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Article

Polyamine-Conjugated Nitroxides Are Efficacious Inhibitors of Oxidative Reactions Catalyzed by Endothelial-Localized Myeloperoxidase

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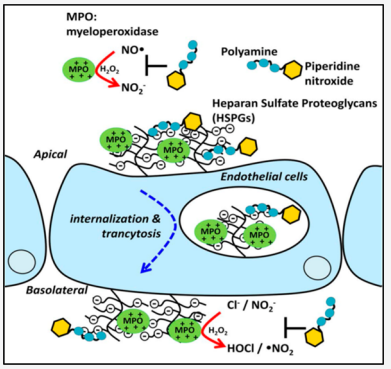
ABSTRACT: The heme enzyme myeloperoxidase (MPO) is a key mediator of endothelial dysfunction and a therapeutic target in cardiovascular disease. During inflammation, MPO released by circulating leukocytes is internalized by endothelial cells and transcytosed into the subendothelial extracellular matrix of diseased vessels. At this site, MPO mediates endothelial dysfunction by catalytically consuming nitric oxide (NO) and producing reactive oxidants, hypochlorous acid (HOCl) and the nitrogen dioxide radical ($^{\bullet}NO_2$). Accordingly, there is interest in developing MPO inhibitors that effectively target endothelial-localized MPO. Here we studied a series of piperidine nitroxides conjugated to polyamine moieties as novel endothelial-targeted MPO inhibitors. Electron paramagnetic resonance analysis of cell lysates showed that polyamine conjugated nitroxides were efficiently internalized into endothelial cells in a heparan sulfate dependent manner. Nitroxides effectively inhibited the consumption of MPO's substrate hydrogen peroxide (H_2O_2) and formation of HOCl catalyzed by endothelial-localized MPO, with their efficacy dependent on both nitroxide and conjugated-polyamine structure. Nitroxides also differentially inhibited protein nitration catalyzed by both purified and endothelial-localized MPO, which was dependent on $^{\bullet}NO_2$ scavenging rather than MPO inhibition. Finally, nitroxides uniformly inhibited the catalytic consumption of NO by MPO in human plasma. These studies show for the first time that nitroxides effectively inhibit local oxidative reactions catalyzed by endothelial-localized MPO. Novel polyamine-conjugated nitroxides, ethylenediamine-TEMPO and putrescine-TEMPO, emerged as efficacious nitroxides uniquely exhibiting high endothelial cell uptake and efficient inhibition of MPO-catalyzed HOCl production, protein nitration, and NO oxidation. Polyamine-conjugated nitroxides represent a versatile class of antioxidant drugs capable of targeting endothelial-localized MPO during vascular inflammation.



INTRODUCTION

Considerable experimental and clinical evidence has established the innate immune heme enzyme, myeloperoxidase (MPO), as a key mediator of endothelial dysfunction.[1,2] This is a major pathogenic event in cardiovascular disease that increases the adverse clinical event risk of coronary artery disease patients.[3] Multiple clinical studies show an inverse relationship between circulating MPO levels and clinical indices of endothelial dysfunction.[4−6] MPO, which is released extracellularly by activated circulating leukocytes during inflammation, transcytoses across the endothelium, accumulating within endothelial cells and the subendothelial extracellular matrix of the vascular wall.[7] Thus, MPO and biomarkers of its oxidative reactions are enriched in the endothelial and subendothelial compartments of vessels of cardiovascular disease patients.[4,8−12] At this site, and in the presence of its substrate hydrogen peroxide (H_2O_2), MPO catalyzes several deleterious oxidative reactions that mediate endothelial dysfunction, including the following: (i) the conversion of chloride ions (Cl^-) into the potent tissue-damaging oxidant hypochlorous acid (HOCl), (ii) the oxidation of nitrite (NO_2^-) into the nitrogen dioxide radical ($^{\bullet}NO_2$) that mediates protein tyrosine nitration, and (iii) the catalytic consumption of the homeostatic signaling biomolecule, nitric oxide (NO).[1,2,13] As MPO catalyzes these deleterious oxidative reactions in a localized manner in endothelial and subendothelial compartments,[7,13] there is a need to discover efficacious MPO inhibitors that are capable of accumulating in these compartments at sufficient concentrations.

Piperidine nitroxides are a class of cell-permeable small molecule antioxidants, which can attenuate oxidative stress via multiple mechanisms,[14] including (i) superoxide dismutase

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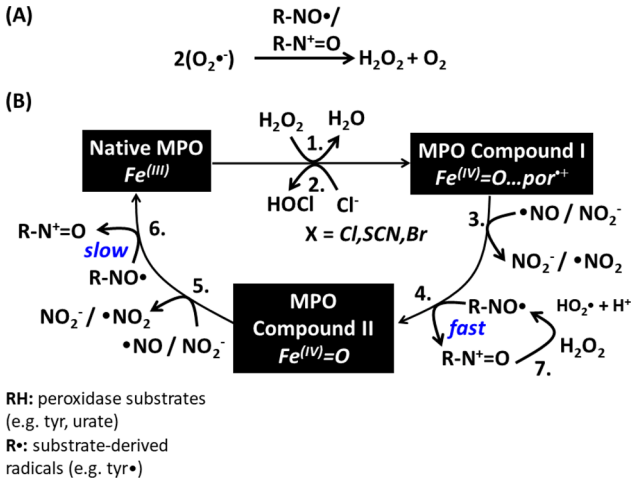
(SOD) mimetic activity (Scheme 1A), (ii) direct radical scavenging, and (iii) participating in radical−radical termination reactions.[14] Nitroxides were also recently identified as a new class of competitive substrate inhibitors of MPO (Scheme 1B, reactions 4 and 6), where they were reported to inhibit HOCl production.[8,15−18] Rees et al.[15] established that piperidine nitroxides competitively react with MPO compound I (Scheme 1B, reaction 4 versus reaction 2) to yield the corresponding nitroxide oxoammonium cation and MPO compound II, which does not participate in MPO's halogenation cycle (Scheme 1B). Kinetic studies confirmed that nitroxides are poor substrates for MPO compound II; thus, in an isolated system with no peroxidase substrates, MPO compound II accumulates and HOCl production is limited.[19] However, in complex biological systems, several physiological substrates (e.g., tyrosine, urate, NO_2^-, $O_2^{\bullet-}$) can reduce MPO compound II to regenerate native MPO, which is capable of participating in HOCl production (Scheme 1B, reaction 5 versus reaction 6).[17,20] As such, the ability of nitroxides to inhibit MPO-catalyzed oxidative reactions in biological environments where competing physiological substrates are available is thought to be substantially limited.[1] For the first time, in this study we address the ability of nitroxides to directly inhibit oxidative reactions catalyzed by MPO that is sequestered within endothelial cells.

Scheme 1. SOD Mimetic Activity and MPO Inhibition by Nitroxides[a]



RH: peroxidase substrates (e.g. tyr, urate)
R•: substrate-derived radicals (e.g. tyr•)

[a](A) Nitroxides are superoxide dismutase mimetics whereby they catalytically dismutate superoxide anion radical ($O_2^{\bullet-}$). (B) MPO consumes H_2O_2 to form MPO compound I (reaction 1), which mediates a two-electron oxidation of halides into the respective hypohalous acid (e.g., Cl^- into HOCl) via the halogenation cycle (reaction 2). MPO also oxidizes small molecule substrates via its peroxidase cycle through two successive one-electron reactions (reaction 3 followed by reaction 5, e.g., one-electron oxidation of NO_2^- into the nitrogen dioxide radical [$^{\bullet}NO_2$] or NO into NO_2^-). Nitroxides (R−NO$^{\bullet}$) are competitive substrates for MPO compound I, resulting in the formation of MPO compound II and the corresponding oxoammonium cation (R—N^+═O) (reaction 4). Efficacious nitroxide MPO inhibitors are poor substrates for MPO compound II such that reaction 6 is slowed, and in the absence of other peroxidase substrates, compound II accumulates, which does not participate in the halogenation cycle. Nitroxides inhibit in a catalytic fashion as the oxoammonium cation can be recycled back to the nitroxide radical through a reaction with H_2O_2 (reaction 7).

