Hypobromous acid and bromamine production by neutrophils and modulation by superoxide

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INTRODUCTION

During inflammation, oxidants are generated by phagocytic white blood cells, such as neutrophils and eosinophils. When stimulated the cells undergo a respiratory burst that consumes oxygen and produces superoxide radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) [1]. MPO (myeloperoxidase) and eosinophil peroxidase are released from neutrophils and eosinophils respectively. The peroxidases use H$_2$O$_2$ to oxidize halides (Cl$^-$, Br$^-$) and thiocyanate (SCN$^-$) to their corresponding hypohalous acids. Eosinophil peroxidase generates mainly hypobromous acid (HOBr) and hypothiocyanite [2-4]. Specificity constants indicate that Cl$^-$ and SCN$^-$ are the major substrates for MPO [5]. On the basis of these specificity constants and rate constants for reactions of substrates with compound I, MPO should use <5% of the H$_2$O$_2$ it reacts with to oxidize Br$^-$ [5,6].

Previous studies have demonstrated that MPO and neutrophils produce HOBr [2,7]. However, the levels detected were low and considered to be inconsequential when compared with those formed by eosinophils. Stimulated neutrophils were shown to brominate nucleobases when stimulated in the presence of Br$^-$.

The authors proposed that the species responsible for bromination was BrCl, formed in a transhalogenation reaction between HOCl and Br$^-$, rather than MPO-generated HOBr [8]. In contrast with these findings, a more recent study has shown that MPO generates significant amounts of HOBr at physiological concentrations of Cl$^-$ (140 mM) and Br$^-$ (100 μM) [9]. In a mouse model of sepsis, levels of 3-bromotyrosine, a marker of HOBr protein modification, were elevated in wild-type mice, but were substantially decreased in MPO-deficient mice. This indicates that bromination was due to MPO [10]. These results suggest that neutrophils may generate HOBr in vivo.

O$_2^-$, which is generated at high rates by both neutrophils and eosinophils, may affect the production of HOBr and its subsequent reactions. O$_2^-$ is known to react with all the redox intermediates of MPO and thereby modulate enzyme activity [11-14]. O$_2^-$ also reacts at diffusion-controlled rates with HOBr to generate bromine radicals (Br*, $k = 3 \times 10^7$ M$^{-1}$ s$^{-1}$) [15,16], suggesting that Br* may be generated by stimulated granulocytes. Given the high concentration of amines (free and protein-bound) at sites of inflammation, HOBr would be expected to generate bromamines and bromamides [17]. These species may in turn react with O$_2^-$ in reactions analogous to those of chloramines [18]. In support of this proposal, the reaction of N-bromosuccinimide with O$_2^-$ is fast and results in the formation of Br* ($k = 4.8 \times 10^6$ M$^{-1}$ s$^{-1}$) [19].

Two studies in the literature support a reaction between O$_2^-$ and HOBr when they are generated by phagocytes [20,21]. McCormick and co-workers demonstrated that stimulated eosinophils produced a reactive radical that required eosinophil peroxidase, O$_2^-$, H$_2$O$_2$ and either chloride or bromide [20]. The data obtained was interpreted in terms of hydroxyl radical (HO$^*$) formation. However, it was acknowledged that bromine radicals might yield an identical spin-trapped product [20]. In a related study, Hazen’s group found that eosinophils and eosinophil peroxidase produced formation of 8-hydroxyguanine in DNA by an analogous reaction that needed bromide, O$_2^-$ and H$_2$O$_2$ [21]. They also proposed that HO$^*$ was formed by a reaction of O$_2^-$ with HOBr. In support of their proposal, others have suggested that O$_2^-$ reacts with HOBr to generate HO$^*$, rather than Br*, in a reaction comparable with that of HOCl with O$_2^-$ [22,23]. However, thermodynamically, the formation of Br* is favoured over HO$^*$ at physiological pH [24].

In the present study, we aimed to determine whether neutrophils are capable of generating HOBr in the presence of physiological...
concentrations of chloride and bromide. We also sought evidence to demonstrate that $\text{O}_2^\cdot$ reacts with HOBBr, bromamines and bromamides. We show that significant amounts of HOBBr are generated by PMA-stimulated neutrophils. An increase in the yield of HOBBr was detected in the presence of SOD (superoxide dismutase) which removes $\text{O}_2^\cdot$. These results can be explained in part by an efficient reaction between $\text{O}_2^\cdot$ and bromamines and bromamides. The induction of biological damage via these pathways may be of considerable importance since bromamines/bromamides and $\text{O}_2^\cdot$ can be generated concurrently with HOBBr at sites of inflammation.

