

# Selective Metabolism of Hypothiocyanous Acid by Mammalian Thioredoxin Reductase Promotes Lung Innate Immunity and Antioxidant Defense\*

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**Background:** Secreted hypothiocyanous acid (HOSCN) kills pathogens but paradoxically is tolerated by many mammalian cells.

**Results:** Mammalian thioredoxin reductase (H-TrxR) reduces HOSCN, whereas bacterial L-TrxR is inhibited by it, corresponding to differential cytotoxicity.

**Conclusion:** Mammalian H-TrxR confers resistance against HOSCN, enabling its use as a selective biocide.

**Significance:** Findings directly link mammalian H-TrxR to innate immunity and inflammatory lung disease.

The endogenously produced oxidant hypothiocyanous acid (HOSCN) inhibits and kills pathogens but paradoxically is well tolerated by mammalian host tissue. Mammalian high molecular weight thioredoxin reductase (H-TrxR) is evolutionarily divergent from bacterial low molecular weight thioredoxin reductase (L-TrxR). Notably, mammalian H-TrxR contains a selenocysteine (Sec) and has wider substrate reactivity than L-TrxR. Recombinant rat cytosolic H-TrxR1, mouse mitochondrial H-TrxR2, and a purified mixture of both from rat selectively turned over HOSCN ( $k_{\text{cat}} = 357 \pm 16 \text{ min}^{-1}$ ;  $K_m = 31.9 \pm 10.3 \mu\text{M}$ ) but were inactive against the related oxidant hypochlorous acid. Replacing Sec with Cys or deleting the final eight C-terminal peptides decreased affinity and turnover of HOSCN by H-TrxR. Similarly, glutathione reductase (an H-TrxR homologue lacking Sec) was less effective at HOSCN turnover. In contrast to H-TrxR and glutathione reductase, recombinant *Escherichia coli* L-TrxR was potently inhibited by HOSCN ( $\text{IC}_{50} = 2.75 \mu\text{M}$ ). Similarly, human bronchial epithelial cell (16HBE) lysates metabolized HOSCN, but *E. coli* and *Pseudomonas aeruginosa* lysates had little or no activity. HOSCN selectively produced toxicity in bacteria, whereas hypochlorous acid was nonselectively toxic to both bacteria and 16HBE. Treatment with the H-TrxR inhibitor auranofin inhibited HOSCN metabolism in 16HBE lysates and significantly increased HOSCN-mediated cytotoxicity. These findings demonstrate both the metabolism of HOSCN by mammalian H-TrxR resulting in resistance to HOSCN in mammalian cells and the potent inhibition of bacterial L-TrxR resulting in cytotoxicity in bacteria. These data support a novel selective mechanism of host defense in mammals wherein HOSCN formation simultaneously inhibits pathogens while sparing host tissue.

Hypothiocyanous acid (HOSCN)<sup>2</sup> is produced when thiocyanate (SCN), an abundant pseudohalide in mammalian extracellular fluids (30  $\mu\text{M}$  in plasma; concentrated up to 100-fold in secretions (1)), is oxidized by  $\text{H}_2\text{O}_2$  in a reaction catalyzed by haloperoxidases (e.g. lactoperoxidase (LPO) (2); myeloperoxidase (MPO) (3); and eosinophil peroxidase (4)). HOSCN is a potent and selective oxidizer of nucleophilic thiols (5) that inhibits and kills multiple species of bacteria (6–8), viruses (9), and fungi (10) but paradoxically is well tolerated by healthy mammalian tissue (11). For example, the human oral cavity is exposed to steady-state concentrations of HOSCN up to 70  $\mu\text{M}$  (12).

MPO also catalyzes a similar halogenation reaction utilizing  $\text{Cl}^-$  instead of SCN to produce hypochlorous acid (HOCl), the active component of bleach. HOCl is linked to host defense and human disease through phagocytic activation, resulting in large amounts of HOCl in inflamed tissue (13). HOCl is a nonselective oxidant that reacts with a number of biological targets, resulting in cytotoxicity (14, 15). However, SCN, which is enriched in secretions, can effectively compete with  $\text{Cl}^-$  for MPO and diminishes HOCl formation in favor of HOSCN (3). SCN also reacts directly with HOCl to form HOSCN (16), which alleviates cytotoxicity (17), and scavenges toxic metabolites of HOCl such as monochloramines, also forming HOSCN (16). Although HOSCN and HOCl both function in host defense, only HOSCN is well tolerated by mammalian tissue. One possible basis of this difference is the selective targeting of nucleophilic thiols (5) and selenols (18) by HOSCN in contrast to the promiscuous oxidizing nature of HOCl.

Selenocysteine-containing proteins (selenoproteins) are found in all kingdoms of life but are most abundant in higher

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<sup>2</sup> The abbreviations used are: HOSCN, hypothiocyanous acid/hypothiocyanate; SCN, thiocyanate; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); NTB, 2-nitro-5-thiobenzoate; GR, glutathione reductase; LPO, lactoperoxidase; MPO, myeloperoxidase; HOCl, hypochlorous acid/hypochlorite; Trx, thioredoxin; H-TrxR, high molecular weight thioredoxin reductase; L-TrxR, low molecular weight thioredoxin reductase; DMSO, dimethyl sulfoxide.

## Mammalian H-TrxR Metabolizes HOSCN

order eukaryotes such as mammals (19). High molecular weight thioredoxin reductase (H-TrxR, 112 kDa) is a critical selenoprotein in mammals that regulates multiple biologic pathways including DNA synthesis and oxidant scavenging through the reduction of thioredoxin (Trx) (20). H-TrxR shares homology with glutathione reductase (GR) but has uniquely evolved an additional redox-active site in its C terminus that in mammals is expressed with a penultimate selenocysteine (Sec) residue. H-TrxR is strikingly different from the low molecular weight L-TrxR (70 kDa (20)) found in bacteria and yeast, sharing only 20% sequence identity (21). Although L-TrxR functions similarly to mammalian H-TrxR to reduce Trx, it lacks many of its other features including Sec expression. Sec has been proposed to broaden the substrate reactivity of mammalian H-TrxR and help it resist oxidative inactivation (22). The high nucleophilicity of Sec predisposes it for rapid, selective reaction with HOSCN (18).

