Serotonin as a physiological substrate for myeloperoxidase and its superoxide-dependent oxidation to cytotoxic tryptamine-4,5-dione

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During inflammatory events, neutrophils and platelets interact to release a variety of mediators. Neutrophils generate superoxide and hydrogen peroxide, and also discharge the haem enzyme myeloperoxidase. Among numerous other mediators, platelets liberate serotonin (5-hydroxytryptamine), which is a classical neurotransmitter and vasoactive amine that has significant effects on inflammation and immunity. In the present study, we show that serotonin is a favoured substrate for myeloperoxidase because other physiological substrates for this enzyme, including chloride, did not affect its rate of oxidation. At low micromolar concentrations, serotonin enhanced hypochlorous acid production by both purified myeloperoxidase and neutrophils. At higher concentrations, it almost completely blocked the formation of hypochlorous acid. Serotonin was oxidized to a dimer by myeloperoxidase and hydrogen peroxide. It was also converted into tryptamine-4,5-dione, especially in the presence of superoxide. This toxic quinone was produced by stimulated neutrophils in a reaction that required myeloperoxidase. In plasma, stimulated human neutrophils oxidized serotonin to its dimer using the NADPH oxidase and myeloperoxidase. We propose that myeloperoxidase will oxidize serotonin at sites of inflammation. In doing so, it will impair its physiological functions and generate a toxic metabolite that will exacerbate inflammatory tissue damage. Consequently, oxidation of serotonin by myeloperoxidase may profoundly influence inflammatory processes.

Key words: myeloperoxidase, neutrophil, platelet, serotonin (5-hydroxytryptamine), superoxide, tryptamine-4,5-dione.

INTRODUCTION

Myeloperoxidase is a haem enzyme of neutrophils that contributes to host defence and is also implicated in the pathogenesis of numerous inflammatory diseases [1]. It uses hydrogen peroxide to oxidize an array of substrates to either hypohalous acids or free radicals [2,3]. The main reducing substrate for myeloperoxidase is generally accepted to be chloride, which is oxidized to hypochlorous acid. This is one of the most reactive oxidants produced *in vivo*. It is produced during inflammation and has been shown to react with proteins in atherosclerotic plaques [4], and those in the airways of individuals with respiratory diseases [5,6]. Under physiological conditions, thiocyanate is oxidized as readily as chloride and is converted into the weak oxidant hypothiocyanite [7,8]. Other substrates include bromide, tyrosine, ascorbate, nitrite, nitric oxide and a plethora of xenobiotics [2,3]. It is becoming increasingly apparent that production of oxidants from these substrates contributes to the development and progression of cardiovascular disease [9].

Serotonin (5-hydroxytryptamine) has the potential to rival chloride and thiocyanate as a physiological substrate for myeloperoxidase. It undergoes one-electron oxidation by the redox intermediates of myeloperoxidase [10,11]. The rate constants for these reactions are the highest measured for any reducing substrate of myeloperoxidase, [11]. Oxidation of serotonin by myeloperoxidase is physiologically important because this neurohormonal factor is a key neurotransmitter and has been implicated in cardiovascular function [12]. It regulates vascular tone and stimulates cardiomyocytes and chemosensitive nerves. Perturbation of the normal physiological concentration of serotonin can result in cardiovascular dysfunction [12]. Serotonin will be available to myeloperoxidase at inflammatory sites where platelets and neutrophils are activated [13]. This will occur during bacterial infections, thrombosis and inflammatory events within the respiratory system. Serotonin may also be oxidized by myeloperoxidase in the brain, where the enzyme has been shown to be associated with microglia and neurons [14,15].

Currently, it is not known whether serotonin competes with other substrates of myeloperoxidase at physiologically relevant concentrations or what products are formed when it is oxidized. Early work by Wrona and Dryhurst [16] showed that horseradish peroxidase could oxidize serotonin to a complex mixture of products, which included the neurotoxin tryptamine-4,5-dione. Furthermore, stimulated neutrophils convert serotonin into a product that becomes bound to proteins in a reaction that requires myeloperoxidase and hydrogen peroxide [17].