**EXPERIMENTAL**

**Materials**

Hypochlorous acid (Reckitt and Colman) concentrations were determined from its absorbance at 292 nm ($\text{pH} 12$, $\varepsilon_{292} = 350 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [25]. CD16 magnetic beads were from Miltenyi Biotec. SOTS-1 [superoxide thermal source 1; di-(4-carboxybenzyl)hyponitrite] [26] was a gift from Dr D. Harman (Department of Chemistry, University of Wollongong, Wollongong, NSW, Australia). All other chemicals were from either Sigma or BDH. All bromamine studies were performed in 0.1 M phosphate buffer (pH 7.4), with the latter prepared using MilliQ water treated with Chelex resin (Bio-Rad) to remove contaminating metal ions. HOBBr was prepared by mixing equal volumes of 20 mM HOCI and 22 mM NaBr [27].

**Reaction of 4-HPAA (4-hydroxyphenylacetic acid) with HOBBr**

4-HPAA (0.5 mM) was treated with an equal volume of HOBBr (2.5–10 $\mu$M) in the presence of taurine (2.5 mM) in 10 mM phosphate buffer (pH 7.4). Taurine was included to trap HOCl produced by neutrophils; it does not affect bromination of 4-HPAA because taurine bromamine readily brominates 4-HPAA (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/417/bj4170773add.htm). After 5 min at room temperature (22–24°C), thiodipropionic acid (10 mM) was added to stop the reaction. Reaction of 4-HPAA (4-hydroxyphenylacetic acid) with HOBr

4-HPAA (0.5 mM) was incubated with MPO (25 nM) in PBS containing taurine (2.5 mM) and Br$^-$ (20–100 $\mu$M) for 10 min at 37°C, before the addition of PMA (100 ng/ml). Cells were then incubated for a further 30 min with gentle mixing every 5 min. SOD (90 units/ml) was added to some samples before the addition of PMA. Reactions were stopped by the addition of catalse (20 $\mu$g/ml), and cells were placed on ice for 5 min. Cells were pelleted by centrifugation at 14 000 $g$ for 5 min. Aliquots of the supernatants were assayed for 3-bromo-4-HPAA by HPLC.

**Preparation and quantification of chloramines/bromamines**

Taurine chloramine formation by PMA-stimulated neutrophils was quantified by TMB (3,3′,5,5′-tetramethylbenzidine) oxidation in the presence of $\Gamma^-$ [30]. Assays were also performed in the absence of $\Gamma^-$ to determine whether taurine bromamine was formed; the latter reacts directly with TMB.

In experiments where the stability of bromamines was examined in the presence of $\text{O}_2^\cdot$, bromamines were prepared by mixing HOBBr (1 mM) with amine (50 mM) in equal volumes, and the spectra were recorded on a PerkinElmer Lambda 40 spectrometer. TNB (5-thio-2-nitrobenzoic acid) (35–45 $\mu$M), prepared as described previously [31,32], was used to assess residual bromamine concentrations after 15 min using $\varepsilon_{412} 14150 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [33]. None of the reagents used to generate $\text{O}_2^\cdot$ (see below) interfered with the TNB assay.

**Measurement of brominated 4-HPAA by LC (liquid chromatography)–ESI (electrospray ionization)–MS**

LC–ESI–MS analyses were performed in the negative-ion mode with a Thermo Finnigan LCQ Deca XP Plus ion-trap mass spectrometer coupled to a Thermo Finnigan Surveyor HPLC system. A C$_18$, Luna column (150 mm $\times$ 2.0 mm, 5 $\mu$m) was used for separation using 50% methanol supplemented with 0.1% HCOOH as a solvent, at 0.2 ml/min, with 50 $\mu$l of sample injected. 4-HPAA and halogenated products were eluted over 30 min using an isocratic method. The electrospray needle was held at 4500 V. Nitrogen was used as a sheath gas, and set at 42 units. The collision gas was helium. The temperature of the heated capillary was 275°C. The full-scan mass spectrum was monitored at m/z 50–500. Deprotonated parent ions corresponding to 4-HPAA ([M$-$H]$^-$ = 151), 3-chloro-4-HPAA ([M$-$H]$^-$ = 185 and 187, 3:1 ratio) and 3-bromo-4-HPAA ([M$-$H]$^-$ = 229 and 231, 1:1 ratio) were monitored.

**Generation of di-4-HPAA by MPO**

4-HPAA (1 mM) was incubated with MPO (10 nM) in 10 mM phosphate buffer (pH 7.4), in the absence or presence of chloride (100 mM) and/or bromide (100 $\mu$M). Reactions were started by
the addition of H$_2$O$_2$ (7.5 μM). The generation of the dimer of 4-HPAA was detected by fluorescence spectroscopy ($\lambda_{ex}$ 330 nm, $\lambda_{em}$ 405 nm) after 30 min at room temperature. Methionine (1 mM) was present to scavenge any HOCl and HOBr produced.