Here we report a new metabolic function for Sec-expressing mammalian H-TrxR, which rapidly turns over HOSCN with physiologically relevant  $K_m$ . Replacement of the Sec residue with Cys and deletion of the eight final C-terminal peptides dramatically decreased enzyme activity. In contrast, recombinant *Escherichia coli* L-TrxR lacked activity and was potently inhibited by HOSCN exposure. Lysates of human bronchial epithelia cells (16HBE), *E. coli*, and *Pseudomonas aeruginosa* were assayed for HOSCN reductase activity, and rapid turnover of HOSCN only occurred in 16HBE lysates. Three clinical isolates of antibiotic-resistant *P. aeruginosa* from cystic fibrosis (CF) patients had limited detectable HOSCN reductase activity an order of magnitude below that of 16HBE cells. In addition, HOSCN exposure was selectively toxic to bacteria but well tolerated by 16HBE cells. Inhibition of H-TrxR with auranofin reduced HOSCN metabolism in 16HBE lysates and increased HOSCN-mediated toxicity, coinciding with increased intracellular thiol oxidation. These data suggest that HOSCN and H-TrxR constitute an important mechanism of host defense in mammals that inhibits pathogens while limiting host tissue injury. To the best of our knowledge, this is the first published finding of hypo(pseudo)halous acid metabolism by mammalian H-TrxR.

### EXPERIMENTAL PROCEDURES

**Sources of Purified Thioredoxin Reductase and Glutathione Reductase**—Rat recombinant cytosolic H-TrxR1 was purchased from Cayman-IMCO. Wild type mitochondrial H-TrxR2, mutant Sec489Cys H-TrxR2, and mutant H-TrxR2 lacking the eight ultimate C-terminal peptides from mouse were produced from semisynthesis as described previously (23). A purified mixture of H-TrxR1 and H-TrxR2 from rat liver was purchased from Sigma. L-TrxR from *E. coli* was purchased from Cayman-IMCO. Purified GR from human red blood cells were purchased from Sigma.

**Enzyme Kinetics and Inhibition Studies**—Oxidoreductase activity *in vitro* and in cell lysates was fit to the Michaelis-Menten kinetics equation:  $Y = V_{max}[S]/(K_m + [S])$ . Inhibition data for bacterial L-TrxR were fit according to the sigmoidal dose response with the variable slope equation:  $Y = \text{Bottom} + ((\text{Top} - \text{Bottom}) / (1 + 10^{\wedge} \text{LogEC}_{50} - X \times \text{Hill slope}))$ . Data

were fit using the least squares method with Prism 5 software (GraphPad).

**Mammalian Cell Culture**—Human bronchial epithelia (16HBE, American Type Culture Collection) were maintained in DMEM supplemented with FBS (Cellgro), 100 nM selenium methylselenocysteine (Sigma), and penicillin-streptomycin (Cellgro). Cells were plated at a density of  $2.0 \times 10^5$  cells/well on 24-well tissue culture plates (Corning) and allowed to adhere overnight. Viability was determined from lactate dehydrogenase release into the medium 24 h after the beginning of exposure as described previously (24).

**Bacterial Strains and Culture**—*E. coli* AB1157 (ATCC), *P. aeruginosa* strain PAO1 (from the Pseudomonas Genetic Stock Center, East Carolina University) and late clinical isolates of *P. aeruginosa* (from the laboratory of Jane L. Burns, University of Washington) were maintained in lysogeny broth (LB). For viability assay, bacteria were diluted to  $1 \times 10^6$  cfu ml<sup>-1</sup> for treatment and then serially diluted from 1:1 to 1:1000 and plated on LB agar for 30 min. Colonies were counted following overnight incubation at 37 °C to determine viability.

**Thioredoxin Reductase Assay**—75 nM H-TrxR or L-TrxR or 500 μg ml<sup>-1</sup> lysate protein was added to 100 μM NADPH in 100 mM potassium-phosphate buffer pH 7.5 with 1 mM EDTA at room temperature. Reaction was initiated with the addition of 20 μM oxidized *E. coli* Trx (Cayman-IMCO) and 1 mM DTNB and followed based on change in  $A_{412}$  ( $\epsilon = 14,150 \text{ M}^{-1} \text{ cm}^{-1}$ ) (25). If HOSCN had been added to the system, 50 μM reduced glutathione (GSH) was added to the DTNB solution beforehand (forming 2-nitro-5-thiobenzoate (NTB)) to quench any remaining HOSCN.

**Preparation of Cell Lysates**—Cells were pelleted, resuspended in PBS, sonicated for 5 s, and placed at -20 °C overnight. The solution was thawed, sonicated for 5 s, vortexed, and centrifuged at  $2,000 \times g$  to pellet debris. The supernatant was centrifuged at  $14,000 \times g$  in a 10-kDa cutoff filter. Protein was measured by Coomassie Blue stain, and the preparation was used immediately.

**Generation of Hypothiocyanous Acid**—HOSCN was generated as described previously with modifications (5). Briefly, 6.5 mM NaSCN and 60 units ml<sup>-1</sup> LPO (EMD-Millipore) were added to  $5 \times 1 \text{ mM}$  aliquots of H<sub>2</sub>O<sub>2</sub> in 10 mM PBS, pH 7.4, over 5 min followed by the addition of 100 units ml<sup>-1</sup> catalase. The solution was centrifuged at  $14,000 \times g$  for 5 min at 4 °C in a 10-kDa cutoff filter to remove proteins and diluted in a known concentration of NTB. HOSCN was determined by the loss of signal at  $A_{412}$  ( $\epsilon = 14,150 \text{ M}^{-1} \text{ cm}^{-1}$ ) (25) and immediately used. This yielded 2 mM HOSCN that was stable on ice for at least 30 min.

**HOSCN Reductase Assay**—75 nM H-TrxR, L-TrxR, or GR or 500 μg ml<sup>-1</sup> lysate protein was added to 100 μM NADPH in PBS at room temperature. Reaction was initiated with the addition of HOSCN and followed based on change in  $A_{340}$  ( $\epsilon = 6220 \text{ M}^{-1} \text{ cm}^{-1}$ ) (26).