In the present study, we have investigated how readily serotonin is oxidized by myeloperoxidase in the presence of alternative physiological substrates. We show that serotonin is a preferred substrate for myeloperoxidase that inhibits hypochlorous acid production. It is converted into a dimer as well as into tryptamine-4,5-dione.

EXPERIMENTAL

Reagents

Myeloperoxidase was purchased from Planta. Its concentration was determined using a ε_{430} of 89 000 $M^{-1} \cdot cm^{-1}$ per haem. Serotonin (5-hydroxytryptamine hydrochloride), SOD (superoxide

Abbreviations used: DPI, diphenyliodonium; DTPA, diethylenetriaminepenta-acetic acid; ESI, electrospray ionization; LC, liquid chromatography; MRM, multiple reaction monitoring; SOD, superoxide dismutase; TMB, 3,3′,5,5′-tetramethylbenzidine.

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dismutase), catalase, creatine phosphokinase (rabbit muscle) xanthine oxidase, PMA, DL-methionine, taurine, TMB (3,3′,5,5′-tetramethylbenzidine), acetaldehyde, DTPA (diethylenetriamin-epenta-acetic acid) and cytochrome *c* were purchased from Sigma Chemical Co. DMSO was purchased from Merck. All of the reagents used for buffers were of analytical grade. Blood was obtained from healthy human volunteers with informed consent, and with ethical approval from the Canterbury Southern Ethics Committee.

Hydrogen peroxide solutions were prepared by diluting a 30 % stock solution (Merck) and calculating its concentration using a ε_{240} of 43.6 $M^{-1} \cdot cm^{-1}$ [18]. Hypochlorous acid solutions were prepared by diluting a concentrated commercial bleach solution and calculating its concentration using a ε_{292} of 350 $M^{-1} \cdot cm^{-1}$ at pH 12 [19]. Stock solutions of serotonin were prepared daily in water. Tryptamine-4,5-dione was synthesized using the method of Jiang and Dryhurst [20]. Briefly, serotonin was allowed to react with a five molar excess of potassium nitrosodisulfonate in water at room temperature (21 °C) for 25 min to form a dark purple solution. The solution (500 μl) was then added to a C_{18} solid-phase extraction cartridge (500 mg column; Alltech) preconditioned with 3 ml of methanol followed by 2 ml of water. The cartridge was then washed with water to remove salts including excess potassium disulfonate. The purified purple product (tryptamine-4,5-dione) was eluted with 0.1 % formic acid/75 % methanol and concentrated by vacuum centrifugation. Its identity and purity were check by infusion MS.

The stable isotope of [α,α,β,β-$^2H_4$]serotonin was purchased from Medical Isotopes. It was used to prepare the stable isotope of the serotonin dimer by dissolving it at 5 mM in 0.01 M phosphate buffer (pH 7.4) along with horseradish peroxidase (5 μg/ml). Oxidation to its dimer was promoted by three additions of hydrogen peroxide (0.5 mM). The reaction was terminated by adding catalase (5 μg/ml). The solution was ultrafiltered using a Vivaspin column (7500 ***g*** at 15 min) at 4 °C. The filtrate was freeze-dried and then dissolved in an aliquot (50 μl) of water. The sample was separated by HPLC using a Combi-RP column (20 mm × 100 mm) with 5 mM ammonium acetate containing 1 % acetic acid (solvent A). The major peak was collected, and the column was then washed with solvent A/acetonitrile (1:1). The fraction was freeze-dried and stored below −20 °C. The dimer concentration was estimated by measurement of absorbance at 301 nm ($\varepsilon = 8128$ $M^{-1} \cdot cm^{-1}$) [21].

Oxidation of serotonin by myeloperoxidase

The oxidation of serotonin was followed by monitoring its absorption spectrum between 200 and 600 nm using a Beckman DU 7500 diode array spectrophotometer. Recordings were an average of ten readings taken over 1 s. Initial rates of oxidation were determined by calculating the increase in absorbance at 317 nm over the initial linear phase of the reaction. The extent of oxidation was also determined by measuring the loss of serotonin fluorescence (λ_{ex}, 280 nm; λ_{em}, 340 nm) using a Hitachi F4500 fluorescence spectrophotometer.

Detection of hypochlorous acid

Hypochlorous acid produced by either purified myeloperoxidase or stimulated neutrophils was trapped with 5 mM taurine and converted into taurine chloramine. This stable oxidant was detected by the iodide-catalysed oxidation of TMB [22].