**O$_2^•^−$-generating systems**

O$_2^•^−$ was generated using the following. (i) The thermal source SOTS-1 (≤ 1.38 mM) which decays spontaneously at 37°C and pH 7.4 to give defined concentrations of O$_2^•^−$ in approx. 40% yield [26,34]. (ii) Potassium superoxide (K$_\text{O}_2$, ≤ 4.54 mM final concentration) in DMSO/50 mM crown-18 polyether at 22°C, pH 7.4. The stock solution of this mixture was infused using a syringe pump into the bromamine/bromamides at 4 μl/min over 90 min for the model compounds and peptides, and 72 μl/min over 5 min for protein-derived bromamines. (iii) An aerobic acetaldehyde (≤ 4.5 mM)–XO (xanthine oxidase) (0.06 unit/ml) system, 22°C, pH 7.4.

**Statistics**

Statistical analysis of the effect of O$_2^•^−$ on bromamines/bromamides compared with controls used one-way ANOVA with Newman–Keuls post-hoc test (GraphPad Prism). Letters that are different indicate statistically distinct results at the P < 0.05 level. Results are means ± S.D. for n ≥ 3 experiments. In neutrophil experiments where data did not fit a normal distribution, the Kruskal–Wallis one-way analysis of ranks was used to determine differences for the effect of O$_2^•^−$ on HOCl compared with HOBr production (SigmaStat).

**RESULTS**

**Production of HOBr by MPO**

4-HPAA was chosen as a trap for HOBr generated by PMA-stimulated neutrophils as it is readily halogenated on its phenolic ring to generate the stable product 3-bromo-4-HPAA (cf. rate constants in [17]). Treatment of 4-HPAA with reagent HOBr resulted in a dose-dependent, linear generation of 3-bromo-4-HPAA over the range 1–10 μM HOBr (see Supplementary Material Figure S1). At concentrations of HOBr >10 μM, the linear relationship was lost, probably due to secondary bromination to give 3,5-dibromo-4-HPAA and other oxidation products (results not shown). This observation is consistent with published rate constants for the bromination of phenols, with secondary bromination, to give the dibromo compound, being approx. 2-fold faster than initial monobromination [17,35].

In experiments where chloride was present in buffers, taurine was also included to scavenge HOCl and prevent it from oxidizing Br$^−$ to HOBr via a transhalogenation reaction [8]. The taurine chloramine, which was produced in this reaction, accumulated and was measured to gauge formation of HOCl. Under the conditions of our assay, taurine chloramine is stable and does not chlorinate phenolic compounds such as 4-HPAA. Taurine also traps HOBr (cf. reaction of N-α-acetyl-lysine and HOBr, $k = 3.6 \times 10^5$ M$^{-1}$ s$^{-1}$ [17]), but in marked contrast with taurine chloramine, the resulting taurine bromamine readily brominates 4-HPAA [7]. Thus, when taurine was present in buffers, it was possible to detect HOCl production by the accumulation of taurine chloramine and HOBr as brominated 4-HPAA. In the presence of 140 mM Cl$^−$, MPO readily brominated 4-HPAA when it was incubated with H$_2$O$_2$ (50 μM) and physiological concentrations of Br$^−$ (20–100 μM). 3-Bromo-4-HPAA was formed at all concentrations of Br$^−$, reaching approx. 6 μM HOBr equivalents with 100 μM Br$^−$ (results not shown). At this concentration approx.

![Figure 1: Generation of HOBr by purified MPO](image)

4-HPAA (0.5 mM) was incubated with MPO (25 nM) in PBS (140 mM Cl$^−$) containing taurine (2.5 mM) and Br$^−$ (20–100 μM). Reactions were started by adding H$_2$O$_2$ (50 μM), in a final volume of 200 μl. After 30 min at room temperature, aliquots (100 μl) were assayed for 3-bromo-4-HPAA by HPLC. The generation of 3-bromo-4-HPAA was expressed as a percentage of the H$_2$O$_2$ consumed by MPO. Results are means ± S.D. for three experiments.

12% of the H$_2$O$_2$ consumed was used to generate 3-bromo-4-HPAA (Figure 1).

**Generation of HOBr by PMA-stimulated neutrophils**

The yield of HOBr generated by neutrophils was examined using cells stimulated with PMA in the presence of 4-HPAA, taurine and Br$^−$. 3-Bromo-4-HPAA was detected under these conditions (Figure 2A, continuous line). The HPLC peak observed from the cells co-eluted with authentic 3-bromo-4-HPAA (Figure 2A, broken line). This peak was confirmed as 3-bromo-4-HPAA, by peak collection and MS. It had the characteristic isotope pattern for 3-bromo-4-HPAA with ions at m/z 229 and 231, in a ratio of 1:1 (Figure 2B).