Additionally, the use of nitroxides as therapeutics is limited by poor *in vivo* bioavailability. High doses (up to millimolar concentrations) of the widely used piperidine nitroxide 4-hydroxy-TEMPO (tempol) are typically employed to achieve beneficial effects in animal models of inflammation.[21] We hypothesized that functionalizing nitroxides with cellular-targeting moieties enhances endothelial internalization and improves their ability to inhibit oxidative reactions catalyzed by endothelial-localized MPO. Accordingly, we report on the chemical synthesis of a novel series of piperidine nitroxides conjugated to cationic polyamines with the aim of increasing their endothelial accumulation. This approach is rationalized first by reports that conjugation of enzymes or small molecules to polyamines enhances endothelial cell uptake and transport across the blood−brain barrier[22,23] and second that cellular uptake of both polyamines and MPO is reportedly mediated by heparan sulfate proteoglycans (HSPGs).[7,24] Thus, we examine whether polyamine conjugation confers enhanced cellular uptake relative to the widely employed unconjugated nitroxide tempol and its dependency on HSPGs. We further examined the influence of polyamine conjugation on inhibition of MPO-catalyzed oxidative reactions in both isolated and endothelial cell systems.

Our studies establish for the first time that nitroxides effectively inhibit oxidative reactions catalyzed by endothelial-localized MPO and we identify novel polyamine-conjugated nitroxides, ethylenediamine-TEMPO and putrescine-TEMPO, as efficacious nitroxides uniquely exhibiting high endothelial uptake and efficient inhibition of MPO-catalyzed HOCl production, protein nitration, and NO oxidation.

EXPERIMENTAL METHODS

General Chemical Synthesis Information. Chemical reagents were commercially purchased and used without further purification. Solvents were purified according to well-established procedures.[25] Methanol was distilled from magnesium and stored over 3 Å molecular sieves under an atmosphere of nitrogen or argon. Chloroform was passed through basic alumina (70% w/v) directly before use. Dichloromethane (DCM) was obtained from a Pure Solv dry solvent system (Innovative Technology, Inc. model no. PS-MD-7). All reactions were followed by analytical thin layer chromatography (TLC) carried out with the use of Fluka PET-foils Silica Gel 60 and compounds visualized by irradiation with short-wavelength UV light and stained with Ninhydrin Spray dip (3.3 g of ninhydrin/100 mL of ethanol). Flash chromatography was performed by using chromatographic silica media LC60A, 40−63 μm, with appropriate solvent systems. Solvent was eluted with a Thomson SINGLE StEP pump at the manufacturer's recommended flow rate (Thomson Instrument Co., Oceanside, CA, USA). Deactivated silica gel was prepared by mixing silica gel with 5% (v/w) of triethylamine. Nuclear magnetic resonance (NMR) spectra were collected on a 300 and 75 MHz Bruker DPX 300 or Bruker Avance III 300 and are reported in parts per million (ppm). NMR spectra of nitroxyl radicals were obtained by adding a drop of phenylhydrazine to form the corresponding nonradical hydroxylamine. Compound purity was >95% as judged by HPLC.

General Synthetic Procedure for Compounds 11a−11e and 12. $Ti(OiPr)_4$ (1.2 equiv) was added dropwise to solid 4-oxo-TEMPO (1 equiv), and the mixture was stirred for 20 min. Boc protected polyamines (2 equiv; **9a−9e**, **10**) were added portionwise, and the reaction mixture was stirred for 3 h. The mixture was diluted with methanol (1.5 M) prior to the addition of $NaBH_3CN$ (1 equiv) and stirring for 24 h. The reaction was stopped by addition of water, and the inorganic white precipitate was removed by filtration and washing with ethyl acetate. This mixture was evaporated *in vacuo* to remove ethyl acetate. The aqueous layer was extracted with ethyl

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acetate, and the combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting orange oil was adsorbed onto silica and purified by flash chromatography (yield 30−74%).

General Synthetic Procedure for Compounds 1−6. Solutions of purified Boc protected nitroxyl radicals in DCM (0.01 M) were stirred with excess trifluoroacetic acid (10 equiv) for 4 h, and the mixture was evaporated under reduced pressure. The resulting dark oil was taken up in methanol/DCM (1:400), treated with anhydrous potassium carbonate (2 g for 200 mL of DCM), and stirred overnight. The mixture was filtered via a Celite plug eluting with DCM and concentrated *in vacuo*, yielding free nitroxyl radicals **1−6** as orange oils. No further purification was necessary.

Biological Materials and Methods. *General Biological Materials.* Purified human neutrophil MPO and H_2O_2 (30% w/v) were obtained from Merck Millipore. MPO was reconstituted (0.6−2 μM) in Milli Q water and stored at 4 °C. The concentrations of H_2O_2 stocks were determined by spectrophotometry (H_2O_2 ε_{240} = 43.6 M^{-1} cm^{-1}). H_2O_2 solutions were diluted in Milli Q water and used fresh each time. The NO donor NOC-9 ($t_{1/2}$ 2.7 min, 22 °C) was from Santa Cruz Biotechnology. NOC-9 solutions were prepared in ice-cold 10 mM NaOH and used fresh. Unless otherwise indicated, all other materials were purchased from Sigma-Aldrich, were of the highest purity available, and were used without further purification. For experiments, Chelex-treated phosphate buffer (0.1 M, pH 7.4; "phosphate buffer") was used. The nitroxides, 4-amino-TEMPO (4AT), 4-carboxy-TEMPO (4CT), 4-hydroxy-TEMPO (4HT) (all from Sigma-Aldrich), and mito-TEMPO (MT) (Santa Cruz Biotech), were of the highest purity available. Novel nitroxides **1−6** were synthesized as described above. The identity and purity (>95%) of the synthesized novel nitroxides were verified via NMR, HRMS, and HPLC.

Preparation of Human Plasma. Plasma was obtained after centrifugation (5000 rpm, 10 min at 4 °C) of freshly isolated heparinized blood donated by healthy consenting adult volunteers as per a protocol approved by the UNSW Human Ethics Review Committee. Aliquots of the isolated plasma were immediately frozen and stored at −80 °C. Plasma aliquots were thawed immediately before experiments and used within 1 h of thawing for MPO NO oxidase experiments.

Endothelial Cell Culture. Primary bovine aortic endothelial cells (ECs; Gelantis or Lonza) were cultured on gelatin-coated (Sigma; 0.05% w/v in PBS, 20 min, 22 °C) tissue culture flasks (75 cm^2, Nunc, Thermo Scientific) in endothelial basal medium (EBM; Lonza) with all supplements added except hydrocortisone (endothelial cell growth medium microvascular, EGM-MV; Lonza). Endothelial cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere and used for experiments between passages 3 and 9. For experiments, ECs were plated on gelatin-coated (0.05% w/v in PBS) 12-well plates (Nunc, Thermo Scientific) and 60 mm Petri dishes (Corning or Nunc, Thermo Scientific) and grown until confluent.

Western Blotting. Purified proteins or cell lysates in 1 × SDS sample buffer were boiled for 5 min at 100 °C and centrifuged for 5 min (12 000 rpm), and proteins were separated by SDS-PAGE using either 3−8% Tris acetate gels or 10% Bis-Tris gels (NuPAGE, Thermo Fisher). Proteins were resolved at 165−180 V over 55−65 min with either NuPAGE SDS Tris acetate running buffer or MOPS buffer, respectively. Resolved proteins were transferred onto nitrocellulose membranes by using the iBlot Gel transfer system according to the manufacturer's instructions (Invitrogen). Membranes were blocked with 5% nonfat dry milk in Tris buffered saline with Tween-20 (TBST) for at least 30 min. Membranes were then replaced with 5% nonfat dry milk in TBST (TBST-milk) containing the required primary antibody (i.e., mouse monoclonal anti-HOCl oxidized protein, clone 2D10G9 (that does not cross-react with epitopes generated by oxidative reactions involving nitrating species, transition metals, or lipid peroxidation),[18] 1:20; mouse monoclonal antitubulin (Sigma), 1:10000; mouse monoclonal anti-3-nitrotyrosine (Merck Millipore), 1:3000; rabbit polyclonal anti-3-nitrotyrosine (Abcam), 1:3000) and incubated overnight at 4 °C. Membranes were washed (3 × TBST) and incubated in TBST-milk with the relevant anti-mouse or anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (1:3000, Abcam) for 1 h at room temperature. Membranes were washed (3 × TBST) and protein bands were detected with the use of ECL Western blotting detection reagents (Amersham Biosciences) and developed with hyperfilm (GE healthcare) or visualized by using ImageQuant LAS4000 (GE Healthcare Life Sciences). Tubulin was used to index equal protein loading. Densitometric analyses of Western blot bands of multiple independent experiments were carried out with ImageJ software.