Isolation of human neutrophils

Neutrophils were isolated from the blood by Ficoll–Hypaque centrifugation at 2000 ***g*** for 10 min, dextran sedimentation and hypotonic lysis of red blood cells [23]. After isolation, neutrophils were resuspended in 10 mM phosphate buffer, pH 7.4 containing 140 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mg/ml glucose.

Effect of serotonin on oxidant production by stimulated neutrophils

Neutrophils (2×10^6 cells/ml) were pre-incubated at 37 °C in PBS with 5 mM taurine and serotonin for 10 min. The reactions were triggered by addition of 100 ng/ml PMA. Reactions were stopped after 30 min by the addition of catalase (20 μg/ml). Neutrophils were pelleted by centrifugation at 2000 ***g*** for 5 min and accumulated taurine chloramine was measured in the supernatants. Superoxide production was measured as SOD-inhibitable reduction of ferricytochrome *c* [24]. Neutrophils (2×10^6 cells/ml) were pre-incubated for 10 min at 37 °C in PBS with catalase (20 μg/ml) and ferricytochrome *c* (100 μM). The reaction was triggered by the addition of PMA (100 ng/ml) and the rate of change in absorbance at 550 nm over the first 1 min was measured ($\varepsilon_{550} = 21\,000$ $M^{-1} \cdot cm^{-1}$) [24].

Oxidation of serotonin by stimulated neutrophils in plasma

Neutrophils (2×10^7 cells/ml) were pre-incubated at 37 °C in PBS and then added to three times the volume of whole plasma. Serotonin (20 μM) was added to this suspension of cells which was then stimulated with a combination of PMA (1 μg/ml) and cytochalasin B (10 μg/ml). Aliquots (100 μl) were immediately withdrawn and added to 1.5 ml microfuge tubes containing nothing, 10 μM DPI (diphenyliodonium), 1 mM sodium azide, catalase (100 μg/ml), SOD (20 μg/ml) or 1 mM thiocyanate. These cells were maintained at 37 °C with repeated mixing for 30 min, then pelleted by centrifugation at 2000 ***g*** for 5 min. The proteins in the resulting supernatants were precipitated by adding four times the volume of ice-cold acetonitrile containing the stable isotope of the serotonin dimer. Finally, these samples were centrifuged to remove protein and assayed by LC (liquid chromatography)–MS for serotonin and its dimer. To follow the time course for the oxidation of serotonin, aliquots from the suspension of stimulated neutrophils were removed at set time points, and cells and proteins were precipitated as described above.

Detection of serotonin oxidation products and conjugates by HPLC and LC–MS

Reaction products of serotonin were separated by HPLC using a Waters 2690 Separation Module coupled to a diode array detector (Waters 996) and GBC LC1250 fluorescence detector. A Luna C_{18} reversed-phase column (25 mm × 4.6 mm, 5 μm particle size) (Phenomenex) was used to separate serotonin from its oxidation products using gradient elution. The mobile phase consisted of eluant A (1 % acetic acid and 1 mM ammonium acetate in water), and eluant B (100 % acetonitrile). The gradients applied were 0–20 min, 100–85 % eluant A; 20–25 min, 85–50 % eluant A; 25–30 min, 50 % eluant A. The flow rate was 0.8 ml/min. Serotonin oxidation products and glutathione conjugates were also separated on a Luna C_{18} (2) column (150 mm × 2.0 mm, 5 μm particle size) (Phenomenex) using a Surveyor HPLC pump (Thermo Corp.). The column was maintained at 30 °C, and products (20 μl injections) were eluted at a flow rate of 0.2 ml/min using a linear gradient of two solvents: solvent A (5 mM ammonium acetate/1 % acetic acid) and solvent B (100 % acetonitrile). The gradient was as follows: 0–5 min, 0 % solvent B; 5–25 min, solvent B increased to 15 %; 25–30 min, solvent B increased to 50 %; 30–35 min, solvent B maintained at

50 %; 35–37 min, solvent B decreased to 0 %. The HPLC was coupled to an ion-trap mass spectrometer (ThermoFinnigan LCQ Deca XP Plus; Thermo Corp.) equipped with an ESI (electrospray ionization) source. The mass spectrometer was operated with positive ionization using full scan mode (scan range 100–2000 *m*/*z*). Spray voltage was set at 5.0 kV, the capillary temperature at 275 °C and the sheath gas flow at 26 units (instrument units). For MS/MS experiments, parent ions were fragmented in the ion trap using 28 % CID (collision-induced energy).