Neutrophil preparations contain up to 10% eosinophils. As Br$^−$ is readily oxidized by eosinophil peroxidase, it was important to eliminate this enzyme as a possible source of HOBr. Cell preparations were depleted of eosinophils by selective binding to GRN, neutrophils (PMN) or eosinophils (EOS) were then isolated eosinophils generated over twice the granulocyte preparation (Table 1). In the absence of Br$^−$, taurine chloramine accounted for all of the hypohalous acids produced by eosinophils. Isolated eosinophils generated over twice the concentration of 3-bromo-4-HPAA as neutrophils. Formation of 3-bromo-4-HPAA by all cell preparations occurred only in the presence of Br$^−$.

Taurine chloramine formation by neutrophils depleted of eosinophils was quantified to determine the contribution HOBr makes to the total yield of hypohalous acids produced by these cells (Table 1). In the absence of Br$^−$, taurine chloramine accounted for all of the hypohalous acid generated. In the presence of Br$^−$, 3-bromo-4-HPAA accounted for approx. 13% of the total reactive halogen species generated.

To confirm that bromination was due to MPO-generated HOBr, neutrophils were incubated with a range of enzyme inhibitors and oxidant scavengers. In the presence of azide, a haem poison that irreversibly inhibits MPO, no bromination was detected. The competitive substrate SCN$^−$ completely inhibited bromination, as did methionine, a scavenger of HOBr, and diphenylpropyleniodonium, an inhibitor of the neutrophil oxidative burst. Catalase, which
of three experiments.

Results are typical of our experiments with neutrophils, some di-4-HPAA will be formed via production of its radical intermediate. However, it is likely to be a minor product of stimulated neutrophils.

decomposes H₂O₂, also prevented the production of HOBr (results not shown).

4-HPAA is a substrate for compound I of MPO (k = 7.7 × 10⁹ M⁻¹·s⁻¹, for tyrosine [36]) and may compete with Cl⁻ and Br⁻ for reaction with the enzyme to generate a radical species. To assess this, we monitored the formation of di-4-HPAA by MPO in the absence and presence of halides (Supplementary Figure S2 at http://www.BiochemJ.org/bj/417/bj4170773add.htm). In the absence of halides, MPO readily converted 4-HPAA into a fluorescent dimer. The addition of 100 μM Br⁻ resulted in a 30% reduction in the amount of dimer generated. Supplementation of the buffer with 100 mM Cl⁻ in the absence or presence of Br⁻ decreased dimer formation by 80%. The effects of the halides was due to their ability to compete with 4-HPAA for oxidation by MPO. It was not due to reaction of hypohalous acids with the dimer because methionine was included in the buffer to scavenge these oxidants. These results indicate that, under the conditions of our experiments with neutrophils, some di-4-HPAA will be formed via production of its radical intermediate. However, it is likely to be a minor product of stimulated neutrophils.

**Effects of O₂⁻ on hypohalous acid production by neutrophils**

SOD was added to neutrophils to determine whether O₂⁻ influences production of the reactive bromine species by these cells. Removal of O₂⁻ by SOD resulted in an approx. 2-fold increase in bromination (Figure 4). In order to ensure that there was sufficient 4-HPAA present to trap all the HOBr formed, and allow the full effect of SOD to be seen, the yield of 3-bromo-4-HPAA formed in the presence of 0.5–2 mM 4-HPAA was examined. The generation of 3-bromo-4-HPAA was dependent on the concentration of 4-HPAA (Figure 4A). An increase in bromination was seen on increasing the concentration of 4-HPAA from 0.5 to 1 mM, but no further increase was seen with 2 mM 4-HPAA, consistent with saturation at this level. SOD addition resulted in an approx. 2-fold increase in bromination at every concentration of 4-HPAA. This indicates that, under the standard conditions (1 mM 4-HPAA), there was sufficient 4-HPAA present to trap all of the HOBr generated.

The effect of SOD was also examined over the physiological concentration range of Br⁻ in plasma (20–100 μM) [37]. 3-Bromo-4-HPAA was detected at all concentrations within this range (Figure 4B), with SOD increasing the yield of bromination at all concentrations of Br⁻.