Measuring Endothelial Cell Nitroxide Uptake by EPR. Confluent endothelial cells were incubated in Hanks Balanced Salt Solution (1 × HBSS, calcium, magnesium, no Phenol Red, Gibco, Life Technologies) and treated with nitroxides (10 μM) for 20 min at 37 or 4 °C. Cells were then washed with ice-cold PBS and harvested into cell lysis buffer (ice-cold 0.1% Triton X-100 in 1 × PBS, 350 μL) and stored at −20 °C until EPR analysis. In some experiments, cells were preincubated with heparin (1250 μg/mL, Sigma) for 45 min prior to addition of nitroxides and incubation for a further 20 min. Measurements of nitroxide concentration and protein levels in cell lysates were carried out with EPR and BCA protein assay (Pierce, Thermo Scientific), respectively. Cellular nitroxide concentration was expressed as nanomoles of nitroxide per microgram of cell protein. For EPR analysis, samples were thawed and diluted by 10% with potassium ferricyanide (11 mM; final concentration ∼1 mM) to oxidize all available nitroxide to its radical and hence EPR detectable form. Samples (250 μL) were added to a flattened aqueous sample cell (WG-814-Q; Wilmad, Buena, NJ, USA), and spectra were acquired on a Bruker X-band EPR coupled to Xenon software (cavity, 4119HS; magnet, X-band ER073; microwave bridge, Bruker EMXplus premium Xbridge). EPR parameters were conversion time (1.3 s), time constant (5.24 s), sweep width (80 G), receiver gain (60 dB), attenuation (10 dB), modulation amplitude (2 G), and sweep time (520 s). The low-field nitroxide spectrum was used to quantify nitroxide concentrations alongside a standard curve using known concentrations of the nitroxide tempol (4HT; 0−1 μM).

Measurement of H_2O_2 Consumption by Isolated MPO. MPO-catalyzed H_2O_2 consumption was measured with an H_2O_2-specific electrode (ISO-HPO-2) interfaced to an Apollo 4000 free radical analyzer (World Precision Instruments) and quantified via LabScribe 3 software. Reactions were run at room temperature (∼22 °C) in stirred air-saturated Chelex-treated 0.1 M phosphate buffer solutions containing NaCl (100 mM). Reactions contained H_2O_2 (25 μM) and methionine (300 μM; added to scavenge HOCl) in the absence or presence of nitroxides (1 μM) and were initiated by the addition of MPO (20 nM). H_2O_2 consumption was recorded for up to 6 min following MPO addition.

Measurement of HOCl Production by Isolated MPO. HOCl production catalyzed by isolated MPO was measured by the 2-nitro-5-thiobenzoic acid (TNB)[9] and iodide-catalyzed tetramethylbenzidine (TMB) assays.[26] For the TNB assay, the TNB reagent was prepared by dissolving DTNB in aqueous 0.05 M NaOH solution to make a final concentration of 1 mM DTNB (TNB is formed via hydrolysis of DTNB in NaOH) and then diluting 1:40 into phosphate buffer. Reactions were run in phosphate buffer and contained MPO (100 nM), taurine (20 mM), and Cl^- (100 mM) in the absence or presence of nitroxides (10 μM) and/or heparin (1 mg/mL), and they were started by H_2O_2 (50 μM) addition. After 5 min the reaction was terminated by the addition of catalase (50 $\mu g\ mL^{-1}$), and an aliquot of reaction mixture was added to TNB reagent in a 96-well microtiter plate. The extent of TNB oxidation to DTNB arising from HOCl-mediated oxidation of taurine into taurine chloramine was quantified after 5 min at 412 nm with a FLUOstar Omega microplate reader (BMG Labtech). Data were expressed as a percent of the level of TauNHCl formed by MPO in the absence of nitroxides. For the TMB assay, reactions were performed in PBS (pH 7.4) containing 140 mM Cl^-, MPO (10 nM), and taurine (5 mM) in the absence or presence of nitroxides (0.1−10 μM). Reactions were initiated by the addition of H_2O_2 (50 μM) and terminated after 6 min by the addition of catalase (20 $\mu g\ mL^{-1}$). An aliquot of reaction mixture was added to

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the TMB reagent (2 mM; in 400 mM acetate buffer, pH 5.4, containing 10% dimethylformamide and 100 μM sodium iodide) with the extent of TMB oxidation due to HOCl-mediated oxidation of taurine into taurine chloramine quantified by measuring the absorbance at 650 nm.

Measurement of Tyrosine Nitration by Isolated MPO. MPO-catalyzed conversion of NO_2^- into $^{\bullet}NO_2$ and the resultant nitration of protein tyrosines was measured as the formation of 3-nitrotyrosine on BSA by Western blotting using a rabbit polyclonal anti-3-nitrotyrosine antibody. Reactions contained MPO (50 nM), BSA (100 μg/mL), and NO_2^- (100 μM), in the absence and presence of nitroxides (0−5 μM) in phosphate buffer, and were initiated by the addition of H_2O_2 (50 μM), followed by incubation at 37 °C for 30 min. The reaction was stopped by the addition of sample loading buffer containing SDS for subsequent Western blot analysis.

Measurement of NO Consumption by MPO. The NO donor NOC-9 ($t_{1/2}$ 2.7 min, 22 °C) was added to human plasma diluted into 0.1 M phosphate buffer (ratio 1:5; pH 7.4, ∼22 °C, air saturated) with rapid mixing to achieve a concentration of ∼500 nM NO (final NOC-9 concentration ∼2 μM), at which time MPO-catalyzed reactions were initiated by the sequential addition of H_2O_2 (10 μM) and MPO (15 nM) at 3.5 and 4 min, respectively, after the addition of NOC-9 in the absence and presence nitroxides (50 μM). Changes in NO concentration were measured continuously with an NO-specific electrode (ISO-NOP), interfaced to a one-channel free radical analyzer (TBR1025) and LabScribe 3 software (World Precision Instruments). The initial rate of NO consumption was measured by performing a linear regression of the slope over a 5 s time period beginning 5 s following the addition of MPO. An NO standard curve was performed as per the manufacturer using sulfuric acid and known concentrations of NO_2^-.

Measurement of H_2O_2 Consumption by Endothelial-Localized MPO (Amplex Red Assay). H_2O_2 consumption by endothelial cells was measured by the Amplex Red assay (Life Technologies). For this, confluent endothelial cells in HBSS (containing 0.2% BSA) at 37 °C were incubated in the absence or presence of MPO (20 nM) for 2 h and washed to remove unincorporated MPO.[27] Cells in HBSS were then nontreated or treated with nitroxides (10 μM) and incubated for 15 min prior to H_2O_2 (30 μM) addition and removal of supernatant (50 μL) at 5, 10, 15, 30, and 60 min, which was mixed in a black-bottomed 96-well microplate (Thermo Fisher) with 50 μL of Amplex Red/HRP solution prepared as per the manufacturer's instructions. The microplate was incubated in the absence of light for 30 min at room temperature, and H_2O_2 levels were quantified by measurement of the fluorescence intensity (excitation 545 nm (530−560 nm)/emission 590 nm) with a FLUOstar Omega plate reader (BMG Labtech). H_2O_2 concentration was determined by using a standard curve generated with known concentrations of H_2O_2 (0−50 μM).

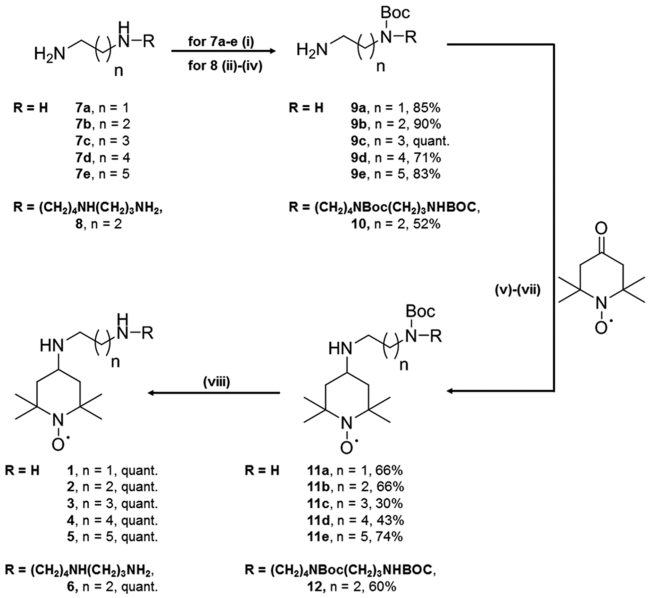
Measurement of Chlorination and Nitration Reactions Catalyzed by Endothelial-Localized MPO. Confluent endothelial cells in HBSS (containing 0.2% BSA) at 37 °C were loaded with MPO (20 nM) for 2 h and then washed with HBSS to remove unincorporated MPO.[27] Cells were nontreated or treated with nitroxides (10 μM) for 15 min prior to the addition of H_2O_2 (50 μM). To study MPO-catalyzed nitration, NO_2^- (100 μM) was added 15 min prior to H_2O_2. Cells were incubated at 37 °C for 60 min and then lysed in 1 × SDS-loading buffer for Western blotting analysis of 3-nitrotyrosine or HOCl-oxidized proteins.