**Oxidase-Peroxidase-coupled System**—16HBE, *E. coli* and *P. aeruginosa* were exposed to an inflammation-mimicking enzyme system containing 6 mM glucose, 50 million units ml<sup>-1</sup> glucose oxidase, 5 units ml<sup>-1</sup> LPO or MPO (EMD-Millipore), and/or 400 μM SCN in 10 mM PBS (pH 6.8, to mimic secretions

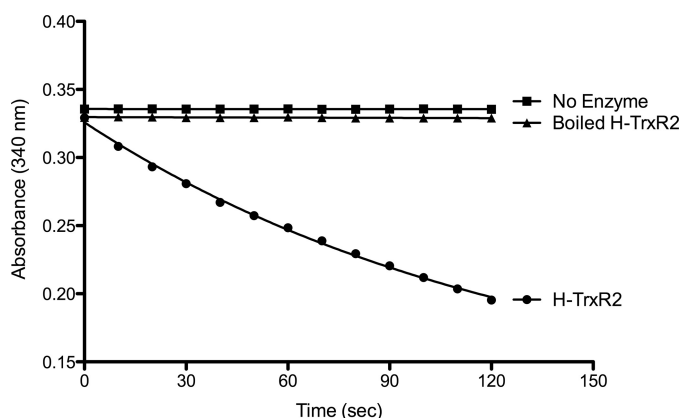


FIGURE 1. **Mammalian H-TrxR catalytically reduces HOSCN.** 75 nM H-TrxR2 or boiled H-TrxR2 or no enzyme was added to 100  $\mu\text{M}$  NADPH and 100  $\mu\text{M}$  HOSCN. The figure is representative of data obtained from each wild type H-TrxR enzyme assayed.

(27–29), or 7.4, to mimic plasma) for 2 h before being returned to full medium (16HBE) or plated on LB agar (bacteria). The identities of the hypohalites formed during the experiment was confirmed as described previously (11).

**Auranofin Exposure**—For lysate studies, 0.5, 1, or 2  $\mu\text{M}$  auranofin was incubated with lysate for 10 min at room temperature before proceeding with Trx and/or HOSCN reductase assay. Cultured cells were conditioned in low serum overnight and treated the following day with up to 2  $\mu\text{M}$  auranofin or DMSO in serum-free medium for 1 h for whole cell inhibition studies.

**HOCl**—Hypochlorous acid was purchased from Sigma. Stock concentration was confirmed by  $A_{290}$  in deionized distilled water ( $\epsilon = 350 \text{ M}^{-1} \text{ cm}^{-1}$ ) (17).

**Acute Oxidant Exposure**—Cells were washed in PBS and exposed to 300  $\mu\text{M}$  HOSCN or HOCl in PBS for 20 min. Cells were harvested immediately or given fresh medium with FBS and antibiotics and harvested after 24 h.

**Intracellular Thiol Measurement**—Cells were harvested and lysed on ice by sonication. Lysates were exposed to 1 mM DTNB in 100 mM potassium-phosphate buffer, pH 7.5, with 1 mM EDTA and incubated 5 min at room temperature in the dark. Total absorbance at  $A_{412}$  ( $\epsilon = 14,150 \text{ M}^{-1} \text{ cm}^{-1}$ ) (25) was blanked against lysate-free buffer and normalized to cell number.

**Statistics**—Graphical and tabular data are expressed as means  $\pm$  S.E. or  $\text{IC}_{50}$  and its 95% confidence interval. Prism 5 (GraphPad) was used to perform and evaluate one-way analysis of variance with Tukey's post test or parametric  $t$  test with variance testing. A  $p$  value of  $p < 0.05$  was considered significant.

## RESULTS

We assayed purified mammalian H-TrxR for reductase activity upon the addition of HOSCN. Mammalian H-TrxR catalytically reduced HOSCN with a physiologically relevant  $K_m$  in an NADPH-dependent manner (Fig. 1, Table 1). Cytosolic H-TrxR1, mitochondrial H-TrxR2, and a purified mixture of both enzymes each displayed similar saturation kinetics for the HOSCN substrate (mean  $K_m = 31.9 \pm 10.3 \mu\text{M}$ ). Furthermore, lysates of the human bronchiolar epithelial cell line (16HBE) were assayed for HOSCN activity, and we observed a similar

**TABLE 1**  
Kinetic parameters of HOSCN reductase activity

Enzyme	$V_0$ at 250 $\mu\text{M}$ $\mu\text{M min}^{-1}$	$k_{\text{cat}}$ $\text{min}^{-1}$	$K_m$ $\mu\text{M}$
H-TrxR1	$26.5 \pm 3.0$	$349.3 \pm 23.1$	$36.9 \pm 17.7$
H-TrxR2	$27.6 \pm 1.4$	$388.3 \pm 14.9$	$46.7 \pm 13.3$
H-TrxR1/2 <sup>a</sup>	$23.0 \pm 2.2$	$333.3 \pm 44.6$	$12.2 \pm 10.8$
H-TrxR2 (Sec489Cys)	$18.5 \pm 1.0$	NA <sup>b</sup>	NA <sup>b</sup>
H-TrxR2 ( $\Delta 8$ )	$16.3 \pm 0.8$	NA <sup>b</sup>	NA <sup>b</sup>
GR	$3.4 \pm 0.2$	$49.7 \pm 4.10$	$165.7 \pm 34.7$
L-TrxR	$0.03 \pm 0.03$	ND <sup>c</sup>	ND <sup>c</sup>
Boiled H-TrxR	$0.18 \pm 0.14$	ND <sup>c</sup>	ND <sup>c</sup>
Blank	$0.01 \pm 0.01$	ND <sup>c</sup>	ND <sup>c</sup>

<sup>a</sup> Purified from rat liver.

<sup>b</sup> NA, not applicable; saturation kinetics were not observed.

