Cyanate-mediated inhibition of neutrophil myeloperoxidase activity

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Cyanate (CNO^-) forms spontaneously in solutions containing urea, and is present in urine and the body fluids of uraemic patients. We have explored the possibility that CNO^- might be one of the unknown substances responsible for the reported impairment, by urine and uraemic plasma, of neutrophil oxidative metabolism (especially as measured by luminol-enhanced chemiluminescence). Luminol-enhanced chemiluminescence generated by human neutrophils derives predominantly from the activity of myeloperoxidase (MPO) which produces hypochlorous acid from H_2O_2 and Cl^- . We hypothesized that CNO^- (which resembles the 'pseudohalide' thiocyanate, an alternative substrate for MPO) might somehow interfere with the activity of MPO. In

support of this, we find: (i) CNO^- inhibits both peroxidative and halogenating activities of MPO and also inhibits the enzyme within intact human neutrophils; (ii) the inhibition is H_2O_2 -dependent, irreversible, accompanied by covalent addition of [^{14}C] CNO^- (or a carbon-containing fragment thereof) to the enzyme; (iii) CNO^- also inhibits $\text{Cl}^-/\text{H}_2\text{O}_2/\text{MPO}$ -mediated bacterial killing. Impairment of this arm of neutrophil bactericidal activity by CNO^- formed from urea may be one factor in the risk of urinary-tract infection associated with urinary stasis and perhaps in the generalized increase in susceptibility to infection in uraemic patients.

INTRODUCTION

Largely uncharacterized substances in the plasma of uraemic patients have been reported to depress neutrophil (PMN) metabolism/respiratory burst (particularly, luminol-enhanced chemiluminescence and bacterial killing) [1–3]. Although the precise substance(s) in uraemic plasma responsible for diminished PMN chemiluminescence and bactericidal activity remain controversial [4,5], it has been repeatedly suggested that the high levels of urea in patients with renal failure might be involved. High concentrations of urea are also present in the urinary bladder, particularly in patients with urinary stasis, a recognized risk factor for urinary-tract infections. It has been speculated that these infections are promoted, in part, by constituents of urine that impair phagocyte activity. One possible inhibitory factor is the hyperosmolality of urine, which may inhibit PMN phagocytosis [6] and oxidative metabolism [7] without affecting PMN viability. However, increased osmolality from inorganic salts may be only partially responsible for inhibition of PMN oxidative activity (particularly as measured by luminol-enhanced chemiluminescence). In fact, Etzioni et al. [8] reported that urea, which can be present in urine in concentrations exceeding 500 mM, is more inhibitory of PMN chemiluminescence than is hyperosmolality caused by inorganic salts.

It occurred to us that the aforementioned inhibitory effects of urea on PMN function might not be due to urea *per se*. Under physiological conditions, cyanate (CNO^-) spontaneously forms from urea and reaches equilibrium in the molar ratio approx. 0.0075:1 (CNO^-/urea) [9]. CNO^- , a nucleophile, reacts with amino and thiol groups of proteins, irreversibly and reversibly,

through carbamoylation reactions [10]. In patients with chronic renal failure, PMN proteins are modified by CNO^- *in vivo*, and it has been suggested that this carbamoylation-mediated protein modification might contribute to PMN dysfunction [11].

CNO^- is chemically similar to thiocyanate (SCN^-), a so-called 'pseudohalide', which can serve as substrate for peroxidases [such as lactoperoxidase, myeloperoxidase (MPO) and eosinophil peroxidase] yielding hypothiocyanous acid (HOSCN) [12]. Therefore we thought that CNO^- also might act as a pseudohalide but that the product of an MPO– CNO^- reaction, if any, might be relatively impotent at bacterial killing and/or might directly inhibit MPO.

The possibility that product(s) of MPO– CNO^- reaction might hinder normal MPO activity was indirectly supported by the previously mentioned decreases, caused by constituents of normal urine, of luminol-enhanced chemiluminescence produced by activated PMNs [18]; luminol-enhanced chemiluminescence reflects primarily MPO generation of hypochlorous acid (HOCl) [13]. The activity of MPO, which normally generates HOCl from Cl^- and H_2O_2 , is important in the bactericidal activity of PMN. Therefore we investigated the effects of CNO^- on MPO activity, PMN oxidative metabolism, and MPO-mediated bactericidal activity. Overall, the results indicate that, particularly at the lower pH possible in urine, physiologically expectable concentrations of CNO^- irreversibly inhibit MPO. This evidently occurs via a mechanism-based H_2O_2 -dependent modification of MPO by CNO^- . Such inhibition may help to explain the lack of effective host killing of pathogens within the urinary tract and perhaps the defective bacterial killing by PMNs from uraemic patients.

Abbreviations used: DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); HBSS, Hanks balanced-salt solution; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OZ, opsonized zymosan; PMNs, polymorphonuclear neutrophils; TNB, 5-thio-2-nitrobenzoic acid.