Statistical Analyses. Data are the mean ± SEM of three or more independent experiments. Statistical differences in column graphs between two treatments were assessed with a Student *t* test and one-way ANOVA with Bonferroni's or Dunnett's post hoc test for more than two treatments and for curves with two-way ANOVA. Statistical differences in IC_{50} values were assessed by using a one-way ANOVA with the Dunnett's post hoc test. Analyses were performed with Prism 6 software, and a *P*-value of <0.05 was considered significant.

RESULTS

Synthesis of Polyamine-Conjugated Nitroxides. Piperidine nitroxides were conjugated to cationic polyamines to enhance their bioavailability at endothelial and subendothelial sites where MPO resides. TEMPO derivatives **1**−**6** were synthesized as per the modular synthetic approach in Scheme 2. Diamines were mono-Boc protected by addition of di-*tert*-butyl dicarbonate to yield compounds **9a**−**9e** in yields of 71% quantitative (quant.), while tri-Boc-spermine (**10**) was synthesized in a one-pot reaction, as previously reported (52% yield).[28] Compounds **11a**−**11e** and **12** were generated by reductive amination of 4-oxo-TEMPO with partially protected polyamines (**9a**−**9e**, **10**) as described[29] (Scheme 2v−vii); 4-oxo-TEMPO was stirred with 1.2 equiv of titanium isopropoxide and 1 equiv of mono-Boc protected diamines or tri-Boc-spermine, followed by reduction with sodium cyanoborohydride to yield **11a**−**11e** and **12** in yields of 30−74%. Final compounds **1**−**6** (Table 1, >95% purity) were obtained after removal of Boc protecting groups with trifluoroacetic acid and subsequent formation of the free amine by stirring in methanol/DCM with potassium carbonate.

Scheme 2. Synthesis of Polyamine-Conjugated Nitroxides[a]



[a](i) Diamine (10 equiv), Boc_2O (1 equiv), $CHCl_3$, 16 h, 0 °C to RT; (ii) $CF_3COOCH_2CH_3$ (1 equiv), MeOH, 1 h, −78 °C; (iii) Boc_2O (4 equiv), MeOH, 20 h, 0 °C to RT; (iv) saturated aqueous NH_3, MeOH, 24 h, RT; (v) $Ti(OiPr)_4$ (1.2 equiv), 20 min, RT; (vi) **9a**−**9e**, 10 (2 equiv), 3 h, RT; (vii) $NaBH_3CN$ (1 equiv), MeOH, 24 h, RT; (viii) TFA (10 equiv), DCM, 4 h, RT, then K_2CO_3, MeOH/DCM, 12 h, RT.

Endothelial Cell Uptake of Nitroxides. The cellular accumulation of nitroxides was examined by incubating confluent cultures of aortic endothelial cells with nitroxides followed by subsequent determination of nitroxide content in cell lysates with electron paramagnetic resonance (EPR) spectroscopy by measuring the signal amplitude of the low-field spectrum (Figure 1A). Figure 1B shows that 4AT and novel nitroxides **1** and **3** exhibited the greatest extent of endothelial cell uptake. Novel nitroxides **4**, **5**, and **6** were internalized to a lesser degree, exhibiting ∼32, 42, and 36%, respectively, of the uptake apparent for novel nitroxide **1** and

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Table 1. Compounds Studied in This Work

Compound Name	Symbol	R:
4-amino TEMPO	4AT	NH_2
4-hydroxy TEMPO	4HT	OH
4-carboxy TEMPO	4CT	COOH
Mito-TEMPO	MT	$NHC(O)CH_2P^+(Ph_3)$
Ethylenediamine-TEMPO	1	$NH(CH_2)_2NH_2$
Propanediamine-TEMPO	2	$NH(CH_2)_3NH_2$
Putrescine-TEMPO	3	$NH(CH_2)_4NH_2$
Cadaverine-TEMPO	4	$NH(CH_2)_5NH_2$
Hexamethylenediamine-TEMPO	5	$NH(CH_2)_6NH_2$
Spermine-TEMPO	6	$NH(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH_2$

4AT. Additionally, 4HT, 4CT, or MT showed comparatively poor uptake exhibiting only ~3, 7, and 14%, respectively, of the uptake of **1** and 4AT. Overall, the efficiency of nitroxide endothelial cell uptake was 4AT = **1** = **3** > **2** > **4** = **5** = **6** > MT > 4CT = 4HT.

We further confirmed that the majority of nitroxide cellular uptake required an energy-dependent process as endothelial uptake of novel nitroxides **3** and **6** was inhibited by 70−80% in endothelial cells cultured at 4 °C versus cells cultured at 37 °C (Figure 1C). The role of endothelial-expressed HSPGs in facilitating endothelial uptake of positively charged nitroxides was next assessed by performing a competitive binding study testing the ability of the heparan sulfate analogue, heparin, to limit cellular nitroxide uptake. Heparin, at an ~10-fold molar excess, inhibited the uptake of 4AT, **3**, or **6** by ~60% (Figure 1D), indicating a significant role for HSPGs in the endothelial uptake of these nitroxides.

Inhibition of MPO Chlorination Activity by Nitroxides. We next tested the ability of nitroxides to inhibit the chlorination activity of isolated MPO. Initially, an H_2O_2 electrode was used to assess nitroxide inhibition of isolated MPO-catalyzed H_2O_2 consumption in the presence of physiological Cl^- levels; inhibition of H_2O_2 consumption in the presence of Cl^- (and absence of other competing substrates) is a direct measure of inhibition of MPO's catalytic activity and an indirect index of inhibition of MPO's chlorination activity (Scheme 1B, reactions 1 and 2). Structure−function activity was observed regarding the efficacy of commercial nitroxides to inhibit MPO-catalyzed H_2O_2 consumption with the order of efficiency 4AT > 4HT > MT > 4CT (Figure 2A). For novel nitroxides, while **1**−**5** effectively inhibited MPO-catalyzed H_2O_2 consumption, **6** showed a lower inhibitory capacity (Figure 2B).

To study the efficacy of nitroxides to inhibit H_2O_2 catabolism by endothelial-localized MPO, we employed the Amplex Red assay to detect H_2O_2 and performed experiments in endothelial cells containing transcytosed MPO, which concentrates within endothelial cells and the subendothelial space.[7,27] Figure 3A shows that H_2O_2 consumption was markedly enhanced in MPO-containing endothelial cells. While nitroxides did not inhibit basal H_2O_2 consumption by control endothelial cells, nitroxides **1**, **3**, 4AT, and 4HT significantly inhibited the MPO-accelerated H_2O_2 loss in endothelial cells by 50−60% (Figure 3). In contrast, **2**, **4**, **5**, **6**, 4CT, and MT did not significantly affect H_2O_2 consumption in MPO-containing endothelial cells (Figure 3B).

The effect of nitroxides on HOCl production catalyzed by isolated or endothelial-localized MPO was next assessed. We quantified the individual IC_{50} values for inhibition of MPO chlorination for both the commercial and novel nitroxides using the sensitive iodide-catalyzed TMB assay (Figure 4). We observed structure−function activity for nitroxide-mediated inhibition of HOCl production catalyzed by isolated MPO, with 4AT being the most efficient, 4HT being intermediate, and MT or 4CT exhibiting reduced or no inhibition,