<sup>c</sup> ND, no activity detected.

apparent  $K_m$  of  $30.8 \pm 10.4 \mu\text{M}$ . Mutant Sec489Cys H-TrxR2 or mutant H-TrxR2 lacking the eight final peptides reduced HOSCN less rapidly than WT and was not saturated at concentrations of HOSCN up to 250  $\mu\text{M}$ , underscoring the importance of the Sec residue in the HOSCN reductase activity of mammalian H-TrxR (Fig. 2A). GR, an H-TrxR homologue lacking the Sec-expressing C-terminal redox motif, was also a poor HOSCN reductase as compared with H-TrxR (Table 1).

We also assayed H-TrxR and GR for reductase activity using the related oxidant HOCl, but no reductase activity was observed. Instead, HOCl rapidly oxidized NADPH independent of the enzymes, and boiling them prior to assay did not blunt the additional NADPH oxidation rates that were observed. This strongly suggests that although H-TrxR and GR are able to metabolize HOSCN, they cannot enzymatically reduce HOCl.

To determine whether oxidative inactivation of H-TrxR or GR occurs over the course of HOSCN metabolism, the enzymes were repeatedly exposed to high concentrations of HOSCN in the presence of NADPH. However, HOSCN reductase activity of H-TrxR and GR was not affected after three consecutive exposures to 150  $\mu\text{M}$  HOSCN over 30 min at room temperature, suggesting that the catalytic activity of the enzymes protects them from terminal oxidation. Activity after the third HOSCN exposure was  $103 \pm 4\%$  for H-TrxR and  $102 \pm 2\%$  for GR. We also assayed the Trx reductase activity of H-TrxR after HOSCN exposure and similarly found no significant inhibition of the activity of the enzyme after exposure to HOSCN (Fig. 2B).

We then tested whether a purified prokaryotic L-TrxR from Gram-negative bacteria was also capable of catalytically reducing HOSCN. Recombinant L-TrxR from *E. coli* was assayed for HOSCN reductase activity, but none was detected. Furthermore, the Trx reductase activity of L-TrxR was inhibited after 5 min of exposure to HOSCN in the presence of NADPH ( $\text{IC}_{50}$ , 2.75; 95% confidence interval, 2.23–3.52  $\mu\text{M}$ ) (Fig. 2B). These data indicate that L-TrxR is not competent to metabolize HOSCN and that its other catalytic functions are directly inhibited by HOSCN exposure.

To check whether another system may exist in bacteria that can reduce HOSCN, laboratory strains of *P. aeruginosa* (PAO1) and *E. coli* (AB1157) and six late disease antibiotic-resistant clinical isolates of *P. aeruginosa* from CF patients (30) were screened for HOSCN reductase activity. No activity was observed in either of the laboratory strains, whereas three of the

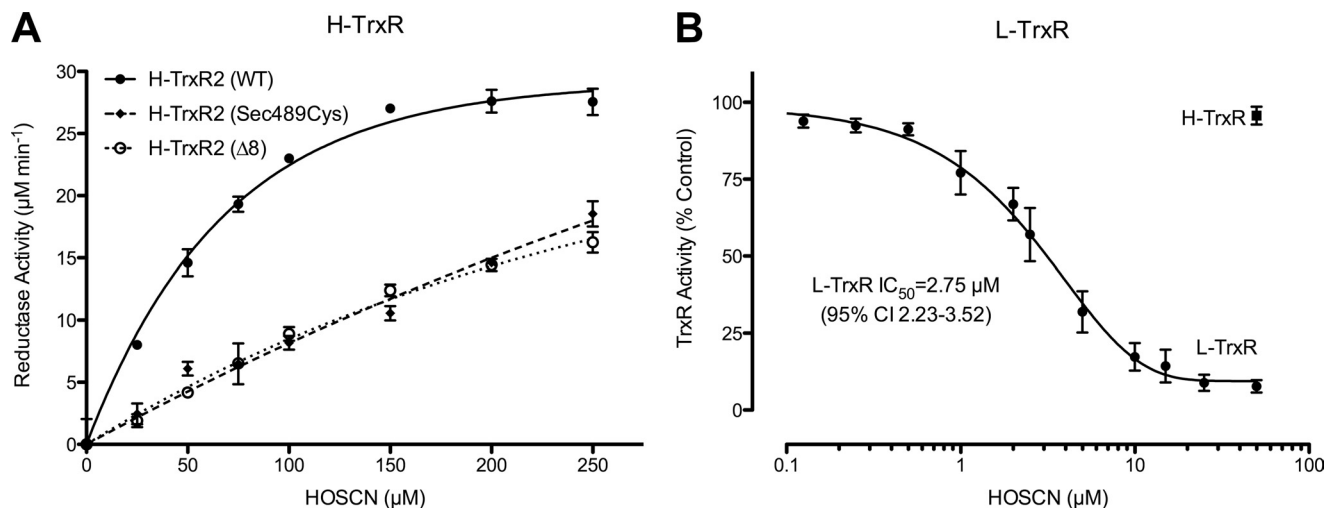


FIGURE 2. **Selective effects of HOSCN on TrxR.** Mammalian H-TrxR metabolizes HOSCN in a Sec-dependent manner, whereas L-TrxR is inhibited by HOSCN. A, 75 nM mouse wild type H-TrxR2 (open circles), Sec489Cys H-TrxR2 (diamonds), or H-TrxR2 lacking the final eight C-terminal peptides ( $\Delta 8$ , closed circles), 80  $\mu\text{M}$  NADPH, and increasing concentrations of HOSCN were used to determine enzyme activity by following consumption of NADPH. Recombinant *E. coli* L-TrxR lacks any detectable activity under these conditions using either NADPH or NADH. B, 75 nM recombinant *E. coli* L-TrxR (circles) or mouse wild type H-TrxR2 (square) and 80  $\mu\text{M}$  NADPH were incubated with increasing HOSCN concentrations for 5 min before the addition of 20  $\mu\text{M}$  *E. coli* Trx, 1 mM DTNB, and 50  $\mu\text{M}$  NTB to quench leftover HOSCN that would interfere with detection of NTB reduction by Trx. The  $\text{IC}_{50}$  and its 95% confidence interval were calculated from curve fitted data.