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MATERIALS AND METHODS

Materials

All reagents, unless otherwise indicated, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Partially purified human MPO with an RZ value (A_{428}/A_{280}) of 0.51 was a gift from Dr. Beulah Gray, University of Minnesota, Minneapolis, MN, U.S.A. The stock concentration was determined spectrophotometrically and calculated using a molar absorption coefficient of $89 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 428 nm. The stock enzyme solution had a concentration of 0.5 mg of MPO/ml or $3.37 \mu\text{mol/l}$ MPO (as tetramer). With guaiacol as substrate (see peroxidase assay below), this enzyme preparation had an activity of 343 units/mg of total protein (1 unit = ΔA_{470} of 1.0/min at pH 7.0 and 25°C in a total volume of 3 ml). This definition of a unit is used throughout this paper. Dextran (3%) in 0.89% NaCl, and Percoll were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Zymosan was washed with, and suspended in, PBS at 20 mg/ml. The zymosan was opsonized by incubation with an equal volume of fresh human serum for 30 min at 37°C with shaking. The opsonized zymosan (OZ) was then washed twice with PBS, suspended at a density of 50 mg/ml and stored at -20°C .

Neutrophil preparation

Human PMNs were obtained from heparinized venous blood of healthy volunteers by the technique of Redl et al. [14]. Briefly, fresh minimally heparinized venous blood was sedimented under unit gravity with 3% dextran in 0.85% NaCl and the supernatant was collected and centrifuged. Contaminating erythrocytes in cell pellets were lysed by brief exposure to ice-cold water after which isotonicity was rapidly restored. PMNs were separated by continuous gradient containing sterile iso-osmotic NaCl and Percoll with a density of 1.075. The PMNs were washed three times in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks balanced-salt solution (HBSS) and resuspended in complete HBSS. The cell preparations usually contained more than 95% PMNs, and cell viability was at least 98% as determined by Trypan Blue exclusion.

Peroxidase assay

Peroxidase activities of MPO and PMN lysates were determined by guaiacol oxidation [15]. Reaction mixtures contained 50 mM sodium phosphate buffer, pH as indicated, 13.5 mM guaiacol and enzyme sources in a total volume of 1 ml. The reactions were initiated by adding $300 \mu\text{M}$ H_2O_2 , and changes in A_{470} were monitored over 3 min at 25°C . Enzyme activity was expressed as the rate of absorbance increase per min.

HOSCN assay

The halogenation activity of MPO was determined by oxidation of the pseudohalide SCN^- . In the presence of SCN^- and H_2O_2 , MPO catalyses the oxidation of SCN^- to yield HOSCN or its conjugate base, hypothiocyanite (OSCN^-) ($\text{pK} \approx 5.3$). HOSCN/ OSCN^- are less reactive with most substrates than are hypochlorous acid/hypochlorite (HOCl/OCl^-) and do not require trapping (such as the use of added taurine to trap HOCl as taurine chloramine). Reactions were carried out as described above in the presence of 10 mM KSCN and in the absence of Cl^- . HOSCN/ OSCN^- accumulation was detected by the method of Aune and Thomas [16]. Briefly, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) was pre-reduced to 5-thio-2-nitrobenzoic acid (TNB) with 2-mercaptoethanol, in a molar ratio of 1:0.9, in 100 mM Na_2PO_4 buffer containing 5 mM EDTA at pH 8.8, then

the pH was adjusted to 7.0 with 100 mM NaH_2PO_4 , pH 4.4. The amounts of TNB oxidized to DTNB by HOSCN/ OSCN^- were determined spectrophotometrically at 412 nm (assuming a molar absorption coefficient of $13600 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Ascorbate oxidation

In the presence of Cl^- and H_2O_2 , MPO generates HOCl which, unlike $\text{O}_2^{\cdot -}$ or H_2O_2 , will oxidize ascorbate. Therefore, in well-defined systems (especially the absence of transition metals), oxidation of ascorbate can be used as a relatively sensitive and continuous indicator of HOCl formation [17]. The reaction mixtures contained 50 mM phosphate, 100 mM NaCl, 0.5 mM EDTA and $100 \mu\text{M}$ sodium ascorbate. The reaction was initiated by adding $300 \mu\text{M}$ H_2O_2 , and ascorbate oxidation was monitored by decrease in A_{266} over 3 min. The millimolar absorption coefficient of ascorbate is $15.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

CNO⁻-mediated inactivation of MPO in intact PMNs

Purified PMNs (0.8×10^6 cells/ml) were suspended in complete HBSS containing 0.2% BSA, pH 7.0. The cells were incubated with the indicated compounds at room temperature (20°C) for 5 min, followed by the addition of 1 mM H_2O_2 , 100 nM PMA or 0.2 mg/ml OZ (final concentrations) to the cell suspensions. The cells were then incubated for 30 min at 37°C and pelleted by centrifugation at $12000 g$ for 1 min. Cell pellets were lysed in 1 ml of 50 mM sodium phosphate, pH 7.0, with 0.2% Triton[®] X-100 and the lysates were sonicated in an ice bath for 1 min to release MPO from granules. Control experiments indicate that this procedure releases more than 95% of total PMN MPO content. We should note that lysates prepared by the addition of cetrimide exhibited somewhat less MPO activity and contained precipitates so that Triton extraction was substituted. For comparison, PMN suspensions with identical treatment without the addition of H_2O_2 or stimuli were incubated, pelleted and lysed under the same conditions. The residual MPO activities in the lysates were determined by guaiacol assay.