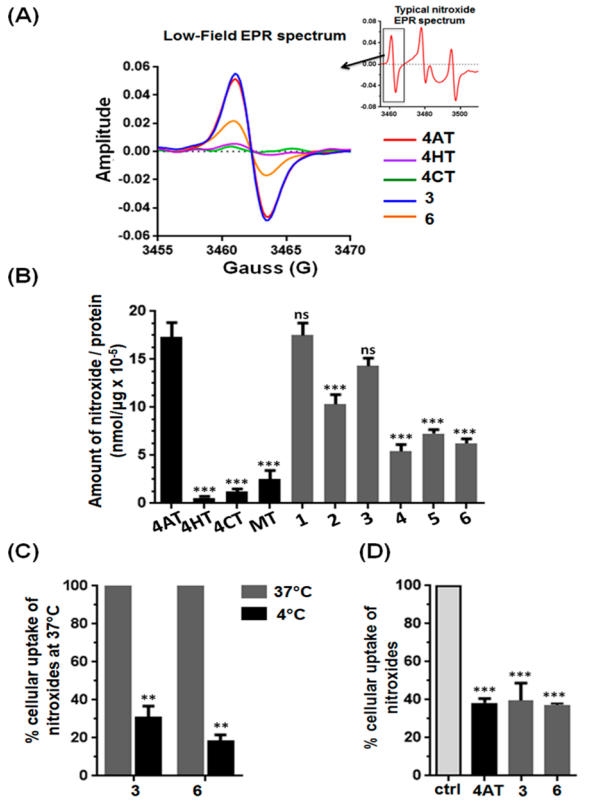

Figure 1. Endothelial cell uptake of nitroxides. Confluent aortic endothelial cells were incubated with nitroxides (10 μM) for 20 min, and the cell lysate content of nitroxides was measured by EPR. (A) Representative low-field EPR spectrum of nitroxides; the signal amplitude was used for quantitation. (B) Nitroxide content in endothelial cells. Data are the mean ± SEM of three independent experiments performed in triplicate for all conditions. ***, $P < 0.001$; ns, nonsignificant; compared to 4AT. (C) Relative contents of **3** and **6** in endothelial cells incubated at either 37 or 4 °C. Data are expressed as a percent of nitroxide uptake at 37 °C and represent the mean ± SEM of three independent experiments for all conditions. **, $P < 0.01$, relative to treatment of cells at 37 °C. (D) Prior addition of heparin (1250 μg/mL or ~100 μM; assuming an MW for heparin of 13 kDa) inhibits nitroxide uptake by endothelial cells. Data are expressed as a percent of nitroxide uptake in the absence of heparin and represent the mean ± SEM of three independent experiments for all conditions. ***, $P < 0.001$, relative to treatment of cells with nitroxides in the absence of heparin; ctrl, maximal nitroxide uptake in the absence of heparin (i.e., 100%).

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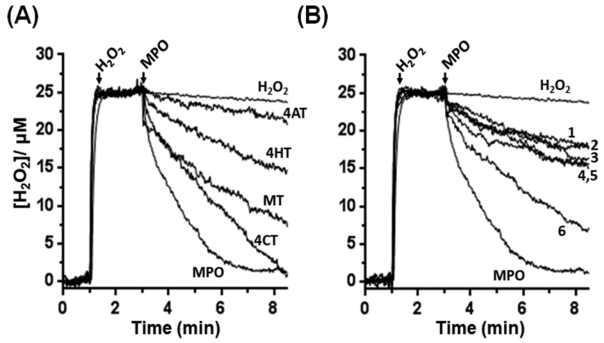


Figure 2. Nitroxides inhibit H_2O_2 consumption by isolated MPO. Effect of (A) commercial or (B) novel nitroxides (1 μM) on H_2O_2 consumption by purified MPO. Reactions were performed in 0.1 M phosphate buffer (pH 7.4) and contained H_2O_2 (25 μM), Cl^- (100 mM), and the HOCl-scavenger methionine (300 μM). Reactions were initiated by the addition of MPO (20 nM) as indicated. The H_2O_2-consumption traces shown are representative of three independent measurements.

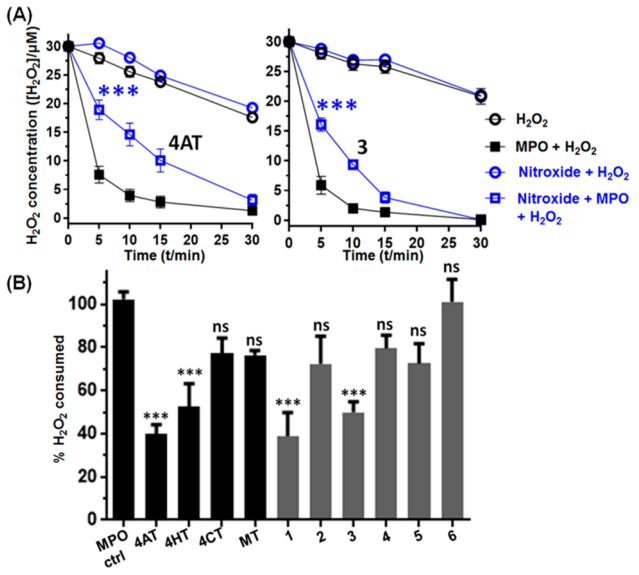
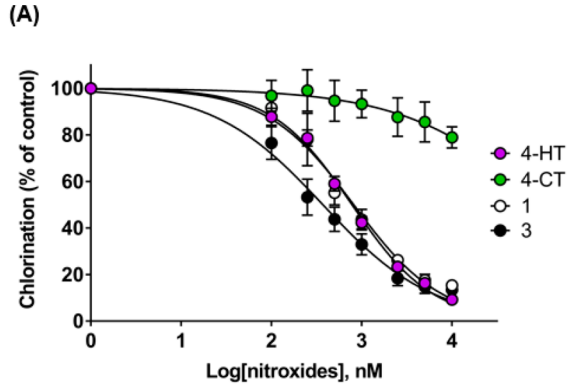


Figure 3. Inhibition of H_2O_2 consumption by endothelial-localized MPO. Confluent endothelial cells were incubated in the absence or presence of MPO (20 nM) for 2 h and the cells washed to remove unbound MPO. Endothelial cells were then incubated in the absence or presence of the relevant nitroxides (10 μM) before H_2O_2 (30 μM) was added, and the time-dependent loss of H_2O_2 in the media was measured at the indicated times using the Amplex Red assay. (A) Inhibition of MPO-dependent H_2O_2 consumption in endothelial cells by 4AT or novel nitroxide **3** over a 30 min time period. (B) Inhibition of H_2O_2 consumption by endothelial-localized MPO by nitroxides at 5 min post H_2O_2 addition. Data are expressed as a percent of maximal H_2O_2 consumption by MPO-containing endothelial cells in the absence of nitroxides (MPO ctrl) at 5 min (100%) and represent the mean ± SEM of three independent experiments. ***, $P < 0.001$; ns, nonsignificant; compared to MPO ctrl.



(B)

Compound name	IC50 (nM)
4-hydroxy TEMPO (4-HT)	782 ± 40
4-Amino TEMPO (4-AT)	298 ± 16*
4-carboxy TEMPO (4-CT)	ND
Mito-TEMPO	4142 ± 377
Ethylenediamine-TEMPO (1)	811 ± 86
Propanediamine-TEMPO (2)	451 ± 44*
Putrescine-TEMPO (3)	392 ± 44*
Cadaverine-TEMPO (4)	742 ± 83
Hexamethylenediamine-TEMPO (5)	689 ± 49
Spermine-TEMPO (6)	796 ± 131

Figure 4. Nitroxide-mediated inhibition of MPO's chlorination activity. Reactions were performed at room temperature in phosphate buffered saline (pH 7.4) containing 140 mM Cl^-, MPO (10 nM), and taurine (5 mM) in the absence or presence of nitroxides (0.1−10 μM), initiated by the addition of H_2O_2 (50 μM) and terminated after 6 min by the addition of catalase (20 μg mL^{-1}). MPO-catalyzed HOCl production (chlorination activity) was then assessed by the sensitive iodide-catalyzed TMB assay. (A) Dose−response curves showing the inhibition of MPO-catalyzed HOCl production by 4HT, 4CT, and novel nitroxides **1** and **3**. Results are expressed as the percent chlorination activity of MPO in the absence of nitroxides (control), which was assigned a value of 100%. (B) Tabulated IC_{50} values of nitroxides (i.e., concentration necessary to inhibit MPO-catalyzed HOCl production by 50%), which were determined from the dose−response curves using nonlinear regression. ∗, $P < 0.05$, relative to the IC_{50} of 4HT using one-way ANOVA with post hoc Dunnett's test. Data represents the mean ± SEM, $n = 4$−12 ($n = 12$ for 4HT, $n = 4$ for all other nitroxides).

respectively (representative curves are shown in Figure 4A). All novel nitroxides **1**−**6** effectively inhibited MPO-catalyzed HOCl production, exhibiting significantly greater or similar efficacy as 4HT (Figure 4), with the most efficacious being **3** (putrescine-TEMPO) and **2** (propanediamine-TEMPO). The order of efficacy of nitroxide inhibition of HOCl production catalyzed by isolated MPO (Figure 4) generally mirrored that apparent for nitroxide-mediated inhibition of MPO-catalyzed H_2O_2 consumption (Figure 2).