For analysis of serotonin and its dimer in plasma, samples were analysed using a Dionex HPLC system coupled to an Applied Biosystems/API 4000™ QTrap triple quadrupole mass spectrometer. Chromatography was performed using a TSKgel® Amide 80, 5 μm column 2.0 mm × 150 mm (Tosoh). Solvent A was 0.1 % formic acid in acetonitrile and solvent B was 0.1 % formic acid in water. The initial conditions (4:1 A/B, v/v) were held for 3 min followed by a linear gradient to 1:1 (v/v) A/B over 7 min. These conditions were maintained for 3 min before returning to starting conditions and re-equilibration for 7 min. Analytes were detected using ESI in the positive-ion mode, and data were collected using MRM (multiple reaction monitoring). Nitrogen was used as the collision gas with a dwell time of 150 ms for each species. The mass transition used for detection of serotonin was 159.9 to 115.1 Da. As reported previously, the parent ion for serotonin (*m*/*z* 177 Da) fragmented in the source to give a major ion with a *m*/*z* of 159.9 Da [25]. Mass transitions for the serotonin dimers were monitored for the doubly charged species; i.e. the *m*/*z* ratios were 176–158.9 Da for the serotonin dimer and 180–162.9 Da for its stable isotope. The electrospray voltage and temperature were 5.5 kV and 300 °C respectively. The declustering potential and collision energy were 26 V and 31 eV for serotonin and 36 V and 17 eV for both serotonin dimer species. Serotonin was quantified by comparing its peak area with those obtained for a series of external standards in plasma. Serotonin dimer levels were quantified by including a known concentration of its stable isotope in the acetonitrile extract and comparing their peak areas with those obtained for a series of external standards of the isotope in plasma.

Reaction of serotonin with the redox intermediates of myeloperoxidase

The absorbance spectrum of myeloperoxidase was monitored between 350 and 700 nm during oxidation of serotonin. Reactions were started by adding 50 μM hydrogen peroxide to 1 μM enzyme and 100 μM serotonin in 50 mM phosphate buffer (pH 7.4). Reaction of serotonin with compound II and compound III were also followed by monitoring absorbance changes of these redox intermediates [2,26]. Compound II (λ_{max}, 456 and 630 nm) was formed by adding 100 μM hydrogen peroxide to 1 μM myeloperoxidase (λ_{max}, 430 and 570 nm), followed by addition of 10 μg/ml catalase to scavenge excess hydrogen peroxide. Compound III (λ_{max}, 456 and 625 nm) was formed in a similar manner by adding 1 mM hydrogen peroxide to the enzyme. Spectral changes of the redox intermediates were monitored upon reducing them with 10 μM serotonin.

RESULTS

Oxidation of serotonin by myeloperoxidase and hydrogen peroxide

In agreement with earlier work [10,11], serotonin was readily oxidized by myeloperoxidase and hydrogen peroxide. The difference spectrum of serotonin during oxidation showed two major peaks with maxima at 238 and 317 nm. There was also a broad shoulder between 350 and 450 nm (Figure 1a). There were no absorbance changes in the absence of myeloperoxidase, hydrogen peroxide or serotonin (results not shown). The initial rate of serotonin oxidation increased towards a maximum and the concentration of serotonin that gave 50 % of the maximum velocity was approx. 250 μM (Figure 1b). The rate of serotonin oxidation increased over physiological pH with a 3-fold enhancement at pH 8 compared with pH 5 (Figure 1c). This latter result suggests that the phenolate is the substrate for myeloperoxidase as is the case with tyrosine [27].