Reaction of [¹⁴C]CNO⁻ with MPO

Limited experiments were performed to determine whether CNO^- might engage in an H_2O_2 -dependent covalent reaction with MPO. For this, $20 \mu\text{g}$ of MPO in 50 mM sodium phosphate buffer, pH 7.0, was incubated with $0.5 \mu\text{Ci}$ (10 nmol) of [¹⁴C] CNO^- (provided as KOCN; specific radioactivity $48.3 \mu\text{Ci}/\mu\text{mol}$; New England Nuclear) in the presence or absence of 1 mM H_2O_2 in a total volume of $50 \mu\text{l}$. After a 10 min incubation at 25°C , $50 \mu\text{l}$ of $2 \times$ sample buffer containing 2-mercaptoethanol (10%), SDS and Bromophenol Blue was added and the samples were heated at 95°C for 5 min. A $20 \mu\text{l}$ portion of this preparation was loaded per lane on SDS/polyacrylamide gels (10%) run in a Mini-Protean[®] II apparatus according to a standardized protocol (Bio-Rad Laboratories). The gels were stained for protein with Coomassie Blue and subjected to autoradiography.

Determination of CNO⁻

CNO^- was assayed by reaction with 2-aminobenzoic acid followed by spectrophotometric determination of the product (quinazoline-2,4-dione) as described by Guilloton and Karst [18]. Briefly, CNO^- -containing solutions were allowed to react with an excess of 2-aminobenzoic acid in 50 mM phosphate

buffer, pH 4.4, at 40 °C for 10 min. After brief cooling to room temperature, an equal volume of 12 M HCl was added and the solutions were placed in a boiling water bath for 1 min. The resulting product, quinazoline-2,4-dione, was measured spectrophotometrically at 310 nm and calculated assuming a molar absorption coefficient of $3.56 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (which was confirmed by a standard curve made with reagent KOCN).

Neutrophil-derived H_2O_2 and HOCl production

Purified PMNs were suspended in complete HBSS buffered with added 10 mM sodium phosphate, pH 7.4. For H_2O_2 assays, 1 mM NaN_3 was added to 0.5×10^6 cells/ml (final volume 1 ml) to inhibit endogenous catalase and peroxidase activities. The cells were stimulated by addition of 100 nM PMA in the presence or absence of KOCN (added 5 min beforehand) and incubated at 37 °C for 1 h. Under these conditions, PMN generation of H_2O_2 is known to be sustained for as long as 2 h [19]. The accumulated H_2O_2 was determined by ferrithiocyanate as described previously [19], with minor modifications [20]. Briefly, 200 μl of 50 % ice-cold trichloroacetic acid was added to 1 ml of PMN suspensions and the proteins sedimented by brief (5 min) centrifugation at 10000 g. Freshly prepared ferrous ammonium sulphate (100 μl of a 20 mM stock) was added to 800 μl of the supernatant, followed by 100 μl of 2.5 M KSCN. Absorbance of the ferrithiocyanate complex was measured spectrophotometrically at 480 nm, and H_2O_2 concentration was calculated by comparison with a standard curve made with reagent H_2O_2 . The reagent H_2O_2 concentration was determined assuming a molar absorption coefficient of $81 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 240 nm [21]. For HOCl determinations, 15 mM taurine was added to 1×10^6 cells/ml to trap HOCl. The accumulated taurine chloramine was determined by reaction with KI and the I_3^- was measured spectrophotometrically at 355 nm [22]. Other conditions were as described for assays of H_2O_2 production.

Bactericidal activity

Staphylococcus aureus, a clinical isolate obtained from the Microbiology Laboratory at Albany Medical College, were maintained on standard blood-agar plates, and suspension cultures were grown in trypticase soy broth for 16 h at 37 °C before use in bactericidal assays. The organisms were washed with complete HBSS three times and resuspended in the same buffer. Density of the organisms was estimated turbidometrically (A_{550} of 1.0 was assumed to equal 8.6×10^8 cells/ml). The diluted bacterial suspensions were pipetted into 96-well plates with a final density of 1×10^7 cells/well. Incubations with various oxidants and MPO were carried out in a total volume of 100 μl /well. All constituents of the incubation mixtures were prepared by dilution in complete HBSS. The plates were incubated at 37 °C for 10 min followed by the addition of 100 μl of trypticase soy broth with 25 $\mu\text{g}/\text{ml}$ catalase to scavenge the residual oxidants. After 30 min, 50 μl of trypticase soy broth containing 25 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and the incubation continued for an additional 90 min at 37 °C. Cumulative MTT reduction, a measure of bacterial viability, was determined spectrophotometrically at 550 nm with a Molecular Devices Thermomax plate reader after lysis of the cells and solubilization of the formazan with 50 μl of a solution containing 10 % SDS, 50 % dimethylformamide and 200 mM acetic acid [23]. The bactericidal activity was expressed as $(A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \times 100$.

RESULTS

In the presence of H_2O_2 , MPO oxidizes halides and pseudo-halides, including Cl^- , Br^- , I^- and SCN^- , to the corresponding hypohalous acids. MPO also causes one-electron peroxidation of numerous electron donors such as guaiacol without the participation of halides. We used the guaiacol assay and an ascorbate-oxidation assay to investigate interactions of CNO^- with MPO and to distinguish the possible effects of CNO^- on the two activities of MPO, namely peroxidation and hypohalous acid production [24].

