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Myeloperoxidase scavenges peroxynitrite: A novel anti-inflammatory action of the heme enzyme

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ABSTRACT

Peroxynitrite, a potent pro-inflammatory and cytotoxic species, interacts with a variety of heme containing proteins. We addressed the question whether (i) the interaction of myeloperoxidase (MPO, an enzyme generating hypochlorous acid from hydrogen peroxide and chloride ions) with peroxynitrite affects the clearance of peroxynitrite, and (ii) if peroxynitrite could modulate the chlorinating activity of MPO. Our results show that this interaction promotes the decomposition of the highly reactive pro-inflammatory oxidant, whereby MPO Compound II (but not Compound I) is formed. The efficiency of MPO to remove peroxynitrite was enhanced by L-tyrosine, nitrite and (−)-epicatechin, substances known to reduce Compound II with high reaction rate. Next, peroxynitrite (added as reagent) diminished the chlorinating activity of MPO in the presence of hydrogen peroxide. Alternatively, SIN-1, a peroxynitrite donor, reduced hypochlorous acid formation by MPO, as measured by aminophenyl fluorescein oxidation (time kinetics) and taurine chloramine formation (end point measurement). At inflammatory loci, scavenging of peroxynitrite by MPO may overcome the uncontrolled peroxynitrite decomposition and formation of reactive species, which lead to cell/tissue damage.

Introduction

Peroxynitrite, a reactive oxidant with a short half-life (approx. 10–20 ms at physiological pH [1,2]), is generated by the diffusion-controlled reaction of nitric oxide (·NO) and superoxide anion (O_2^-) with a rate constant of 1×10^{10} M^{-1} s^{-1} at pH 7.0 [3,4]. ·NO is a relatively stable radical, while O_2^- has much shorter half-life and undergoes spontaneous dismutation with a second-order rate constant of 2×10^5 M^{-1} s^{-1} at pH 7.4 [5].

Peroxynitrite is a potent pro-inflammatory and cytotoxic molecule [6–8], which exerts a variety of inflammatory effects such as inhibition of antioxidants [9] and ion channels [10,11], lipid peroxidation [12,13], thiol oxidation and tyrosine nitration of proteins [14] as well as modulation of cyclooxygenase activity [15,16]. Under inflammatory conditions, the excessive production of ·NO and O_2^- favors elevated levels of peroxynitrite that trigger dysregulation of cellular signaling pathways, activation of inflammatory stress and cell/organ injury in further consequence [17–19]. Peroxynitrite interacts with a variety of redox-active heme proteins including hemoglobin [20], myoglobin [20], catalase [21,22], lactoperoxidase [23], cytochrome P450 [24], nitric oxide synthase [25], cyclooxygenase-2 [26], and myeloperoxidase (MPO)[1] [27,28], respectively.

In activated neutrophils, dimeric MPO contributes to the inactivation and killing of phagocytosed microorganisms [29,30]. Under inflammatory conditions, cationic MPO protein becomes attached to negatively charged proteins and membrane epitopes [30–33]. Most importantly, in the presence of hydrogen peroxide (H_2O_2), MPO catalyzes the oxidation of small molecules including tyrosine, tryptophan, nitrite, ·NO, sulfhydryls, phenol and indole derivatives, xenobiotics and others [34–39]. As reported for the other peroxidases, the heme moiety of MPO undergoes characteristic conversions from the ferric state to the oxo-ferryl states: Compound I (which has an additional porphyryl radical function) and Compound II [40]. A unique property of MPO is its ability to oxidize chloride ions (Cl^-) by the abstraction of two electrons to form hypochlorous acid (HOCl); during this reaction Compound I is reduced to the ferric state [41,42].

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[1] *Abbreviations used:* MPO, myeloperoxidase; H_2O_2, hydrogen peroxide; Cl^-, chloride ions; HOCl, hypochlorous acid; APF, aminophenyl fluorescein; KCN, potassium cyanide; PBS, phosphate buffered saline.

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At inflammatory loci, both, the release of MPO from leukocytes as well as the formation of peroxynitrite might coincide. Peroxynitrite is known to interact with ferric MPO as well as with Compound I, whereby in both cases Compound II is formed [27]. Reports about the functional effects of peroxynitrite on MPO are scarce. Peroxynitrite diminishes the consumption of H_2O_2 by MPO and causes a heme depletion that could be prevented by Cl^- [28]. Any effects of peroxynitrite on the chlorinating activity of MPO remain unknown. The same holds for factors modulating the clearance of peroxynitrite by MPO.

Therefore, we aimed to investigate the effects of MPO on peroxynitrite decomposition in order to determine conditions favouring enhanced removal of peroxynitrite. Furthermore, we addressed the question how peroxynitrite modulates the chlorinating activity of MPO.

Materials and methods

Materials

Peroxynitrite solutions (Cayman-81565, Hamburg, Germany) were prepared to minimize nitrite formation [43]. Stock solutions were prepared in 0.01 M NaOH to avoid protonation and homolytic decay of peroxynitrous acid. The concentration of peroxynitrite solutions was determined by measuring absorbance at 302 nm (ε_{302} = 1705 M^{-1} cm^{-1}) [44]. For experiments, final pH values were measured (using pH meter) and adjusted to 9.0 by adding required volume of peroxynitrite stock solution. Purified human MPO was purchased from Planta Natural Products (Vienna, Austria). For denaturation, MPO was heated to 95 °C for 15 min. H_2O_2 (Sigma–Aldrich-216763, Munich, Germany) was diluted in cold water and the concentration was determined using ε_{240} = 43.6 M^{-1} cm^{-1} [45]. H_2O_2 solutions were used within 2 h after dilution.