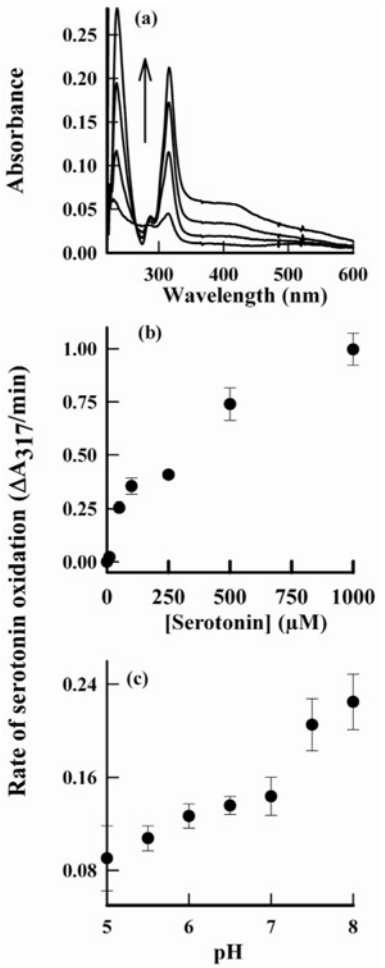


Figure 1 Oxidation of serotonin by myeloperoxidase

(**a**) Reactions were started by adding 50 μM hydrogen peroxide to 10 nM myeloperoxidase, 100 μM serotonin and 100 μM DTPA in 50 mM phosphate buffer (pH 7.4). Spectra of oxidized compared with unoxidized serotonin were recorded at 5, 10, 40 and 120 s after starting the reaction. The arrow indicates the direction of the spectral changes. The effects of (**b**) the concentration of serotonin and (**c**) pH on the initial rate of serotonin oxidation was recorded at 317 nm. Other conditions were as described in (**a**). Results are means ± S.D. for triplicate experiments.

To assess whether serotonin is a potential substrate for myeloperoxidase *in vivo*, we determined how readily chloride and other physiological substrates of myeloperoxidase competed with serotonin and inhibited its oxidation. Chloride was a poor inhibitor of the rate of oxidation of serotonin. At concentrations between 100 and 150 mM, chloride decreased the initial rate of serotonin oxidation by only approx. 15 % (Figure 2a). Other substrates at their physiological concentrations including bromide, urate, nitrite, tyrosine or thiocyanate either alone or all in combination with chloride failed to prevent oxidation of serotonin (Figure 2b). Only ascorbate blocked oxidation.

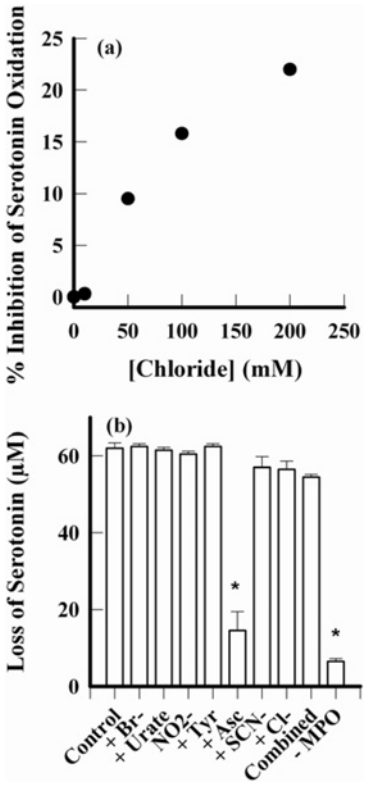


Figure 2 Effect of other alternative substrates on the oxidation of serotonin by myeloperoxidase

(**a**) The initial rate of serotonin oxidation was determined at various concentrations of chloride under the reactions conditions given in Figure 1. Methionine (1 mM) was added to scavenge hypochlorous acid. Results are representative of two separate experiments. (**b**) Alternative substrates of myeloperoxidase were added to myeloperoxidase (MPO) and serotonin under the conditions given in Figure 1. The concentrations of substrates were 100 μM bromide, 500 μM urate, 100 μM nitrite (NO2-), 100 μM tyrosine (Tyr), 50 μM ascorbate (Asc), 100 μM thiocyanate (SCN-) or 100 mM chloride. Reactions were started by adding hydrogen peroxide. After 10 min, the concentration of serotonin was measured by diluting the reaction mixture 100-fold in water and recording its fluorescence (λ_{ex}, 280 nm; λ_{em}, 340 nm). Results are means + S.D. for triplicate experiments. Comparisons with the control were made using Student's *t* test. $^*P < 0.05$.