We compared the effects of SOD on production of both HOCl and HOBr to understand how O₂⁻ suppresses detection of HOBr.
When cells were stimulated with PMA, they generated 30 μM HOCl [IQR (interquartile range) 24–52; n = 17]. Addition of SOD to neutrophils caused a 1.3-fold (IQR 1.2–1.7; n = 17) increase in their production of HOCl (Figure 5). At the same time, it promoted an increase in production of 3-bromo-4-HPAA was 2.0 (IQR 1.7–2.4; n = 17). O2−/H17033− was added to cells in the absence of taurine, the median increase of 3-bromo-4-HPAA were formed in the absence (3.7 μM; n = 3) and presence of taurine (6.3 μM; n = 3). When SOD was added to cells in the absence of taurine, the median increase in production of 3-bromo-4-HPAA was 2.0 (IQR 1.7–2.4; n = 4) (Figure 5). This increase was not statistically different from that seen in the presence of taurine. These results indicate that O2−/H17033− has a greater effect on the production of reactive bromine species than on production of HOCl. Since production of all the reactive halogen species is reliant on MPO, the greater effect of O2−/H17033− on bromination must be independent of this enzyme.

Stability of bromamines in the absence and presence of O2−/H17033−

In the above experiments, it is possible that SOD enhanced bromination by preventing reactions of O2−/H17033− and taurine bromamine in addition to its effects on HOBr and MPO. Therefore we investigated the potential of O2−/H17033− to promote the decay of bromamines and bromamides.

The stability of taurine and N-α-acetyl-lysine bromamines was investigated at 22 °C and pH 7.4 in the absence and presence of O2−/H17033− for various time periods, using the TNB assay. Under these conditions, both bromamines were long-lived in the absence of added agents (Figures 6 and 7). In contrast, when O2−/H17033− was generated (using either SOTS-1 or a KO2/crown-18 polyether system) the rate of decay of both bromamines was enhanced, with the extent of decomposition dependent on the O2−/H17033− concentration (Figures 6 and 7). Control experiments where individual components of these systems were omitted did not result in enhanced decay rates. The role of O2−/H17033− in the enhanced rate of decomposition of both taurine and N-α-acetyl-lysine bromamines was examined by including SOD (1000 units/ml) in the reaction mixtures where SOTS-1 was used to generate O2−/H17033−. The presence of this enzyme decreased the rate of decomposition of both bromamines (Figure 8), consistent with the enhanced rate of decay being O2−/H17033−-dependent. As SOD did not afford complete protection against bromamine decay, the reaction of H2O2, generated by this enzyme, with bromamines was investigated. Addition of bolus H2O2 (0.5 mM) to solutions of the bromamines resulted in some loss of the bromamine from N-α-acetyl-lysine, but this was significantly less than that detected with the O2−/H17033−-generating system (56% remaining after incubation with H2O2, compared with 8% with O2−/H17033−). H2O2-mediated degradation is therefore a relatively minor and slow process.

Attempts to use the acetaldehyde–XO system to generate O2−/H17033− were unsuccessful as both acetaldehyde and XO alone caused decomposition of taurine bromamine. XO preparations are known to contain trace metal ions [26,38], and these species may be responsible for the decay of bromamine in the presence of enzyme alone. To investigate this possibility, the metal chelator
Figure 6  Decomposition of taurine bromamine over time in the absence and presence of \( \text{O}_2^{**} \)-generating systems

(A) Concentration of taurine bromamine (0.5 mM) detected by assay with TNB after incubation in the absence and presence of the \( \text{O}_2^{**} \)-generating compound SOTS-1 (1.38 mM) at 37°C and pH 7.4 and decomposed SOTS-1 (incubated at 37°C for 24 h before addition to the reaction mixture) for 5 (black columns) or 60 (white columns) min. (B) As (A) except in the absence and presence of an \( \text{O}_2^{**} \)-generating system consisting of \( \text{KO}_2 \) (4.54 mM) in DMSO/50 mM crown-18 polyether at 22°C and pH 7.4 over 90 min.

DETAPAC (diethylenetriaminepenta-acetic acid), was included in the reaction systems (Supplementary Figure S3 at http://www.BiochemJ.org/bj/417/bj4170773add.htm). The addition of DETAPAC did not prevent the decay of taurine bromamine in the presence of XO. This implies that the loss of bromamines with acetaldehyde and XO is independent of metal ions.