TABLE 2

Viability after HOSCN exposure and HOSCN reductase activity

Strain	Viability	Apparent $V_{\text{max}}$
<i>E. coli</i> : AB1157	0	ND <sup>a</sup>
<i>P. aeruginosa</i> : PAO1	0	ND <sup>a</sup>
<i>P. aeruginosa</i> : AMT0027L <sup>b</sup>	0	ND <sup>a</sup>
<i>P. aeruginosa</i> : AMT0105L <sup>b</sup>	0	ND <sup>a</sup>
<i>P. aeruginosa</i> : AMT0294L <sup>b</sup>	0	ND <sup>a</sup>
<i>P. aeruginosa</i> : AMT0009L <sup>b</sup>	4.2 $\pm$ 2.1	0.8 $\pm$ 0.3
<i>P. aeruginosa</i> : AMT0058L <sup>b</sup>	0.7 $\pm$ 0.4	1.2 $\pm$ 0.2
<i>P. aeruginosa</i> : AMT0145L <sup>b</sup>	24.8 $\pm$ 2.5	1.4 $\pm$ 0.1
<i>Homo sapiens</i> : 16HBE <sup>c</sup>	96.3 $\pm$ 1.6	14.7 $\pm$ 1.0

<sup>a</sup> ND, no activity detected. Limit of detection = 0.5 nmol  $\text{mg}^{-1} \text{min}^{-1}$ .

<sup>b</sup> Late disease antibiotic-resistant clinical isolate from cystic fibrosis patients (34).

<sup>c</sup> Human bronchial epithelial (16HBE) cells provided for comparison.

clinical isolates of *P. aeruginosa* tested were observed to have slight but detectable HOSCN reductase activity (Table 2), indicating that some infectious bacteria may have an unidentified means of metabolizing HOSCN, but activities were an order of magnitude below the activity detected in 16HBE lysates.

To test whether either HOSCN or HOCl exposure was toxic to whole cells of mammalian or bacterial origin, 16HBE, *P. aeruginosa*, and *E. coli* were exposed to an inflammation-mimicking haloperoxidase-coupled enzyme system that generates a steady-state exposure of HOSCN or HOCl (120  $\mu\text{M h}^{-1}$ ) in the presence of LPO or MPO (LPO+SCN: 119  $\pm$  21  $\mu\text{M h}^{-1}$  HOSCN; MPO: 121  $\pm$  12  $\mu\text{M h}^{-1}$  HOCl; MPO+SCN: 121  $\pm$  21  $\mu\text{M h}^{-1}$  HOSCN). HOSCN was the only detectable product of MPO and SCN in PBS, accounting for >99% of  $\text{H}_2\text{O}_2$  consumed. Sustained exposure to HOCl was toxic to bacteria and 16HBE cells, but HOSCN selectively produced toxicity only in the bacteria (Fig. 3, Table 2). The enzyme system was tested on cells at both pH 6.8, mimicking secreted fluids such as saliva and airway epithelial lining fluid (27–29), and pH 7.4, mimicking plasma. HOSCN was most effective at the secretion-mimicking pH 6.8, where it totally blocked colony formation in most bacterial strains (Fig. 3, Table 2). Interestingly, three CF clinical

strains of *P. aeruginosa* that had shown small amounts of HOSCN metabolism were also more resistant to HOSCN-mediated loss of viability, particularly AMT0145L (Table 2). In contrast with HOSCN, HOCl not only eliminated colony formation but also produced significant toxicity in 16HBE at both pH values, highlighting its potency and lack of selectivity as an oxidant (Fig. 3).

Auranofin is a selective inhibitor of mammalian H-TrxR (31). We used auranofin to assess the role of mammalian H-TrxR in whole cell sensitivity to HOSCN. First, 16HBE lysates were treated with 0.5, 1, or 2  $\mu\text{M}$  auranofin or vehicle (DMSO) for 10 min at room temperature and assayed for HOSCN reductase activity. Auranofin treatment reduced apparent  $V_{\text{max}}$  up to 60.7  $\pm$  3.8%, demonstrating that mammalian H-TrxR is directly involved in HOSCN metabolism and must be redox-active to function (Fig. 4A). No effect was observed on apparent  $K_m$ , which is consistent with a decrease in active H-TrxR concentration following auranofin treatment (32). Next, 16HBE cells were treated with auranofin or vehicle in serum-free medium for 1 h, and H-TrxR inhibition was confirmed at up to 62.4  $\pm$  5.3% after treatment by Trx reduction assay (Fig. 4B). Cells were then given an acute exposure of 300  $\mu\text{M}$  HOSCN or HOCl for 20 min. Auranofin treatment significantly increased intracellular thiol oxidation in HOSCN-exposed cells but did not affect the control or HOCl groups (Fig. 4C). Similarly, cytotoxicity after 24 h was minimal with HOSCN given after vehicle treatment but was greatly potentiated when HOSCN was given after auranofin treatment. The effects of HOCl on intracellular thiol concentration and cytotoxicity were independent of auranofin treatment (Fig. 4D).

## DISCUSSION

The conserved active sequence of H-TrxR and GR (CVN-VGC) located in the FAD-binding domain (21) is likely to be responsible for ultimate catalytic reduction of HOSCN, demonstrated by the activity of GR, but the addition of the C-termi-