Positively charged and polyamine-based nitroxides bind with high affinity to negatively charged heparan sulfates,[24] which are abundant in the subendothelial space where MPO is sequestered.[30] As heparan sulfates may interfere with the access of positively charged and novel nitroxides into the MPO active site, the effect of a molar excess of heparin (a heparan sulfate analogue) on the inhibitory capacity of these nitroxides toward MPO was tested using the TNB assay (Figure 5). While heparin addition did not affect the ability of novel nitroxide **1** to inhibit MPO-catalyzed HOCl production, it abolished the MPO inhibitory capacity of **4** and **6** and partially

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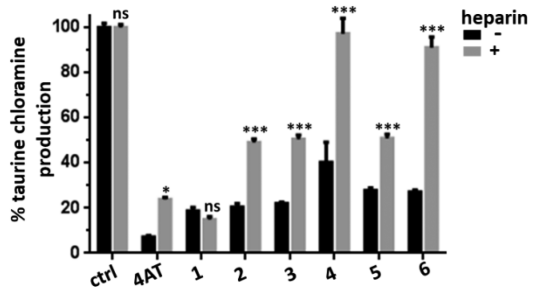

Figure 5. Effect of heparin on nitroxide-mediated inhibition of MPO's chlorination activity. Reactions contained MPO (100 nM), taurine (20 mM), and Cl^- (100 mM) in the absence (ctrl) or presence of nitroxides (10 μM) and the absence (black bars) or presence (gray bars) of heparin (1 mg/mL; 77 μM based on average MW of 13.000 kDa) and were initiated by the addition of H_2O_2 (50 μM). Following 5 min incubation reactions were terminated by the addition of catalase (50 μg mL^{-1}), and MPO-catalyzed HOCl production was assessed via the TNB assay. Results are expressed as a percent of taurine chloramine production formed by MPO in the absence of nitroxides (ctrl) that was assigned a value of 100%. The results represent the mean ± SEM of three independent experiments. ***, $P < 0.001$; *, $P < 0.05$; ns, nonsignificant; compared to values obtained in the absence of heparin for each treatment.

attenuated the inhibitory activity of **2**, **3**, **5**, and 4AT by ~2.4-, ~2.3-, ~1.8-, and ~3.3-fold, respectively (Figure 5).

Prior works by us[27] and others[7] have established that incubation of confluent aortic endothelial cells with MPO results in the transcytosis and accumulation of the enzyme primarily in the subendothelial space, where it catalyzes the chlorination and nitration of extracellular matrix proteins, including fibronectin. To study the efficacy of nitroxides to inhibit HOCl production by endothelial-localized MPO, the formation of HOCl-oxidized proteins in MPO-containing endothelial cells was measured via Western blotting using a well-characterized anti-HOCl-oxidized protein antibody (2D10G9; it does not cross-react with epitopes generated by oxidative reactions involving nitrating species, transition metals, or lipid peroxidation reactions[18]). Consistent with our prior data,[27] exposure of MPO-containing endothelial cells to low micromolar levels of H_2O_2 (50 μM) afforded the detection of high molecular weight HOCl-oxidized proteins, derived from cross-linked aggregates of oxidized extracellular matrix proteins formed within the subendothelium where transcytosed MPO accumulates[27] (Figure 6). Nitroxides differentially inhibited the formation of HOCl-oxidized protein by endothelial-localized MPO; novel nitroxides **1**, **2**, **3**, and **5**, as well as 4AT and 4HT, all significantly inhibited HOCl production, while **4**, **6**, 4CT, and MT showed reduced inhibition (Figure 6).

Inhibition of MPO-Catalyzed Protein Nitration by Nitroxides. A significant reaction catalyzed by MPO *in vivo* is the conversion of NO_2^- into $^{\bullet}NO_2$ that reacts with protein tyrosines to form 3-nitrotyrosine, a hallmark of oxidative or nitrosative tissue damage during inflammation and cardiovascular disease.[7,31] We initially examined the dose-dependent inhibition by nitroxides of tyrosine nitration of bovine serum albumin (BSA) catalyzed by isolated MPO and determined by Western blotting with an anti-3-nitrotyrosine antibody. All nitroxides effectively inhibited BSA tyrosine nitration catalyzed by MPO, with the novel nitroxides and 4AT exhibiting significantly greater efficacy compared to 4HT and 4CT (Figure 7).

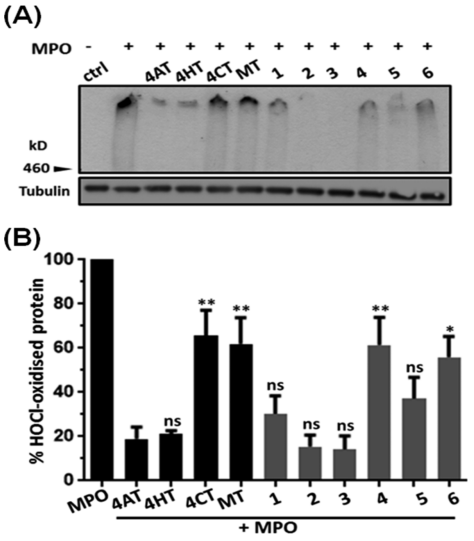

Figure 6. Effect of nitroxides on HOCl production by endothelial-localized MPO. Confluent endothelial cells were incubated in the absence or presence of MPO (20 nM) for 2 h and cells washed to remove unbound MPO. Endothelial cells were then incubated in the absence or presence of nitroxides (10 μM) for 15 min before H_2O_2 (50 μM) was added and endothelial cells were incubated for 1 h. Cell lysates were then assessed for HOCl production using a monoclonal anti-HOCl oxidized protein antibody (clone 2D10G9) and Western blotting. (A) Representative Western blot of three independent blots, which was cropped to show the high molecular weight protein bands detected above 460 kDa. (B) Densitometric analyses of three independent experiments/Western blots were performed with ImageJ software. Data are expressed as a percent of the intensity of the HOCl-oxidized protein bands formed in MPO-containing cells exposed to H_2O_2 in the absence of nitroxides (MPO). Data represent the mean ± SEM of three independent experiments. **, $P < 0.01$; *, $P < 0.05$; ns, nonsignificant; compared to 4AT.

Prior work shows that endothelial-localized MPO catalyzes 3-nitrotyrosine formation in the subendothelial matrix of diseased arteries *in vivo* and of endothelial cells *in vitro*.[7,12,27] We therefore next tested the ability of nitroxides to inhibit 3-nitrotyrosine formation catalyzed by endothelial-localized MPO using Western blot and a 3-nitrotyrosine antibody. Treatment of MPO-containing endothelial cells with H_2O_2 in the presence of NO_2^- resulted in the formation of high molecular weight proteins immunoreactive for 3-nitrotyrosine (Figure 8). Marked differences were noted regarding the ability of nitroxides to inhibit 3-nitrotyrosine formation in MPO-containing endothelial cells; while 4AT did not inhibit 3-nitrotyrosine levels, 4HT, 4CT and MT inhibited 3-nitrotyrosine formation by ~70, 88, and 45%, respectively (Figure 8). For the novel nitroxides, **1** and **3** inhibited protein nitration by ≥80%, **4** and **6** inhibited protein nitration by 60−75%, and **2** inhibited protein nitration by ~30%. In contrast, **5** did not significantly inhibit protein nitration catalyzed by endothelial-localized MPO.

Inhibition of the NO Oxidase Activity of MPO by Nitroxides. MPO's NO oxidase activity is a key oxidative mechanism impairing NO bioactivity in inflammation, e.g., endotoxin-challenged rodents,[13] human coronary artery disease patients,[32,33] and myocardial infarction patients.[4] We tested the ability of novel nitroxides to inhibit MPO-catalyzed NO consumption in diluted human plasma in the presence of physiological steady-state levels of NO (~500 nM) delivered

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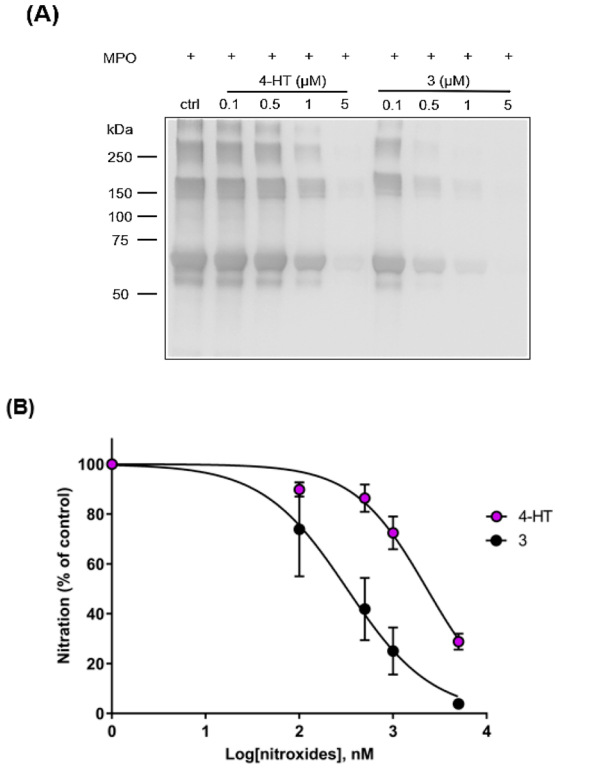