Stability of bromamides in the absence and presence of \( \text{O}_2^{**} \)

The generality of \( \text{O}_2^{**} \)-mediated decomposition of N-bromo compounds was explored further using bromamines generated from the side-chain amide of \( N \)-acetylglutamine, the backbone of cyclo-(Ala), and the stable bromamide \( N \)-bromosuccinimide. Incubation of the \( \text{KO}_2 \)/crown-18 polyether system with \( N \)-acetylglutamine bromamide resulted in stimulated decomposition in a concentration-dependent manner at 22°C and pH 7.4. The SOTS-1 system also stimulated decomposition of the cyclo-(Ala)\(_2\) bromamide and \( N \)-bromosuccinimide. SOTS-1 that had been decomposed before its addition (by incubation at 37°C for 24 h) did not stimulate decomposition (results not shown). The effect of the \( \text{KO}_2 \)/crown-18 polyether system on the stability of the bromamides derived from cyclo-(Ala)\(_2\) and succinimide could not be examined due to direct reaction of these N-bromo compounds with DMSO/crown-18 polyether (results not shown). The

Figure 7  Decomposition of \( N \-\alpha \)-acetyl-lysine bromamine over time in the absence and presence of \( \text{O}_2^{**} \)-generating systems

(A) As Figure 6(A) except with \( N \-\alpha \)-acetyl-lysine bromamine (0.5 mM). (B) Dependence of the concentration of \( \text{O}_2^{**} \), generated by a system consisting of \( \text{KO}_2 \) (6.25–50 mM) in DMSO/50 mM crown-18 polyether at 22°C and pH 7.4 over 90 min, on the levels of residual \( N \-\alpha \)-acetyl-lysine bromamine (0.5 mM). Statistical analysis was by one-way ANOVA with Newman–Keuls post-hoc test; different letters indicate statistically distinct results at the \( P < 0.05 \) level. Results are means ± SD, representative of at least three experiments.

Figure 8  Inhibition of \( \text{O}_2^{**} \)-dependent decomposition of \( N \-\alpha \)-acetyl-lysine and taurine bromamines by SOD

Concentration of \( N \-\alpha \)-acetyl-lysine (black columns) and taurine (white columns) bromamines detected by TNB assay after incubation with, or without the \( \text{O}_2^{**} \)-generating compound SOTS-1 (1.38 mM) at 37°C and pH 7.4 for 60 min in the absence or presence of added SOD (1000 units/ml).

potential inhibition of these stimulated decay reactions by SOD could not be determined due to the occurrence of a rapid direct reaction of the N-bromo species with SOD (results not shown).
HOBr (physiological concentrations of Br formed on reaction of HOCl with amine and amide groups [18,40]. These reactions are likely with eosinophil peroxidase [11–14]. O2 reacts with all the redox intermediates of MPO, and similar reconversion with the release of MPO and eosinophil peroxidase concurrently with the release of MPO and eosinophil peroxidase respectively. It could potentially modulate the formation of oxidants by these peroxidases and their subsequent reactions, as it reacts with all the redox intermediates of MPO, and similar reactions are likely with eosinophil peroxidase [11–14]. O2 also reacts rapidly with HOCl (k = 7.5 × 106 M⁻¹s⁻¹) [22,23] and HOBr (k = 3 × 10⁵ M⁻¹s⁻¹) [15,16]. Data have also been presented for the reaction of O2 with chloramines and chloramides, formed on reaction of HOCl with amine and amide groups [18,40].

In the present study, we have quantified HOBr production from physiological concentrations of Br⁻ by both isolated MPO and neutrophils, and have examined how this process is affected by O2. We have shown that, when neutrophils are stimulated with PMA, they generate considerable quantities of HOBr, and, at physiological concentrations of halides, HOBr accounts for at least 13% of the total hypohalous acids they produce. Others have shown that neutrophils produce HOBr at levels similar to those that we measured [2,41]. However, we are the first to demonstrate that production of HOBr is underestimated due to reactions of O2. Thus, neutrophils are capable of generating HOBr at sites of inflammation, and this oxidant is not an exclusive product of eosinophils. Previous investigators have emphasized how eosinophils are much more efficient than neutrophils at generating HOBr [2,41]. Although this is correct, it is now apparent that neutrophils can be a major source of HOBr in vivo because they are generally present in greater numbers at inflammatory sites than eosinophils. For example, inflammation in the airways of children with cystic fibrosis is dominated by neutrophils, and MPO is the major peroxidase present in their bronchoalveolar lavage fluid [42]. We have measured biomarkers of peroxidase activity in bronchoalveolar lavage fluid from children with cystic fibrosis and observed positive correlations between levels of 3-bromotyrosine and neutrophils, MPO and 3-chlorotyrosine. There was no correlation between 3-bromotyrosine and eosinophils (E. Thomson, R. Senthilmohan and A. J. Kettle, unpublished work). These results strongly suggest that the formation of 3-bromotyrosine, a biomarker for HOBr, was a neutrophil-dependent process.