CNO^- -dependent inhibition of MPO-mediated peroxidation

As shown in Figure 1, at neutral pH, CNO^- inhibits MPO-catalysed guaiacol oxidation. Importantly, when the pH of the reaction buffer (50 mM sodium phosphate) is decreased, the inhibitory potency of CNO^- increases substantially. In the broad range of physiologically possible pH, the IC_{50} values of CNO^- for MPO are approx. 30.4, 63.2, 321.0 and 493.6 μM at pH 5.4, 6.2, 7.0 and 7.8, respectively. [We should mention that, although the optimal pH for the guaiacol assay is around 6, the apparent MPO-mediated reaction rate (in the absence of added CNO^-) decreases slightly from pH 6.2 to pH 7.8.] As detailed below, very similar results were obtained with a qualitatively different assay of MPO halogenating activity (ascorbate oxidation).

We further investigated the influence of the concentration of guaiacol on CNO^- -mediated inhibition of MPO activity. In the presence of a constant concentration of H_2O_2 (300 μM), the IC_{50} of CNO^- decreases from 321 to 131 μM when the guaiacol concentration is lowered from 13.5 to 1.35 mM (Figure 2). Therefore the concentration of peroxidatic substrate affects CNO^- inhibition of MPO, with higher concentrations of the electron donor guaiacol antagonizing CNO^- inhibition. It should be noted that, when up to 1 % (w/v) BSA or 10 mM glycine is included in the assay mixture, the IC_{50} of CNO^- for MPO-catalysed guaiacol oxidation is unaffected. This indirectly supports the idea that CNO^- or its reactive intermediate selectively targets MPO rather than non-specifically reacting with all proteins in the mixture.

Figure 1 Inhibition of MPO by CNO^- at various pH values

The peroxidase activity of MPO in the presence of KOCN was assayed by guaiacol as described in the Materials and methods section. Each assay mixture contained 17 m-units of MPO (as assayed by the standard guaiacol reaction at pH 7.0) in a total volume of 1 ml. Enzyme activity is expressed as percentage of controls (without KOCN) at each pH. The absolute activities of MPO observed at these different pH values were 47×10^{-3} , 55×10^{-3} , 50×10^{-3} and $47 \times 10^{-3} \Delta A/\text{min}$ (in a reaction volume of 1 ml) at pH 5.4, 6.2, 7.0 and 7.8 respectively.

Figure 2 Influence of guaiacol concentration on the inhibition of MPO peroxidative activity by CNO^-

Assay conditions were as in Figure 1 at pH 7.0, except guaiacol concentration was varied.

Figure 3 Effects of CNO^- on MPO-catalysed HOSCN production

The reaction mixtures contained 10 mM KSCN as substrate, 50 m-units of MPO (as assayed by guaiacol) and 0.1% BSA in 50 mM sodium phosphate buffer, pH 7.0 (total volume 1 ml). The reaction was initiated by adding 300 μM H_2O_2 and stopped by adding 50 μg of catalase after 10 min incubation at 20 °C. The accumulated HOSCN/OSCN⁻ was determined by TNB oxidation, as described in the Materials and methods section. HOSCN/OSCN⁻ formation is expressed as percentage of control (without KOCN), which was 36.6 nmol/10 min.

CNO^- -mediated inhibition of hypohalous acid production by MPO

When HOCl production was estimated using taurine chloramine formation, CNO^- was also found to inhibit HOCl production (or perhaps taurine chloramine formation via an enzyme-bound chlorinating intermediate [25]) by MPO with IC_{50} values similar to those observed for guaiacol oxidation (results not shown). We observed that NaOCl can react with CNO^- but does not prevent the formation of taurine chloramine and does not react with the latter. However, as a further test of the inhibitory potency of CNO^- , we took advantage of the fact that HOSCN does not react with CNO^- and employed KSCN as substrate to examine the effects of CNO^- on the halogenation activity of MPO by measuring HOSCN accumulation. As shown in Figure 3, in the presence of 1 mM H_2O_2 and 10 mM KSCN (pH 7.4), CNO^- inhibits MPO-catalysed HOSCN formation. The approximate IC_{50} of CNO^- for MPO under these conditions is about 500 μM , comparable with that obtained with both the guaiacol assay and (not shown) the taurine chloramine assay at this pH. These

Figure 4 Effects of KOCN on MPO/ H_2O_2 /Cl⁻-catalysed ascorbate oxidation

Reaction mixtures contained 100 μM sodium ascorbate, 0.5 mM EDTA, 100 mM NaCl and 50 mM sodium phosphate buffer with pH as indicated. The activities of MPO present were 8.5 m-units and 170 m-units, for pH 5.4 and pH 7.0 samples respectively. The decrease in A_{286} (representing the oxidation of ascorbic acid to dehydroascorbate) was recorded over 3 min, at 20 °C, after addition of H_2O_2 . Enzyme activity was expressed as percentage of control ascorbate oxidation without added KOCN. The MPO/Cl⁻-dependent ascorbate-oxidation rates in the absence of KOCN were about 5 nmol/min at pH 5.4 and 10 nmol/min at pH 7.0. Note that under these conditions (i.e. in the presence of Cl⁻), MPO oxidizes ascorbate 40 times faster at pH 5.4 than at pH 7.0. Therefore, despite the large differences in amounts of MPO added, the actual rates of ascorbate oxidation in the absence of CNO^- were 6.0 and 9.6 nmol/min per ml at pH 5.4 and 7.0 respectively.

results further indicate that concentrations of SCN^- up to 10 mM are unable to competitively block CNO^- -mediated inhibition of MPO. Furthermore CNO^- inhibition of MPO is similar regardless of the alternative substrates employed.