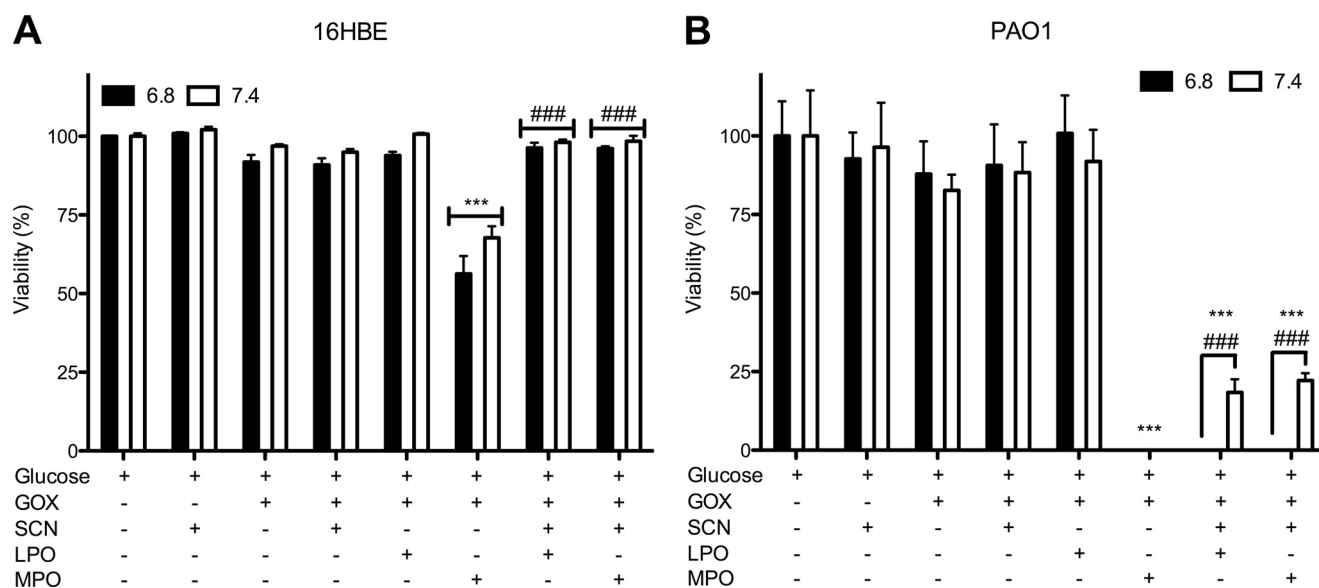


FIGURE 3. **Selective toxicity of HOSCN.** Cells were exposed to 6 mM glucose, 50 milliunits  $\text{ml}^{-1}$  glucose oxidase (GOX), 5 units  $\text{ml}^{-1}$  MPO or LPO, and 400  $\mu\text{M}$  SCN for 2 h in PBS (black bars, pH = 6.8; white bars, pH = 7.4) at 37 °C. This system produces 120  $\mu\text{M h}^{-1}$  of HOSCN (glucose oxidase, LPO, or MPO and SCN) or HOCl (glucose oxidase and MPO without SCN). A, 16HBE cells were given fresh medium after exposure and assessed for viability by lactate dehydrogenase release relative to control 22 h after oxidant exposure. B, *P. aeruginosa* (PAO1) cultures were given 1 unit  $\text{ml}^{-1}$  catalase after exposure and serially diluted on LB agar overnight for cfu viability determinations, normalized to the control group. \*\*\*,  $p < 0.001$  as compared with control. ###,  $p < 0.001$  as compared with MPO without SCN treatment or comparing the same group for two different pH values.

nal redox motif of mammalian H-TrxR appears to greatly enhance the enzyme's  $k_{\text{cat}}$  and  $K_m$  for HOSCN in a Sec-dependent manner, further demonstrated by the dramatic decrease in rate and affinity for HOSCN reduction in the C-terminal mutant enzymes. Thus Sec should enhance HOSCN metabolism at physiologically relevant concentrations (normally below 100  $\mu\text{M}$  in the secretory environment (12)). By contrast, GR is a capable but relatively poor HOSCN reductase and is not likely to function in this role *in vivo*. HOSCN has been shown to react much more rapidly with strongly acidic thiolates than with their weaker counterparts (e.g. NTB versus GSH) (5) and also reacts rapidly with nucleophilic selenols such as Sec, which are ionized at physiologic pH (33). The high nucleophilicity of Sec confers chemoselective reactivity to the reaction of HOSCN with mammalian H-TrxR providing a means for its selective metabolism. However, during inflammation, HOSCN may accumulate in large enough concentrations that some of it will react with less reactive thiol targets as well, which is a possible mechanism by which the cell may "sense" and react to inflammation in the extracellular space (34).