Of particular interest is the observation that the yield of HOBr increased when the cells were stimulated in the presence of SOD to remove O2. This result indicates that O2 plays a key role in modulating HOBr production and its subsequent reactions. Appreciating how O2 affects production of HOBr is reliant on understanding how much O2 is generated by neutrophils and the rates of its reactions with reactive halogen species relative to their other reactions. Under the reaction conditions used in these studies, neutrophils would have generated approx. 200 nM O2/s [43]. Using the kinetic model developed recently to probe oxidant production inside neutrophil phagosomes, this rate of generation would give a steady state concentration of O2 of approx. 600 nM [44]. O2 affects production of HOCl by reacting with MPO, HOCl and taurine chloramine. Most of the HOCl (approx. 99%) would have reacted with taurine (5 mM) to form taurine chloramine (k = 10⁷ M⁻¹s⁻¹) [45]). Only a negligible amount (approx. 1%) would have reacted with O2 to form HO (k = 8 × 10⁵ M⁻¹s⁻¹). The taurine chloramine that accumulated (median = 30 μM) in the reaction systems described in Figure 5 would also have reacted slowly with O2 (k ≈ 5 × 10⁷ M⁻¹s⁻¹) [18]). Therefore, by assuming an average concentration of 10 μM taurine chloramine over the course of the reaction, it can be expected that approx. 5 μM taurine chloramine would have decayed over 30 min by reacting with O2. Consequently, the neutrophils would have produced 35 μM HOCl, although only 30 μM was detected as taurine chloramine. Addition of SOD to the cells would be expected to increase detection of taurine chloramine production to 35 μM or 1.2-fold. This value is close to a 1.3-fold increase in the detection of taurine chloramine when SOD was added to neutrophils (Figure 5). The similarity of these values suggests that reactions of O2 with MPO have only a small influence on generation of hypohalous acids by neutrophils under the reaction conditions we employed.

In the presence of taurine, SOD increased formation of 3-bromo-4-HPAA 1.8-fold (Figure 5). This large increase in detection of HOBr cannot be attributed to the effect that O2 has on the activity of MPO, which was small. Rather, it indicates that O2 reacts with reactive bromine species. Under the reaction conditions, roughly half of the HOBr (approx. 43%) generated by neutrophils would be expected to react with taurine (cf. reaction of N-acetylated-l-lysine and HOBr, k = 3.6 × 10⁷ M⁻¹s⁻¹) [17]), a similar amount (approx. 51%) with O2 (k = 3 × 10⁶ M⁻¹s⁻¹) [15,16]), and the residual (approx. 6%) with 4-HPAA (1 mM; k = 2 × 10⁵ M⁻¹s⁻¹) [17]). Hence, it is kinetically expected that SOD should increase formation of 3-bromo-4-HPAA by preventing the reaction of O2 with HOBr.

On the basis of this kinetic argument, it is plausible that O2 reacts with HOBr formed by neutrophils and eosinophils at sites of infection and inflammation. In support of our proposal, it has been demonstrated that radical products are produced by eosinophils in a reaction that requires eosinophil peroxidase,
H₂O₂, O₂− and bromide [20]. In conjunction with these studies, our results provide compelling evidence that O₂− reacts with bromine species to produce reactive free radicals.

There was an overall increase in the amount of hypohalous acid (H OCI and HOCl, or HOX) generated by the cells when bromide was added to the system (Table 1). Others have shown that HOCl production by PMA-stimulated neutrophils is unaffected by increasing concentrations of bromide (5–100 μM) [2]. Using purified enzyme, van Dalen et al. [5] demonstrated that, at physiological concentrations of chloride, MPO is not saturated and that the addition of bromide increases the turnover rate and efficiency of the enzyme [5]. Hence, the addition of bromide to neutrophils is expected to increase the ability of MPO to convert H₂O₂ into hypohalous acids and explains why HOCl production was not inhibited.

We showed that, under the conditions of our experimental system with neutrophils, 4-HPAA would have been a minor substrate for compound I of MPO that was converted into 4-HPPA radicals. This is expected based on the relative rate constants for reaction of Cl− (k = 2.5 × 10⁷ M⁻¹·s⁻¹ [6]), Br− (k = 1.1 × 10⁶ M⁻¹·s⁻¹ [6]) and 4-HPAA (k = 7.7 × 10⁵ M⁻¹·s⁻¹, the rate for tyrosine [36]) with compound I. The phenoxyl radicals of 4-HPAA would either dimerize or react with O₂− as described previously for oxidation of phenols by MPO in the presence of O₂− [46,47]. They are also likely to react with Br− radicals generated in our experimental system to produce 3-bromo-4-HPAA. Bromamine radicals (E = 1.6 V) are likely to oxidize 4-HPAA and other phenols such as tyrosine to phenoxyl radicals (E = 0.9 V) [48]. Thus it is possible that, under physiological conditions where tyrosine and bromamine radicals are generated by peroxidases, bromination occurs via a radical combination mechanism as well as by direct reaction of HOBr with the phenolate ion of tyrosine.

