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Original Contribution

Inhibition of myeloperoxidase- and neutrophil-mediated oxidant production by tetraethyl and tetramethyl nitroxides

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ABSTRACT

The powerful oxidant HOCl (hypochlorous acid and its corresponding anion, $^{-}$OCl) generated by the myeloperoxidase (MPO)–H_2O_2–Cl^- system of activated leukocytes is strongly associated with multiple human inflammatory diseases; consequently there is considerable interest in inhibition of this enzyme. Nitroxides are established antioxidants of low toxicity that can attenuate oxidation in animal models, with this ascribed to superoxide dismutase or radical-scavenging activities. We have shown (M.D. Rees et al., *Biochem. J.* 421, 79–86, 2009) that nitroxides, including 4-amino-TEMPO (4-amino-2,2,6,6-tetramethylpiperidin-1-yloxyl radical), are potent inhibitors of HOCl formation by isolated MPO and activated neutrophils, with IC_{50} values of ~1 and ~6 μM respectively. The utility of tetramethyl-substituted nitroxides is, however, limited by their rapid reduction by biological reductants. The corresponding tetraethyl-substituted nitroxides have, however, been reported to be less susceptible to reduction. In this study we show that the tetraethyl species were reduced less rapidly than the tetramethyl species by both human plasma (89–99% decreased rate of reduction) and activated human neutrophils (62–75% decreased rate). The tetraethyl-substituted nitroxides retained their ability to inhibit HOCl production by MPO and activated neutrophils with IC_{50} values in the low-micromolar range; in some cases inhibition was enhanced compared to tetramethyl substitution. Nitroxides with rigid structures (fused oxaspiro rings) were, however, inactive. Overall, these data indicate that tetraethyl-substituted nitroxides are potent inhibitors of oxidant formation by MPO, with longer plasma and cellular half-lives compared to the tetramethyl species, potentially allowing lower doses to be employed.

Activated neutrophils, monocytes, and some macrophages release the heme enzyme myeloperoxidase (MPO)[1] both intraphagosomally and extracellularly. This enzyme uses H_2O_2 and halide/pseudohalide ions (Cl^-, Br^-, SCN^-) to generate the potent oxidants hypochlorous acid (HOCl/$^{-}$OCl), hypobromous acid (HOBr/$^{-}$OBr), and hypothiocyanous acid (HOSCN/$^{-}$OSCN), respectively [1–3]. The production of these species is important in immune defense against invading pathogens, as these oxidants, particularly HOCl and HOBr, show potent bactericidal activity [4,5]. The related enzyme eosinophil peroxidase (EPO), released extracellularly by activated eosinophils, uses H_2O_2 and Br^- or SCN^- to generate predominantly HOBr and HOSCN to kill parasites [6,7]. A number of other heme peroxidases (e.g., lactoperoxidase and peroxidases present in the oral cavity and the stomach) also generate oxidants of this family [2,8–11]. Inappropriate formation of these oxidants has, however, been implicated in a number of major human inflammatory diseases, including atherosclerosis, asthma, rheumatoid arthritis, cystic fibrosis, kidney disease, and some cancers [1–3,12].

In light of these data, there is considerable interest in the development of therapeutically useful MPO inhibitors [12–14] and a number of novel materials have been examined (e.g., [13–18]). A number of recent studies have identified species that act as effective peroxidase substrates for MPO, thereby preventing rapid cycling of the enzyme via its halogenation cycle that generates HOCl/HOBr/HOSCN, and/or generate metabolites that induce irreversible heme modification [15,17].

Nitroxides are stable free radicals that have multiple biological effects, including acting as protective agents against oxidation in animal models of inflammation (e.g., [19–24]), though the

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EPO, eosinophil peroxidase; MPO, myeloperoxidase; TEEPO, 2,2,6,6-tetraethylpiperidine-1-oxyl radical; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl radical; TNB, 5-thio-2-nitrobenzoic acid

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mechanisms responsible are not fully understood. It has been reported that nitroxides can: (a) act as superoxide dismutase (SOD) mimetics [25–27], (b) reversibly or irreversibly scavenge radicals (e.g., carbon, oxygen, nitrogen, sulfur, and protein radicals [26,28–30]), and (c) reduce the oxyferryl ($Fe^{IV}=O$) center of heme proteins [19,31–34]. Recently we, and others, have also shown that a number of nitroxides can inhibit the chlorinating and nitrating activities of MPO [33–35]. In some cases this has been ascribed to radical scavenging (e.g., decreased protein nitration by MPO–H_2O_2–NO_2^- [33] and oxidative damage generated by MPO–H_2O_2–phenol [33,36]), but in other cases this inhibition seems to occur via the nitroxide interacting with compound I and compound II of the enzyme [33–35]. Whether inhibition also occurs with EPO is unknown.