CNO^- also profoundly inhibits Cl⁻-dependent ascorbate oxidation at low concentrations (IC_{50} 8 and 51 μM at pH 5.4 and 7.0 respectively) (Figure 4). Although compound II of MPO can directly oxidize ascorbate without forming other reactive intermediates (e.g. HOCl [26–28]), the rate of ascorbate oxidation is greatly accelerated in the presence of Cl⁻. Such Cl⁻-dependent ascorbate oxidation is due to HOCl formation [17], and therefore reflects MPO-mediated HOCl production. With regard to the apparent inhibition of HOCl production, we are reasonably certain that the CNO^- inhibition of this reaction is not an artifact resulting from the interception of HOCl by CNO^- , under the same experimental conditions and in the presence of both CNO^- and ascorbate, HOCl (10–100 μM) reacts stoichiometrically with ascorbate (i.e. not with CNO^-) even when concentrations of CNO^- are 10-fold higher than ascorbate (100 μM –1 mM). A hypothetical oxidizing CNO^- intermediate, if any is indeed formed by MPO, appears not to react with ascorbate because there is no detectable CNO^- -dependent increase in ascorbate consumption in the presence or absence of Cl⁻.

Finally, when 100 μM CNO^- is present in a system containing relatively large amounts of MPO (340 m-units of MPO, 300 μM H_2O_2 , 50 mM phosphate, pH 7.0, in a total volume of 1 ml with the reaction proceeding for 15 min with or without 100 mM NaCl), there is no significant loss of CNO^- [by direct measurements of residual CNO^- after the incubation (results not shown)]. This indicates that MPO is unable to catalytically consume CNO^- and, under these conditions, CNO^- does not simply behave as an HOCl scavenger. Thus CNO^- effectively inhibits both the peroxidatic and halogenating reactions of MPO and does so with approximately the same IC_{50} .

Table 1 H₂O₂-dependent inhibition of MPO by CNO⁻ in the presence or absence of added glycine, BSA or 2-mercaptoethanol

MPO stock (3.4 units) was incubated with the indicated concentrations (0–5 mM) of CNO⁻ in the absence or presence of 1 mM H₂O₂ and in the absence or presence of 20 mM glycine, 1% (w/v) BSA or the indicated concentrations of 2-mercaptoethanol in 50 mM sodium phosphate buffer, pH 7.0, and a total volume of 100 μ l for 10 min at 20 °C. The samples were then further diluted in phosphate buffer (final dilution factor 1:500) for guaiacol assay of residual MPO activity. Under these conditions, the maximum residual CNO⁻ concentration (10 μ M) is insufficient to cause detectable inhibition of MPO. Results shown are representative of three independent experiments.

Conditions	10 ³ × MPO activity (ΔA /min)			
	0	0.2 mM	1.0 mM	5.0 mM
No H ₂ O ₂	54.7	55.1	52.3	51.5
H ₂ O ₂ (1 mM)	45.1	38.5	26.5	11.7
H ₂ O ₂ (1 mM) + glycine (20 mM)	46.5	39.5	28.7	10.5
H ₂ O ₂ + BSA (1%)	46.4	37.3	23.2	11.1
H ₂ O ₂ + 2-mercaptoethanol (0.5 mM)	55.8	—	19.8	—
H ₂ O ₂ + 2-mercaptoethanol (1.0 mM)	55.2	—	18.0	—

Irreversible inactivation of MPO by CNO⁻

Inhibition of MPO by CNO⁻ appears to be mechanism-based and irreversible. As shown in Table 1, when purified MPO is incubated with up to 5 mM KOCN alone for 10 min, there is little change in MPO activity. Furthermore, in the presence of 1 mM H₂O₂ alone, less than 20 % of MPO activity is lost after 10 min incubation. However, in the presence of both CNO⁻ (1–5 mM) and H₂O₂, up to 80 % of MPO activity is lost after only 10 min incubation. Importantly, the addition of either thiol reducing agents such as 2-mercaptoethanol or large amounts of glycine or albumin does not prevent MPO inactivation by CNO⁻ and H₂O₂ (Table 1). These results indicate that MPO inactivation by CNO⁻ requires H₂O₂ and that such inactivation does not involve reversible thiol oxidation or the formation of a free CNO⁻ intermediate reactive with external protein or free glycine. Moreover, the inactivation appears to be irreversible because the inhibition of MPO activity persists after dilution of samples such that there is only 2–10 μ M residual CNO⁻ present (a concentration that does not affect guaiacol oxidation at pH 7.0). Overall, these results support the idea that CNO⁻ inhibits MPO through a mechanism-based H₂O₂-dependent reaction rather than through simple carbamylation of ϵ -amino or other functional groups of MPO (a reaction that should be unaffected by added H₂O₂).

