *g* for 5 min at 4 °C), and free $S^{14}CN^-$ label was removed by washing pellets twice with 5% (w/v) TCA and twice with ice-cold acetone. Protein pellets were air-dried and resuspended in 100 µl of formic acid before addition to 5 ml of Ultima Gold scintillant (PerkinElmer, Waltham, MA, USA) and liquid scintillation counting on a Packard Tri-Carb liquid scintillation counter (2100TR; PerkinElmer). To assess whether $S^{14}CN^-$ incorporation was reversible, dithiothreitol (DTT; 1.25 mM) or $H_2O$ was added 15 min post-$HOS^{14}CN$ incubation before precipitation with TCA. Control experiments to assess nonspecific binding of $S^{14}CN^-$ to the protein were performed using solutions prepared in the absence of LPO in each case.

### *Dimedone specificity studies with $S^{14}CN^-$ incorporation*

Equimolar $HOS^{14}CN$ was added to GAPDH (25 µM) for 30 min before further incubation in the presence of dimedone (5 mM) or ethanol as control for 2 h at 22 °C. Protein was precipitated and washed and $^{14}C$ incorporation assessed as described above.

### *Quantification of protein thiols*

The extent of protein thiol oxidation was assessed using the thiol-specific reagent ThioGlo-1 (Merck) as described previously [25,33]. Thiol concentrations were quantified by fluorescence spectroscopy at $\lambda_{ex}$ 360 nm and $\lambda_{em}$ 530 nm using glutathione (GSH; Sigma–Aldrich) to construct a standard curve.

### *Enzyme activity assays*

GAPDH activity was measured by monitoring the formation of NADH after addition of glyceraldehyde 3-phosphate (GAP) [35]. Cell lysate (20 µl) was combined with 120 µl of phosphate buffer (28.5 mM sodium pyrophosphate, 38 mM sodium phosphate buffer, pH 7.4) and 60 µl of $NAD^+$/GAP substrate solution (1.2 mM GAP, 2.5 mM $NAD^+$) on a 96-well plate. The plate was shaken briefly and GAPDH activity measured as the rate of absorbance increase at