Compound name	IC50 (nM)
4-hydroxy TEMPO (4-HT)	2305 ± 289
4-Amino TEMPO (4-AT)	666 ± 274*
4-carboxy TEMPO (4-CT)	2617 ± 1248
Ethylenediamine-TEMPO (1)	822 ± 214*
Propanediamine-TEMPO (2)	480 ± 96*
Putrescine-TEMPO (3)	323 ± 98*
Cadaverine-TEMPO (4)	354 ± 85*
Hexamethylenediamine-TEMPO (5)	572 ± 84*
Spermine-TEMPO (6)	649 ± 52*

Figure 7. Effect of nitroxides on MPO-catalyzed BSA nitration. Reactions contained MPO (50 nM), BSA (100 μg/mL) and NO_2^- (100 μM) in the absence (ctrl) or presence of nitroxides (0.1−5 μM) and were initiated by H_2O_2 addition (50 μM). After incubation at 37 °C for 30 min the reactions were terminated and the level of 3-nitrotyrosine on BSA determined by Western blot. (A) The Western blot shown is representative of three to four independent blots. 3-Nitrotyrosine levels were quantified by densitometric analysis of the 68 kDa band by ImageJ software. No immunoreactivity was present for BSA + H_2O_2 + NO_2^- in the absence of MPO (data not shown). (B) Representative dose−response curves showing the inhibition of MPO-catalyzed BSA nitration by 4HT and 3. Results are expressed as percent nitration by MPO in the absence of nitroxides (ctrl), which were assigned a value of 100%. (C) Tabulated IC_{50} values (nitroxide concentration required to inhibit MPO-catalyzed BSA nitration by 50%) of nitroxides. *, $P < 0.05$ relative to IC_{50} of 4HT using one-way ANOVA with a post hoc Dunnett's test. Data represents the mean ± SEM of three to four independent experiments for all conditions. IC_{50} values were determined from dose−response curves using nonlinear regression.

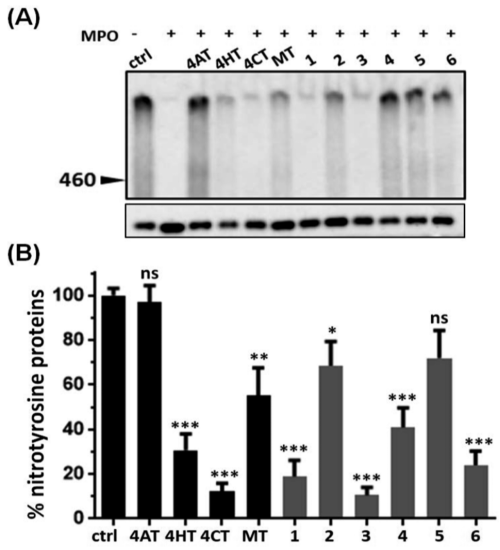

Figure 8. Nitroxides inhibit protein nitration by endothelial-localized MPO. Confluent endothelial cells were incubated in the absence or presence of MPO (20 nM) for 2 h and cells washed to remove unbound MPO. Cells were treated with nitroxides (10 μM) and NO_2^- (100 μM) for 15 min prior to H_2O_2 (50 μM) addition and incubation for 1 h. 3-Nitrotyrosine levels in cell lysates were assessed by Western blotting using an anti-3-nitrotyrosine antibody. (A) Representative Western blot of three independent blots, which has been cropped to show the high molecular weight 3-nitrotyrosine bands detected above 460 kDa. (B) Densitometric analysis of Western blots was performed with ImageJ software. Data are expressed as a percent of the intensity of the 3-nitrotyrosine bands formed in MPO-containing endothelial cells exposed to H_2O_2 in the presence of NO_2^- and absence of nitroxides (ctrl). Data represent the mean ± SEM of three independent experiments. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, nonsignificant; compared to MPO-containing endothelial cells exposed to H_2O_2 in the presence of NO_2^- and absence of nitroxides (ctrl).

by the NO donor NOC-9. Novel nitroxides **1**−**6** and 4AT all significantly inhibited the initial rates of MPO-catalyzed NO consumption in human plasma by 50−60% (Figure 9).

DISCUSSION

In this study we characterized several piperidine nitroxides including a novel series of polyamine-conjugated piperidine nitroxides (Table 1) for their propensity to (i) accumulate into endothelial cells and (ii) inhibit several oxidative reactions catalyzed by both isolated and endothelial-localized MPO that are mechanistically linked as causes of endothelial dysfunction during cardiovascular disease including MPO-catalyzed H_2O_2 metabolism, conversion of Cl^- into HOCl or NO_2^- into $^{\bullet}NO_2$, and MPO's NO oxidase activity. We provide the first evidence that the presence of cationic amino ($-NH_2$) groups para to the nitroxyl radical significantly enhance cellular internalization of piperidine nitroxides in an HSPG-dependent manner and that nitroxides effectively inhibit MPO-catalyzed oxidative and nitrosative damage in the complex biological environment of endothelial cells. This work also reveals important structure−function information useful for the further development of polyamine-conjugated nitroxides. Collectively, this work provides evidence supporting that one mechanism by which piperidine nitroxides preserve endothelial function *in vivo* is MPO inhibition. Moreover, our studies provide the foundation to support further development of endothelial-targeted MPO

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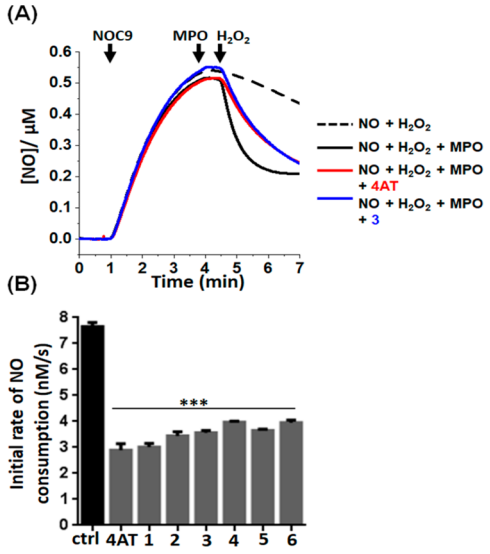

Figure 9. Nitroxides inhibit MPO-catalyzed NO oxidation in human plasma. Effect of novel nitroxides **1**−**6** or 4AT (50 μM) on the initial rate of NO consumption catalyzed by purified MPO (15 nM) in diluted human plasma. (A) Representative traces of NO consumption for 4AT and **3**. The NO donor NOC-9 (~500 nM) was added to plasma (diluted 1:5 in 0.1 M phosphate buffer, pH 7.4) followed by addition of H_2O_2 (10 μM) and MPO (15 nM) at 3.5 and 4 min post addition of NOC-9. (B) Initial rates of MPO-catalyzed NO consumption in the absence (ctrl) or presence of nitroxides. Data are the mean ± SEM of at least three independent experiments for all conditions. ***, $P < 0.001$ relative to the absence of nitroxides (ctrl).

inhibitors useful for combating endothelial dysfunction during vascular inflammation.

Our study aimed to enhance the bioavailability of nitroxides at endothelial sites via conjugation to polyamines. We found that nitroxides exhibited differences in endothelial cell uptake, which related to the lipo/hydrophilicity and/or charge of the compounds.[34] As such, hydrophilic nitroxides with a net positive charge (**1**−**6**, 4AT) were more efficiently internalized in comparison to either hydrophilic neutral nitroxides (4HT), negatively charged nitroxides (4CT), or lipophilic positively charged nitroxides (MT). Tempol (4HT) is the most widely studied nitroxide, and commonly, high doses of 4HT are necessary to provide *in vivo* protection in animal models,[21,35] which is attributed to its low cellular uptake and poor *in vivo* bioavailability.[21,35,36] Our EPR data are in line with these reports, as endothelial cell uptake of 4HT was <5% of positively charged nitroxides **1**, **3**, or 4AT (Figure 1B). We also examined endothelial uptake of the mitochondrial-targeted nitroxide, MT, which Dikalova et al. report is internalized ~5-fold > 4HT.[37] Our data similarly show that MT is internalized into endothelial cells ~4-fold > 4HT. We also show that the novel polyamine-conjugated nitroxides **1**−**6** were internalized 2−7-fold > MT. Overall, our studies show that conjugation of TEMPO nitroxides to polyamines, and the presence of a positive charge in the para position to the nitroxyl radical, increases endothelial internalization above that of the commonly studied 4HT.