The precise nature of the inhibitory reaction between MPO and CNO⁻ remains uncertain. However, we do have evidence that CNO⁻ prevents formation of the reactive MPO compound II (FeIV) intermediate. Thus, as shown in Figure 5(A), 'resting' MPO has the expected absorbance maximum at 428 nm which, on addition of H₂O₂, shifts to 454 nm (reflecting the formation of compound II [29,30]). In contrast, in the presence of added CNO⁻, this spectral shift is absent (Figure 5B). (It should be noted that the absorbance shift at 568 → 628 nm was also absent in the presence of CNO⁻.) In the presence of both H₂O₂ and Cl⁻, MPO progressively loses the haem absorption peak at 400–450 nm, probably reflecting HOCl-dependent haem destruction. However, similar spectra recorded in a Cl⁻-containing system in the presence of CNO⁻ displayed no loss of haem absorption (not shown). Overall, these results indicate that CNO⁻ prevents formation of MPO compound II and, in a Cl⁻-containing system, may also prevent the formation of compound I.

Figure 5 Absorbance spectra of MPO under various conditions

All samples contained 3 nmol of MPO in 50 mM sodium phosphate buffer, pH 7.0 (total volume 1 ml). (A) MPO before (---) and after (—) the addition of 300 μ M H₂O₂. Note the shift of absorbance maximum from 428 to 454 nm. (B) MPO alone (---), after the addition of 1 mM KOCN (---) and after sequential additions of 1 mM KOCN and 300 μ M H₂O₂ (—). Note the lack of 428 → 454 nm spectral shift in the latter sample.

Figure 6 Polyacrylamide-gel electrophoretograms of partially purified MPO incubated with [¹⁴C]CNO⁻, with (+) or without (–) 300 μ M H₂O₂ as described in the Materials and methods section

Gels were stained for protein with Coomassie Blue (left) and subjected to autoradiography (right). Molecular masses (kDa) are indicated on the left. Note the distinct labelling of the higher-molecular-mass α -subunit in samples incubated with added H₂O₂.

Finally, the inactivation of MPO by CNO⁻ evidently involves formation of a covalent CNO⁻ adduct with the enzyme. As would be expected, incubation of MPO or albumin with [¹⁴C]CNO⁻ leads to formation of small amounts of covalent adducts via non-oxidative carbamylation. However, in the case of MPO, but not albumin, addition of H₂O₂ causes a substantial increase in protein-associated radioactivity. Interestingly, as shown in Figure 6, it is the larger (haem-containing) α -subunit of MPO that is preferentially labelled by [¹⁴C]CNO, especially during incubations with added H₂O₂.

Figure 7 Inactivation of MPO in intact neutrophils by KOCN

Purified human PMNs (0.8×10^6 /ml) were suspended in complete HBSS with added 10 mM phosphate, pH 7.0, without (open bars) or with 1 mM (hatched bars) or 4 mM (stippled bars) KOCN, and treated with H₂O₂ (1 mM), PMA (100 nM) or OZ (0.2 mg/ml) for 30 min. The cells were pelleted and then lysed in 100 μ l of 50 mM phosphate containing 0.2% Triton X-100. Aliquots of the lysates (10 μ l) were assayed for peroxidase activity by the guaiacol reaction. Each treatment was carried out in quadruplicate, and results are expressed as percentage of activity in cells incubated without any addition (means $\pm$ S.D.). The effect of treatment (none versus 1 mM CNO⁻ versus 4 mM CNO⁻) assessed by analysis of variance was significant for each condition (control $P = 0.049$, H₂O₂ $P < 0.0001$, PMA $P < 0.001$, OZ $P = 0.0067$). A two-way analysis of variance of the effects of CNO⁻ ($P < 0.0001$) and condition (i.e. control, H₂O₂, PMA or OZ) ($P < 0.0001$) on MPO activity also demonstrates highly significant CNO⁻ dose-dependence of MPO inhibition.

Inhibitory effects of CNO⁻ on MPO activity of intact PMNs

When isolated human PMNs are exposed to CNO⁻ for 30 min and then washed, there is little decrease in PMN-associated MPO activity. However, MPO is inactivated when PMNs are exposed to exogenous H₂O₂, or stimulated by PMA or OZ, in the presence of CNO⁻ (Figure 7). Therefore this CNO⁻-mediated inactivation of MPO within intact PMNs evidently requires either exogenous H₂O₂ or active production of H₂O₂ by stimulated PMNs. These observations also imply that PMNs, as well as the azurophilic granules containing MPO, are permeable to exogenous CNO⁻. Previous results from our laboratory [20] indicate that such maximal PMA stimulation of PMNs for 30 min induces release of only about 3% of total intracellular MPO. Therefore MPO inactivation occurs primarily within the azurophilic granules of the intact cells.

On the other hand, in contrast with earlier reports, we find that in PMNs exposed to CNO⁻, PMA-stimulated H₂O₂ production is unaffected. Thus, as shown in Figure 8, even high concentrations of CNO⁻ have no effect on H₂O₂ production by PMA-stimulated PMNs, whereas relatively low concentrations of CNO⁻ effectively block HOCl generation in duplicate samples of the same PMNs. Therefore it would appear that CNO⁻ has little effect on the (O₂⁻/H₂O₂-generating) metabolism of PMA-stimulated PMNs while specifically impairing MPO-dependent HOCl production.