These data suggest that nitroxides may modulate MPO (and possibly EPO)-mediated damage in vivo, and it has been established that some of these species are well tolerated and show low toxicity in rodents [19,20,32]. Topical application of nitroxides has also been used to prevent radiation-induced alopecia in guinea pigs [37] and humans [38]. Nitroxides are, however, rapidly reduced in vivo to the corresponding hydroxylamines [39,40], which are much less effective inhibitors of MPO [34], and this may limit their activity and/or result in a requirement for high in vivo doses. Interestingly, it has been recently shown that novel members of this family with tetraethyl, instead of tetramethyl, substituents flanking the nitroxide function (i.e., TEEPO compared to TEMPO; for structure and nomenclature see Table 1) are reduced at a slower rate [41–43], with this ascribed to steric shielding and a reduced redox potential of the nitroxide function [44]. In light of these data we have investigated: (a) whether tetraethyl-substituted nitroxides (and related species) modulate oxidant formation by isolated MPO, isolated EPO, and activated human neutrophils and (b) the rates of reduction of these species by human plasma and unstimulated and stimulated human neutrophils to determine whether these species have a longer biological half-life and, hence, might be possible therapeutic agents in diseases that involve MPO-induced damage.

Materials and methods

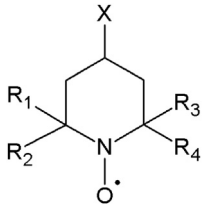
Materials

All buffers and aqueous solutions were prepared using Nanopure water filtered through a four-stage Milli-Q system (Millipore Waters, Lane Cove, Australia). Unless otherwise stated, 0.1 M potassium phosphate buffer, pH 7.4, was used to prepare reaction mixtures. MPO (Planta Natural Products, Vienna, Austria) was resuspended in Nanopure water and stored at 4 °C. Catalase, dimethyl sulfoxide (DMSO), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), Hanks' balanced salt solution without phenol red (HBSS), red blood cell lysing buffer Hybri-Max, sodium bromide, sodium chloride, sodium hydroxide, sodium thiocyanate, and phorbol myristate acetate (PMA) were obtained from Sigma–Aldrich (Sydney, Australia). 4-Oxo-TEMPO and 4-hydroxy-TEMPO were from Sigma–Aldrich, 4-amino-TEMPO was from Alexis Biochemicals (Lausen, Switzerland). 4-Hydroxy-TEEPO was prepared according to the method of Rajca (see supplementary material and [45]). 4-Oxo-TEEPO was synthesized and purified according to a previously reported method [42]. Initial batches of 4-amino-TEEPO were synthesized from 4-hydroxy-TEEPO as described under Method 1 in the supplementary material; subsequent samples were synthesized using Method 2 (see supplementary material) using a minor adaptation of a recently published method for 4-amino-TEMPO [46]. Dispirooxo-TEMPO was synthesized as described previously [47]. Polymorphprep density gradient solution was from Axis-Shield (Oslo, Norway). Stock solutions of hydrogen peroxide (Merck, Darmstadt, Germany) were prepared by dilution of a concentrated stock into Nanopure water and used immediately. Stock solutions of PMA (1 mg ml^{-1}) were prepared in DMSO, stored at −80 °C, and diluted with HBSS before use.

Quantification of oxidants using the TNB assay

Oxidants generated by MPO, EPO, and stimulated neutrophils were quantified using TNB as described previously [48]. Briefly, HOCl or HOBr generated by isolated MPO, EPO, or activated

Table 1
Nitroxide structures.



Side chains					Name
R_1	R_2	R_3	R_4	X	
$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	$-OH$	4-Hydroxy-TEMPO[a]
$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	$-NH_3^+$	4-Amino-TEMPO[b]
$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	$=O$	4-Oxo-TEMPO[c]
$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	$-OH$	4-Hydroxy-TEEPO[d]
$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	$-NH_3^+$	4-Amino-TEEPO[e]
$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	$=O$	4-Oxo-TEEPO[f]
$-CH_2CH_3$	$-CH_2CH_3$	$-CH_3$	$-CH_3$	$=O$	4-Oxo-2,2-diethyl-6,6-dimethylpiperidin-1-yloxyl
$-CH_2CH_2OCH_2CH_2-$		$-CH_2CH_2OCH_2CH_2-$		$=O$	Dispirooxo-TEMPO[g]

[a] Also known as 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-yloxyl.
[b] Also known as 4-amino-2,2,6,6-tetramethylpiperidin-1-yloxyl.
[c] Also known as 4-oxo-2,2,6,6-tetramethylpiperidin-1-yloxyl.
[d] Also known as 4-hydroxy-2,2,6,6-tetraethylpiperidin-1-yloxyl.
[e] Also known as 4-amino-2,2,6,6-tetraethylpiperidin-1-yloxyl.
[f] Also known as 4-oxo-2,2,6,6-tetraethylpiperidin-1-yloxyl.
[g] Also known as 7-aza-3,11-dioxa-15-oxodispiro[5.1.5.3]hexadec-7-yl-7-oxyl.