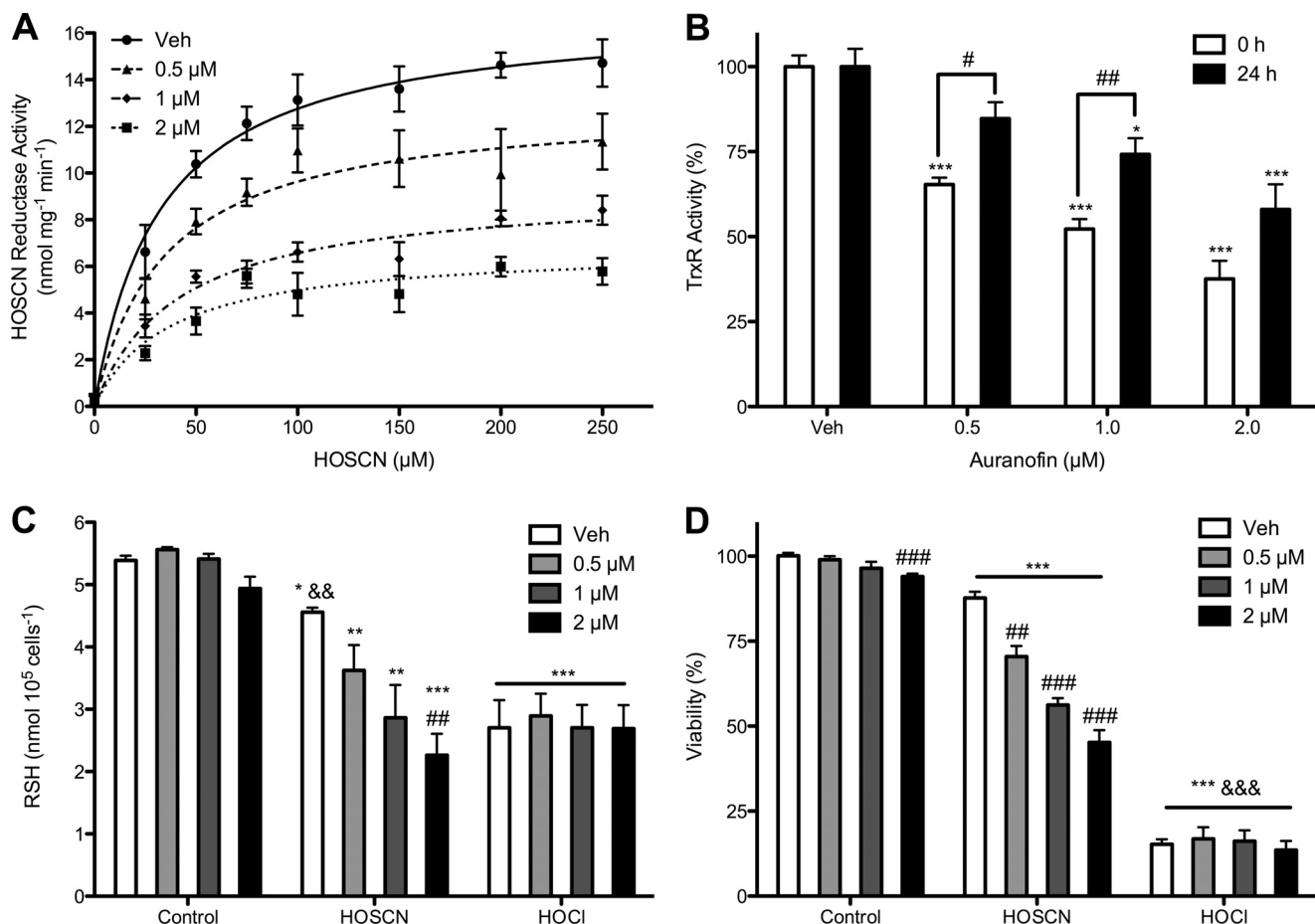
Although HOSCN is strongly acidic and reacts exclusively with thiolates and selenolates in biologic systems, HOCl is a milder acid with greater reduction potential ( $\text{p}K_a = 7.53$ ,  $E'_{\text{pH}7} = +1280$  mV for HOCl versus  $\text{p}K_a = 4.85$ ,  $E'_{\text{pH}7} = +560$  mV for HOSCN (35)) that reacts rapidly with thiols, selenols, amines (16), and phenyl rings (36) and can also undergo Fenton chemistry with the potential to generate hydroxyl radicals (13). Further, we observed no direct NADPH oxidation by HOSCN but did observe this feature in HOCl. This highly reactive chemistry makes HOCl a poor candidate for selective reaction with or even translocation to (37) intracellular H-TrxR for catalytic reduction, whereas HOSCN, as a much more selective oxidant, can more readily traffic to and react with H-TrxR.

We found no significant inhibition of H-TrxR by HOSCN exposure after 5 min of incubation with NADPH, although HOSCN had no other targets to react with in this system. This finding is in conflict with the observations of Skaff *et al.* (18). However, their experiments did not include the NADPH co-factor initially, which gives H-TrxR no means of reducing its substrate. This could result in terminal oxidation reactions of selenium and sulfur that would not normally occur *in vivo* or even just in the presence of co-factor. When NADPH is included, we have not seen evidence of H-TrxR inhibition by HOSCN.<sup>3</sup>

L-TrxR is evolutionarily divergent from H-TrxR, lacking Sec, differing in active sequence (CATC) and active site location (NADPH-binding domain), and having only half as many redox-active moieties per monomer (21). HOSCN is a potent oxidizer of catalytic thiols and appears to oxidatively inactivate L-TrxR due to the inability of the enzyme to turn over HOSCN, unlike the case of H-TrxR or GR. Previously, HOSCN had been shown to potently inhibit the activity of bacterial hexokinase (38), and our research indicates that L-TrxR may be another important antimicrobial target of HOSCN. L-TrxR inhibition hampers bacterial growth and reproduction due to the important role it plays in cell replication through Trx-mediated reduction of ribonucleotide reductase required for *de novo* deoxyribonucleotide synthesis (39). Interestingly, antibacterial effects of L-TrxR inhibition have also been observed with ebselen treatment (40). The L-TrxR gene is generally well conserved across Gram-negative bacteria, suggesting that this mechanism is not likely to vary much across those species (e.g. *E. coli* and *P. aeruginosa* share 69% identity (see UniProt database)).

<sup>3</sup> G. W. Snider and R. J. Hondal, unpublished data.

## Mammalian H-TrxR Metabolizes HOSCN



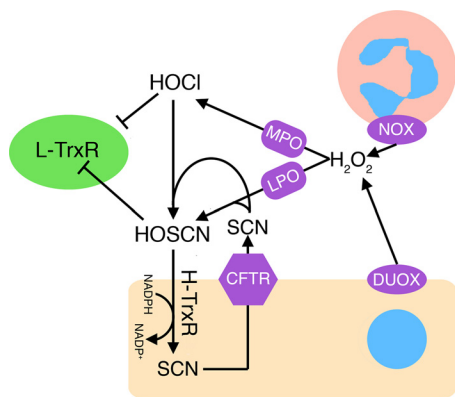
**FIGURE 4. Inhibition of mammalian H-TrxR with auranofin inhibits HOSCN metabolism and sensitizes mammalian cells to HOSCN-mediated toxicity.** *A*, 16HBE lysate (500 μg ml<sup>-1</sup> protein) was treated with DMSO (circles) or 0.5 (triangles), 1 (diamonds), or 2 μM auranofin (squares) for 10 min before adding increasing concentrations of HOSCN. HOSCN reductase activity was determined spectrophotometrically. *B*, 16HBE cells were treated with DMSO (vehicle) or 0.5, 1, or 2 μM auranofin for 1 h in serum-free medium before replacement with fresh medium, and TrxR activity was assessed immediately (0 h) or 24 h later. *C*, after treatment with vehicle (Veh) or auranofin as in *B*, cells were exposed to 300 μM HOCl or HOSCN for 20 min. Intracellular levels of reduced thiols were immediately measured in fresh lysates with the addition of 1 mM DTNB and quantitated spectrophotometrically. *D*, viability was assayed by lactate dehydrogenase release 24 h after the same oxidant exposure as in *C*. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  as compared with control or between indicated groups. #,  $p < 0.05$ , ##,  $p < 0.01$ , ###,  $p < 0.001$  as compared with vehicle or between indicated groups. &&,  $p < 0.01$ , &&&,  $p < 0.001$  between HOSCN and HOCl for indicated dose of auranofin.