5-Amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride (SIN-1, Cayman-82220), aminophenyl fluorescein (APF, Cayman-10157), potassium cyanide (KCN, Sigma–Aldrich-60178), 4-aminobenzoic acid hydrazide (4-ABAH, Sigma–Aldrich-A41909), sodium nitrite ($NaNO_2$, Sigma–Aldrich-71759), taurine (Sigma–Aldrich-T0625), 5,5′-dithiobis(2-nitrobenzoic acid) (TNB, Sigma–Aldrich-D8130), L-tyrosine (Sigma–Aldrich-93829), (−)-epicatechin (Sigma–Aldrich-68097), superoxide dismutase (SOD, Sigma–Aldrich-S7571) and catalase (Sigma–Aldrich-C40) were diluted in 10 mM phosphate buffered saline (PBS, Sigma–Aldrich-P4417, pH 7.4).

Kinetic study of peroxynitrite decomposition

Peroxynitrite decomposition was followed by measuring the absorbance at 302 nm every min at 37 °C for indicated time periods using a Varian Cary 50 UV–vis spectrophotometer (Mulgrave, Australia). The effects of MPO alone (either in its native or denatured form) or in the presence of KCN, 4-ABAH, $NaNO_2$, (−)-epicatechin or L-tyrosine on the decomposition of peroxynitrite were examined. Absorbance values from at least three experiments were recorded and plotted as a function of time. Curves were fitted mono-exponentially to determine the corresponding decomposition rate constants (k_{obs}). The k_{obs} values were averaged and plotted against the proton ($[H^+]$) or MPO concentration to determine corresponding catalytic rate constant (k_{cat}).

Kinetic studies of the APF oxidation by MPO, SIN-1 or peroxynitrite

APF was added to MPO alone or in the presence of SIN-1/peroxynitrite at 37 °C and the formation of fluorescein from APF was measured every 30 s for indicated time periods. Two min after starting the experiments, H_2O_2 was added using an injection device followed by measurement of fluorescence intensities for another 30 min.

Furthermore, the effects of SIN-1/peroxynitrite on APF oxidation were assessed by addition of APF to peroxynitrite or SIN-1 (alone or supplemented with SOD or catalase) at 37 °C followed by recording fluorescence intensities at indicated time periods.

All fluorescence measurements were performed in 96-well plates (a final volume of 250 µl/well) using a fluorescence microplate reader Tecan Infinite 200 PRO (Männedorf, Switzerland). Fluorescence intensity was monitored at 488/522 nm (excitation/emission wavelengths) [46]. For normalization, fluorescence intensity of APF alone was used.

Detection of taurine chloramine

The chlorination of taurine by the MPO–H_2O_2–Cl^- system was followed by measuring formation of taurine chloramine using TNB [47]. Briefly, taurine (10 mM) was pre-incubated with MPO (10 nM in PBS, pH 7.4) in the absence or presence of SIN-1 (10–50 µM in PBS, pH 7.4, kept at 37 °C for 2 h) for 1 min at 37 °C. Then, 25–150 µM H_2O_2 was added to induce the chlorinating activity of MPO. After 5 min the reaction was stopped by addition of 1000 U/ml catalase. After adding 60 µM TNB, formation of taurine chloramine was estimated at 412 nm using ε = 14,100 M^{-1} cm^{-1} [47]. Absorbance values were normalized to added H_2O_2 concentrations to calculate the efficiency of MPO to form HOCl.

Statistics

All values are represented as mean ± SEM and n represents the number of experiments. Statistical significances were tested by Student's *t*-test or one-way ANOVA with adequate post hoc tests (Tukey or Dunnett), using IBM SPSS 20 software. All p values $\leqslant$0.05 were considered statistically significant and all tests were 2-sided.

Results

Influence of pH and MPO on peroxynitrite decomposition

As peroxynitrite is highly unstable at physiological pH [1], we aimed to reveal the decomposition kinetics of this anion under basic conditions. For these experiments 200 µM peroxynitrite was used to get detectable absorbance values. Fig. 1A shows pH-dependent self-decomposition of peroxynitrite. At pH 9.0, about 37% peroxynitrite decomposed during 60 min, while at lower pH (8.0–8.5) faster decomposition was observed. However, at physiological pH peroxynitrite decomposed within a few seconds.

Next, curves from Fig. 1A were fitted mono-exponentially and k_{obs} were calculated. Fig. 1B shows an inverse relation between k_{obs} and pH. At pH 9.0, k_{obs} was 0.01 ± 0.001 min^{-1}, which increased with decreasing pH to 0.376 ± 0.0156 min^{-1} at pH 7.4. However, no linear correlation between $[H^+]$ and k_{obs} values was found (Supplemental Fig. 1A).

Due to the high stability of peroxynitrite at pH 9.0, this condition was chosen to investigate the effects of MPO on peroxynitrite decay. MPO accelerated the decomposition of peroxynitrite in a concentration-dependent manner (Fig. 1C). In all experiments the concentration of Cl^- was 0.14 M. An increase of MPO concentration (up to 40 nM) was paralleled by increased k_{obs} values ranging from 0.013 ± 0.0021 min^{-1} to 0.025 ± 0.0017 min^{-1} at pH 9.0 (Fig. 1D). Data analysis revealed a linear correlation between MPO concentration and k_{obs} resulting a k_{cat} value of 6.25 ± 0.01 × 10^3 M^{-1} s^{-1} for rate-determining step of MPO-derived peroxynitrite decomposition (Supplemental Fig. 1B).