Effects of CNO⁻ on MPO-mediated bactericidal activity

In an attempt to assess the functional importance of CNO⁻-dependent MPO inhibition, we chose *S. aureus* as a target organism because PMN killing of this species is highly dependent on MPO activity [31,32]. When bacterial suspensions are exposed to reagent HOCl for 10 min and then placed in 50% trypticase soy broth (which will consume all residual HOCl), the half lethal dose of HOCl is about 3.8 μ M as measured by MTT reduction,

Figure 8 Effects of KOCN on oxidant production by PMA-stimulated PMNs

PMNs were suspended (at 0.5×10^6 cells/ml and 1.0×10^6 cells/ml for H₂O₂ and HOCl assays respectively) in complete HBSS containing 10 mM added sodium phosphate at pH 7.4. The samples for the HOCl assay also contained 15 mM taurine and those for the H₂O₂ assay contained 1 mM NaN₃. The cells were preincubated with KOCN at the indicated concentrations for 5 min and then stimulated by addition of 100 nM PMA (final concentration). The incubation was carried out for 1 h at 37 °C. The accumulated H₂O₂ and taurine chloramine were determined as described in the Materials and methods section. All samples were in triplicate, and results are expressed as means $\pm$ S.D. Control values for taurine chloramine accumulation were 21.4 ± 4.9 nmol/h per 10^6 PMNs and for H₂O₂ they were 88 ± 4.6 nmol/h per 10^6 PMNs.

whereas even 1000-fold higher concentrations of H₂O₂ cause no detectable killing. Important to the following experiments, when bacterial suspensions are challenged with HOCl in the presence of 0.125–2 mM CNO⁻ the half lethal dose of HOCl is unaffected, indicating that these low levels of CNO⁻ will not directly scavenge significant amounts of preformed HOCl.

As shown in Figure 9, when bacteria are exposed to a non-toxic concentration of H₂O₂ (100 μ M) in the presence of added MPO (17 m-units/100 μ l), more than 50% of the organisms are killed. However, when increasing concentrations of CNO⁻ are added before the addition of H₂O₂, MPO-mediated bactericidal activity is greatly reduced. The inhibitory effect of CNO⁻ is evident at CNO⁻ concentrations as low as 60 μ M. Under these

Figure 9 Effects of KOCN on bacterial killing by MPO/H₂O₂/Cl⁻ system

MPO (50 ng or 17 m-units/microtitre well) was added to suspensions of *S. aureus* (1×10^7 cells/well) in complete HBSS and incubated with KOCN for 5 min. The reaction was initiated by adding H₂O₂ and allowed to proceed for 10 min at 20 °C. The reaction was quenched by addition of trypticase soy broth containing catalase, and bacterial viability was assessed by measurement of MTT reduction as described in the Materials and methods section.

conditions, the IC_{50} of CNO^- for inhibition of bacterial killing is approx. 700 μM , comparable with the IC_{50} for CNO^- inhibition of MPO activity *per se* at pH 7.0. Considering that the amount of MPO in the assay system (17 m-units in a 0.1 ml volume) is sufficient to consume all H_2O_2 within 1–2 min, we speculate that the actual IC_{50} of CNO^- for MPO-mediated bactericidal activity should be even lower *in vivo*.

DISCUSSION

Two situations in which high levels of urea are present, within the urinary bladder especially during prolonged urinary stasis and in uraemia, are characterized by increases in susceptibility to bacterial infections. Whereas many predisposing factors are probably involved, in both these situations impaired phagocyte functions (especially diminished PMN oxidative activities and bacterial killing) have been invoked. As reviewed at the outset, the high osmolality of urine may inhibit PMN oxidative activity (especially as reflected by luminol-enhanced chemiluminescence), but the increased osmolality from inorganic salts *per se* may be of lesser importance than that contributed by urea [8]. Similar correlations between plasma urea concentrations and defective PMN oxidative activities/bacterial killing have been detected in patients with uraemia.

CNO^- : a possible urea-derived inhibitor of bacterial killing

It was speculated earlier that cyanic acid ($H-O-C\equiv N$) or its predominant isoform isocyanic acid ($H-N=C=O$), breakdown products of urea, might play a role in the intoxication of uraemia [33]. In aqueous solution, urea undergoes reversible conversion into CNO^- and NH_3 . At body temperature and physiological pH, about 0.5–0.75 % of urea (in a 1 M urea solution) would be present as CNO^- at equilibrium [9]. There is good evidence, albeit much of it indirect, that the expected elevations in CNO^- do occur in body fluids of uraemic humans and animals and in concentrated urine. Elevated plasma CNO^- concentrations are present in nephrectomized rabbits [34]. CNO^- , being nucleophilic, can also be detected via its reaction products with proteins. CNO^- reacts with α -amino, ϵ -amino, thiol and hydroxy moieties of amino acids, peptides and proteins [10]. Such carbamoylation reactions are known to inactivate enzymes [10,35] and alter protein stability [36–38]. Important to the present work, carbamoylated haemoglobin, presumably formed by reaction with urea-derived CNO^- , has been found in patients with renal failure [39,40]. In addition, Kraus et al. [11] have observed that PMN proteins from uraemic patients are modified by CNO^- *in vivo*.