Some oral and lactic streptococci have been identified that are capable of HOSCN reductase activity (38). Commensal bacteria that live in SCN-rich environments such as these may create a selective pressure against colonization by HOSCN-sensitive transient microbes. Conversely, we have observed that some clinical isolates of *P. aeruginosa* may adaptively develop HOSCN reductase activity of their own, however slight. The identity of the bacterial HOSCN reductase(s) is not clear at the time of this writing, although the homology of GR (which is much better conserved between mammals and bacteria than thioredoxin reductase (21)) with H-TrxR, and our discovery that it can also turn over HOSCN, albeit less effectively than H-TrxR (Table 1), suggests it is a potential candidate for this role. P. H. Courtois and M. Pourtois (42) reported an NAD(P)H-dependent HOSCN oxidoreductase in *Streptococcus sanguinis* that has not been further characterized to our knowledge.

We have demonstrated selective toxicity to bacteria and resistance by the bronchiolar epithelial cell line 16HBE using the same conditions of HOSCN exposure. In contrast, HOCl causes significant injury in both bacteria and 16HBE cells. This

provides strong rationale for the selectivity of mammalian haloperoxidases for SCN over Cl<sup>-</sup> despite the great excess of Cl<sup>-</sup> relative to SCN in all bodily fluids. The specificity constants of MPO and eosinophil peroxidase for SCN are 730- and 2,780-fold, respectively, greater than for Cl<sup>-</sup>, so relatively small increases in SCN dramatically affect the ratio of HOCl to HOSCN formation during inflammation (3, 43). The epithelial haloperoxidase LPO is also a poor Cl<sup>-</sup> oxidizer and primarily utilizes SCN (29). In secreted fluids where SCN is concentrated up to 100-fold over the plasma, HOSCN is likely to dominate H<sub>2</sub>O<sub>2</sub> consumption by halogenation. Because SCN originates from the epithelium, there may be a gradient of increasing SCN with proximity to secretory cells, limiting oxidative stress by scavenging HOCl. However, neutrophils can release MPO bound to neutrophil extracellular traps (44) and thus may enable bactericidal HOCl formation safely away from the epithelium.

CF lung disease is characterized by chronic airway infection with *P. aeruginosa*, resulting in chronic inflammation and lung destruction. The disease is hereditary, resulting from the absence or dysfunction of the cystic fibrosis transmembrane



**FIGURE 5. Selective metabolism of HOSCN by secretory epithelium and host defense implications.** MPO and LPO generate HOSCN from apically exported SCN and  $H_2O_2$  from phagocytic NADPH oxidase (NOX) or epithelial dual oxidase (DUOX). HOCl generated from MPO reacts directly with SCN to form HOSCN as well. HOSCN is reduced back to SCN by mammalian H-TrxR, but not by bacterial L-TrxR, which is a target of the antimicrobial effects of HOSCN. Recycled SCN in mammalian epithelium can be resecreted through apical anion transporter proteins such as the CFTR.

conductance regulator (CFTR) protein (45). Interestingly, CFTR is also a major characterized apical transporter of SCN in the airway (46). Some studies have found decreased SCN in CF secretions and positively correlated SCN levels with lung function (17, 41, 47). Because even small changes in SCN concentration can dramatically affect the oxidant production of MPO and other haloperoxidases (3), deficient apical SCN transport could dramatically change the outcomes of infection and inflammation in the airway by shifting toward HOCl production, resulting in worse tissue inflammation and injury. The discovery of low levels of HOSCN turnover in the lysates of late clinical isolates of *P. aeruginosa* that have colonized CF patients for several years (Table 2) suggests that HOSCN may still be utilized in CF host defense, although it does not demonstrate whether the system is fully intact. It is tempting to speculate whether the potential dysregulation of SCN in the CF airway may aid early bacterial colonization and the development of bacterial resistance to HOSCN-mediated killing.

We observed that auranofin treatment of 16HBE lysates and whole cells dramatically decreased HOSCN reductase activity concurrent with a decrease in the Trx reductase activity of H-TrxR and resulted in enhanced toxicity after acute HOSCN exposure. These data suggest that HOSCN penetrates cells and can be rapidly turned over by H-TrxR, sparing intracellular thiols and preventing cytotoxicity. When HOSCN metabolism by H-TrxR is impaired, thiol oxidation and toxicity significantly increase. Some HOSCN-mediated thiol oxidation occurred without auranofin that was not highly toxic, demonstrating that HOSCN may react with sensitive thiol targets in addition to H-TrxR even in healthy cells and that an oxidative threshold needs to be crossed for HOSCN to elicit major acute damage. Although HOCl toxicity was auranofin-independent, it remained greater than HOSCN toxicity even after maximal auranofin treatment and equal intracellular thiol oxidation. This was likely due to the additional non-thiol-based mechanisms of HOCl toxicity involving reaction with amines (16), phenyl rings (36), and free radical generation (13).

These findings are the first evidence for the selective metabolism of HOSCN by mammalian H-TrxR and provide a direct mechanism for HOSCN tolerance observed in mammalian cells as well as an evolutionary rationale for mammalian haloperoxidase selectivity for SCN over  $Cl^-$ . Because HOSCN is a selective oxidant and mammalian cells have evolved a way to neutralize it, it makes sense that mammalian biology would favor HOSCN formation over HOCl. H-TrxR would also play a pivotal role in recycling HOSCN to SCN for secretion back into the secretory lumen to replenish the haloperoxidase system. Our data also demonstrate that bacterial L-TrxR is potentially inhibited by HOSCN, providing mechanistic evidence for the antibacterial properties of HOSCN. Therefore HOSCN and its precursor SCN may be crucial molecules in mammalian innate immunity that inhibit invading pathogens while buffering host tissue against injury (Fig. 5).

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