As HSPGs are important for endothelial binding and transcytosis of MPO and polyamine cellular uptake,[7,24] we examined the influence of HSPGs on the internalization of 4AT and certain polyamine-conjugated nitroxides. Our finding that the heparan sulfate analogue heparin inhibited endothelial internalization of these nitroxides by ~60% supports a significant role for HSPGs. For polyamine-conjugated nitroxides this likely reflects the reported role for HSPGs in polyamine cellular uptake.[24] The remaining 40% of endothelial nitroxide uptake that was refractory to heparin may reflect HSPG-independent routes, e.g., polyamine import via cationic amino acid transporters.[38] Although HSPGs are important for polyamine-conjugated nitroxide and MPO endothelial uptake, the extent to which nitroxides and MPO follow analogous or different intracellular transport routes is unknown and requires further investigation.

With regard to inhibition of chlorination activity by isolated MPO, our data showed that positively charged (4AT, **1**−**6**) or neutral (4HT) nitroxides efficiently inhibited MPO-catalyzed H_2O_2 metabolism and HOCl production, while negatively charged (4CT) nitroxides were less efficient, findings that were consistent with studies by Rees et al.[15] Previous studies indicate that MPO poorly metabolizes negatively charged substrates due to the presence of a carboxy group in the peroxidase substrate channel (most likely from the glutamine 242 residue).[39] This may explain the poor inhibitory activity of 4CT relative to 4HT and 4AT. On the other hand, the efficacy of nitroxides to inhibit oxidative reactions catalyzed by endothelial-localized MPO appears to be influenced by the interaction of a number of factors including nitroxide bioavailability (i.e., endothelial cell uptake) and capacity to inhibit MPO enzyme activity, as well as competitive binding to other cellular components such as HSPGs. This was most evident with the novel nitroxides. Despite exhibiting similar inhibition of isolated MPO chlorination activity, they showed structure−function activity for the inhibition of endothelial-localized MPO. Novel nitroxides **1** (ethylenediamine-TEMPO) and **3** (putrescine-TEMPO) (i) avidly accumulated into cultured endothelial cells (Figure 1), (ii) efficiently inhibited H_2O_2 consumption and HOCl production by endothelial-localized MPO (Figures 3−6), and (iii) largely maintained their inhibitory capacity toward MPO in the presence of exogenous heparan sulfates (Figure 5). In contrast, novel nitroxides **4** (cadaverine-TEMPO) and **6** (spermine-TEMPO) poorly inhibited H_2O_2 consumption and HOCl production by endothelial-localized MPO (Figures 3 and 6), which diverged from their capacity to effectively inhibit isolated MPO (Figures 2 and 4). This could be accounted for by the fact that endothelial cell uptake of **4** and **6** was lower than that of **1** or **3** (Figure 1B). Moreover, exogenous addition of heparan sulfates abrogated the inhibitory activity of **4** or **6** toward isolated MPO (Figure 5). This indicates that the HSPG-binding affinity of certain polyamine-conjugated nitroxides is a key determinant of their ability to interact with and inhibit MPO within endothelial cells and subendothelial matrix that is enriched with HSPGs. Consistent with this, putrescine exhibits a lower heparin binding affinity than spermine[40] and exogenous heparin abolished the ability of **6** (spermine-TEMPO) to inhibit MPO's chlorination activity, but to a lesser extent for **3** (putrescine-TEMPO) (Figure 5). Taken together, our data show for the first time that low micromolar concentrations of certain novel polyamine-conjugated (**1** and **3**) and commercial nitroxides (4AT and 4HT) can effectively access and inhibit HOCl production catalyzed by endothelial-localized MPO. We further reveal that the structure−function relationships reflect differences in endothelial internalization, access to MPO's active site (i.e., efficacy of inhibition of isolated MPO), and HSPG-binding affinity.

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While nitroxides were recently identified as a novel class of competitive, "compound II trapping" MPO inhibitors, reservations exist regarding their efficacy as MPO inhibitors in complex biological environments replete with MPO compound II reductants capable of reforming native MPO (Scheme 1 B, reaction 5), which is available to re-enter the halogenation cycle (Scheme 1 B, reactions 1 and 2). Despite this, our data establish that low micromolar concentrations of nitroxides efficiently inhibited MPO within cultured endothelial cells in which endogenous MPO compound II substrates (e.g., ascorbate, $O_2^{\bullet-}$) are available. The efficacy of nitroxides within the complex endothelial environment may reflect the unique catalytic nature of nitroxides as reversible MPO inhibitors[15] and their SOD mimetic activity and hence ability to scavenge $O_2^{\bullet-}$, which can otherwise reduce MPO compound II to limit nitroxides' inhibitory action toward MPO. This advocates that nitroxides have the capacity to effectively inhibit the chlorination activity by endothelial-localized MPO *in vivo*.

MPO is a versatile enzyme that also impacts endothelial function through its nitration reactions that proceed via the enzyme's peroxidase cycles.[7,12] Our studies showed that nitroxides' capacity to inhibit MPO-mediated protein nitration was not related to their ability to inhibit MPO activity and was instead due to direct $^{\bullet}NO_2$ scavenging, a conclusion consistent with Vaz et al.[16,41] With respect to inhibition of protein nitration by endothelial-localized MPO, we found conflicting results in particular for 4AT, which did not inhibit protein nitration by endothelial-localized MPO. While the reasons for this are unclear, the efficacy of nitroxides to prevent protein nitration catalyzed by endothelial-localized MPO likely relates to the rate of reaction of $^{\bullet}NO_2$ with the relevant nitroxide versus alternative biomolecule targets present in endothelial cells, as well as the rate of reaction of nitroxides with other locally produced MPO-derived substrate radicals (e.g., urate radicals).

Finally, MPO NO oxidase activity is regarded as an important oxidative reaction impairing endothelial function.[13] Thus, we examined whether the novel nitroxides inhibited this important reaction. All the nitroxides tested inhibited the NO oxidase activity of MPO in human plasma with similar efficacies. The lack of structure–function activity shown by the various nitroxides supports the conclusion of our recent work[17] indicating that inhibition of MPO's NO oxidase activity by nitroxides in plasma occurs independent of their effects on the turnover of MPO's peroxidase cycle and instead reflects nitroxide scavenging of MPO-derived substrate radicals that otherwise consume NO.[17]

CONCLUSIONS

Piperidine nitroxides possess multiple antioxidant functions. In this study, we show for the first time that functionalized nitroxides can access endothelial-sequestered MPO and efficiently inhibit local oxidative reactions (HOCl production, protein nitration) causally linked to endothelial dysfunction in vascular disease. Nitroxides also inhibit MPO's NO oxidase activity in human plasma. Of the novel nitroxides, ethylenediamine-TEMPO (**1**) and putrescine-TEMPO (**3**) emerged as promising MPO-targeted nitroxides as conjugation of these polyamines to TEMPO uniquely conferred both a high endothelial cell uptake and efficient inhibition HOCl production and protein nitration catalyzed by endothelial-localized MPO, as well as NO oxidation in plasma. This contrasted with a lead agent, 4AT, that strongly inhibited MPO-catalyzed HOCl production but not protein nitration in endothelial cells. Also, the enhanced cellular internalization of **1** and **3** may allow them to be used at lower concentrations *in vivo* than the commonly used 4HT to treat endothelial dysfunction during inflammatory vascular disease. Finally, unlike other polyamine-conjugated nitroxides (**4** and **6**), heparan sulfate binding to **1** and **3** exerted reduced effects on their MPO inhibitory capacity, meaning they retain their efficacy of action in the subendothelium that is rich in HSPGs and where MPO is known to accumulate. Our findings therefore advocate further testing of the protective actions of ethylenediamine-TEMPO (**1**) and putrescine-TEMPO (**3**) in preclinical models of cardiovascular disease where MPO plays a pathogenic role.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.1c00094.

Complete syntheses and characterization of all compounds and compound purity statements (PDF)

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Notes

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■ ABBREVIATIONS

4AT, 4-amino-TEMPO; 4CT, 4-carboxy-TEMPO; 4HT, 4-hydroxy-TEMPO; 3-NO_2Tyr, 3-nitrotyrosine; Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; ctrl, control; Cl^-, chloride; DCM, dichloromethane; EPR, electron paramagnetic resonance; HOCl, hypochlorous acid; HSPGs, heparan sulfate proteoglycans; H_2O_2, hydrogen peroxide; MPO, myeloperoxidase; MT, mito-TEMPO; $^{\bullet}NO_2$, nitrogen dioxide radical; NO_2^-, nitrite; NO, nitric oxide; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; TLC, thin layer chromatography; $Tyr^{\bullet}$, tyrosyl radical; TMB, tetramethylbenzidine; TNB, 2-nitro-5-thiobenzoic acid; TLC, thin layer chromatography

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