We have demonstrated that the stability of bromamines/bromamides, generated on reaction of HOBr with amines/amides, is affected by O₂−, with this radical enhancing the decay of the N-bromo species in a SOD-inhibitable manner. These data are consistent with O₂−-playing multiple roles in modulating the concentration and nature of the oxidants generated by MPO at sites of neutrophil activation.

The results obtained in the present study indicate that other radicals may be generated at sites of inflammation in addition to those previously considered. It has been shown that O₂− reacts rapidly with HOBr to generate Br− as a result of one-electron reduction of the O-Br bond and subsequent rapid fragmentation of the transient radical-anion (Reaction 1) [15]. This reaction may account, at least in part, for the lower yield of HOBr detected in the presence of O₂− compared with its absence. The potential occurrence of Reaction 1 is in contrast with the behaviour of HOCl where reduction occurs to give HO− and Cl− [22,23].

\[ \text{O}_2^- + \text{HOBr} \rightarrow \text{O}_2 + [\text{HOBr}]^- \rightarrow \text{HO}^- + \text{Br}^- \quad (\text{Reaction 1}) \]

The enhanced decomposition of bromamines/bromamides in the presence of O₂− is ascribed to a similar one-electron reduction and subsequent fragmentation of the N-Br bond, although whether this occurs via Reaction 2 or Reaction 3 (i.e. whether aminyl/amidyl radicals, RNH• species, or Br• are generated) cannot be established from the current data. Previous studies have provided evidence for the occurrence of an analogous process to Reaction 3 (i.e. aminyl/amidyl radical formation) in the case of chloramines and chloramides incubated with O₂− [18,40,49].

\[ \text{O}_2^- + \text{RNHBr} \rightarrow \text{O}_2 + [\text{RNHBr}]^- \rightarrow \text{RNH}^- + \text{Br}^- \quad (\text{Reaction 2}) \]

\[ \text{O}_2^- + \text{RNHBr} \rightarrow \text{O}_2 + [\text{RNHBr}]^- \rightarrow \text{RNH}^- + \text{Br}^- \quad (\text{Reaction 3}) \]

Both Br• and aminyl radicals are reactive species and can undergo further reactions that may exacerbate biological damage. Bromine radicals can abstract hydrogen atoms, and reaction with excess Br− yields the bromine radical anion (Br•−), which is a powerful one-electron oxidant. Aminyl and amidyl radicals can initiate hydrogen atom abstraction reactions, and are believed to be key intermediates in the fragmentation of proteins [18], carbohydrates [40,49] and extracellular matrix proteoglycans [50,51] induced by HOCl/HOBr. The exact nature of the species generated on decomposition of the bromamines/bromamides is the subject of current investigations. However, our results suggest that RNH• and/or Br• may contribute to oxidative damage induced by activated neutrophils and eosinophils.

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REFERENCES


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26 Ingold, K. U., Young, M. J. and Dorian, L. (1997) Invention of the first azo concept, synthesis and chemical properties. J. Am. Chem. Soc. 119, 12364–12365


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SUPPLEMENTARY ONLINE DATA

Hypobromous acid and bromamine production by neutrophils and modulation by superoxide

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Figure S1 Bromination of 4-HPAA by HOBr in the presence of taurine

4-HPAA (0.5 mM) was treated with an equal volume of HOBr (2.5–10 μM) in the presence of taurine (2.5 mM) in 10 mM phosphate buffer (pH 7.4). After 5 min at room temperature, the reaction was stopped by the addition of 10 mM thiodipropionic acid. Aliquots (100 μl) were assayed for 3-bromo-4-HPAA by HPLC. Results are means ± ranges, representative of three experiments.

Figure S2 Generation of di-4-HPAA by MPO in the absence and presence of halides

4-HPAA (1 mM) was incubated with MPO (10 nM) and H2O2 (7.5 μM) in 10 mM phosphate buffer (pH 7.4) for 30 min at room temperature. The generation of dimer was detected by fluorescence spectroscopy. Reactions were started by the addition of H2O2. Methionine (1 mM) was present to scavenge any HOCl produced. When added, the concentrations of chloride and bromide were 100 mM and 100 μM respectively. Results are means ± ranges, representative of two experiments.

Figure S3 Effect of DETAPAC on the metal-catalysed degradation of taurine bromamine in the absence and presence of an O2•−-generating source

Concentration of taurine bromamine detected by assay with TNB after incubation with, or without the O2•−-generating system comprising 4.3 mM acetaldehyde (AA) and 0.06 unit/ml XO at 22°C and pH 7.4 over 30 min, in the absence or presence of 0.1 mM DETAPAC.

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