CNO^- irreversibly inhibits PMN MPO activity

Because CNO^- is chemically related to thiocyanate (SCN^-), a known pseudohalide and preferred substrate for eosinophil peroxidase, lactoperoxidase and salivary peroxidase, we studied the interaction of CNO^- with MPO. We find that, in the presence of H_2O_2 , CNO^- strongly inhibits MPO activities (both peroxidative and halogenating).

Both H_2O_2 and $HOCl$ will inactivate MPO under certain conditions [41]. In the absence of halide, low concentrations of H_2O_2 convert native MPO into compound II, which can be reactivated by reducing agents. In the presence of Cl^- , however, the enzyme may be inactivated irreversibly by its own product, $HOCl$, through destruction of the haem moiety (evident from the loss of haem absorption at 400–450 nm) as well as through the oxidation of critical amino acid residues. We find that MPO, in

both Cl^- -free and Cl^- -containing systems, is rapidly inactivated by CNO^- . In fact, CNO^- causes almost instant inhibition of luminol-enhanced chemiluminescence in systems containing MPO/ H_2O_2 / Cl^- (results not shown). The inactivation requires H_2O_2 and is apparently irreversible. The necessity for H_2O_2 is supported by experiments with intact PMNs in which largely intracellular MPO is also irreversibly inactivated by CNO^- , but only if the cells are stimulated to generate H_2O_2 or exogenous H_2O_2 is added.

Mechanism of inactivation of MPO by CNO^-

Our experimental results point to an H_2O_2 -dependent formation of an irreversible complex between MPO and CNO^- . Perhaps the most direct evidence for this is the H_2O_2 -promoted formation of a [^{14}C]CNO adduct with the (haem-containing) α -subunit of MPO (Figure 6). Furthermore CNO^- blocks the expected H_2O_2 - and H_2O_2 / Cl^- -mediated spectral shifts in MPO, reflecting the formation of compound II and haem destruction.

Of possible relevance to the inhibitory reaction, Boffa et al. [42] found that a cytochrome *P*-450 fraction will, in the presence of H_2O_2 , convert CNO^- into a short-lived dialysable metabolite that inhibits protein synthesis. However, in MPO/ H_2O_2 incubation mixtures, we find no evidence for a diffusible oxidatively activated form of CNO^- . Thus we are unable to detect appreciable loss of CNO^- in the presence of H_2O_2 and MPO. Furthermore, in the absence of Cl^- , CNO^- does not cause MPO-mediated ascorbic acid oxidation [whereas, in the presence of Cl^- , CNO^- potently inhibits MPO/ H_2O_2 / Cl^- -mediated ascorbic acid oxidation as well as luminol-enhanced chemiluminescence (not shown)]. Finally, in mixtures of MPO and albumin, there is no evidence of an H_2O_2 -dependent incorporation of CNO^- into albumin (although MPO is labelled by [^{14}C]CNO⁻ in an H_2O_2 -dependent fashion). These results indicate that, if a redox-active intermediate of CNO^- does form, it is not reactive with external compounds and probably does not escape the catalytic site of MPO.

Implications for PMN function

In previous reports by others, CNO^- at high concentrations inhibited glucose utilization (when present at 7.5 mM [43]) and superoxide generation (at concentrations of 36–120 mM) by stimulated PMNs [11]. In contrast, we find that, at concentrations up to 10 mM, CNO^- does not affect H_2O_2 production by PMA-stimulated PMNs. On the other hand, CNO^- strongly inhibits PMN chlorinating activity at much lower concentrations, as measured by taurine chloramine formation (largely effected by released, extracellular MPO activity) and by ascorbic acid oxidation. We excluded the possibility that CNO^- simply quenches chlorinating species ($HOCl$ or taurine chloramine), thereby preventing taurine chloramine accumulation because: (1) addition of $NaOCl$ to solutions containing both taurine and $KOCN$ results in stoichiometric taurine chloramine recovery; (2) taurine chloramine does not react with CNO^- at neutral pH (as determined by taurine chloramine spectral change and retention of oxidative potential). We therefore conclude that MPO is far more susceptible to CNO^- inhibition than is the PMN NADPH oxidase system. Finally, to the extent that MPO activity is important in the bactericidal actions of PMNs, CNO^- has the potential to impair bacterial killing. We find that, at neutral pH, 1–2 mM CNO^- will almost completely prevent MPO-mediated killing of *S. aureus*.

Conclusion

In summary, CNO^- , one likely component of so-called 'uraemic toxins', irreversibly inactivates PMN MPO. The inactivation appears to be mechanism-based and requires the presence of H_2O_2 , suggesting that MPO inhibition does not simply arise from direct carbamoylation by CNO^- . If an enzymically generated form of 'activated' CNO^- (e.g. HOOCN) is produced, this reactive species has either a very short half-life or low oxidation potential, rendering MPO *per se* the preferential target of subsequent reactions. H_2O_2 -dependent inactivation of MPO by CNO^- occurs in the intracellular environment of activated PMNs, and such inactivation would probably diminish MPO-dependent bactericidal activity of PMNs. Indeed, we observe that relatively small amounts of added CNO^- will prevent MPO/ H_2O_2 -mediated killing of at least one target organism, *S. aureus*. We speculate that impairment of PMN bactericidal capacity by CNO^- may help explain both the high risk of urinary-tract infection associated with urinary stasis and perhaps the generalized increase in susceptibility to infection in uraemic patients.

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