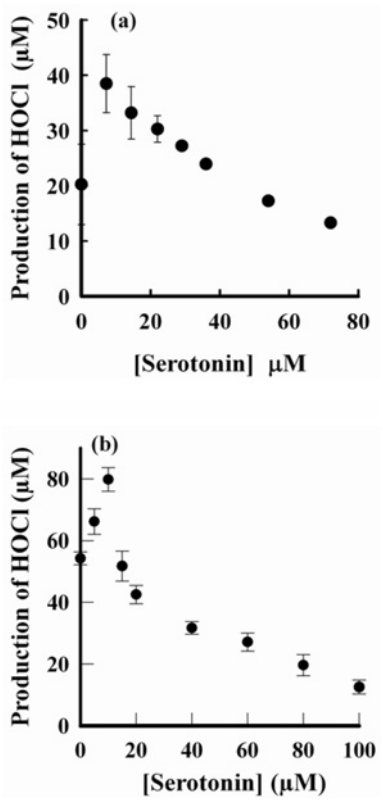


Figure 3 Effect of serotonin on production of hypochlorous acid by myeloperoxidase and human neutrophils

(**a**) Hydrogen peroxide (50 μM) was added to 10 nM myeloperoxidase in PBS containing 5 mM taurine. After 5 min, reactions were stopped by adding 20 μg/ml catalase and the concentration of accumulated taurine chloramine was measured. Results are means ± S.D. for triplicate experiments. (**b**) Neutrophils (2×10^6 cells/ml) were incubated at 37 °C in PBS containing 5 mM taurine and various concentrations of serotoinin. They were stimulated with PMA and, after 30 min, reactions were stopped and the amount of accumulated taurine chloramine was measured. Results are means ± ranges for duplicate experiments and are representative of data obtained for cells from two individuals.

Serotonin had dual effects on hypochlorous acid production by purified myeloperoxidase (Figure 3a) and isolated neutrophils (Figure 3b). Below 20 μM, it enhanced production, whereas at higher concentrations, it progressively inhibited the enzyme. This was not due to serotonin-scavenging hypochlorous acid because, under the conditions of the assay, hypochlorous acid reacted preferentially with taurine. Also, taurine chloramine did not react with serotonin and neither did serotonin reduce oxidized TMB (results not shown). Thus there was no artefactual inhibition of the detection of taurine chloramine. Serotonin did not affect production of superoxide by stimulated neutrophils (results not shown). Consequently, in these two systems, serotonin could have acted only by reacting with myeloperoxidase and influencing its ability to produce hypochlorous acid. Collectively, these results suggest that serotonin is a physiological substrate for myeloperoxidase and should influence production of hypochlorous acid by the enzyme.

Influence of superoxide on the oxidation of serotonin by myeloperoxidase

Myeloperoxidase normally functions in the presence of superoxide [2]. Superoxide is a substrate for the enzyme [28] and also reacts with radicals produced by peroxidases [29]. We therefore wanted to determine whether superoxide influenced the oxidation of serotonin by myeloperoxidase. Superoxide had a marked effect on the changes of the absorption spectrum of serotonin when it was oxidized by myeloperoxidase and a xanthine oxidase system (Figure 4). With the complete system, there was a loss in absorbance at approx. 280 nm and increases in absorbance at 315 and 535 nm (Figure 4a). However, when SOD was added to remove superoxide, the absorbance spectrum was dominated by an increase at approx. 315 nm (Figure 4b). These results indicated that superoxide influenced the type of products formed by myeloperoxidase.

The products derived from serotonin were identified using HPLC and MS (Figure 5). Two main products eluted at approx. 7 (peak 2) and 16 (peak 3) min, whereas unreacted serotonin was detected at 15.5 min (peak 1) (Figure 5a). Glutathione (GSH) was added to the reaction because it forms adducts with quinones. In the presence of GSH, the product at 16 min was replaced by another at 19 min (peak 4, Figure 5a). The earlier eluting product (peak 2) was not affected by GSH. The products were identified by their UV spectra and by MS. Serotonin had an $[M + H^+]$ ion with a *m*/*z* of 177 mass units. The absorbance spectrum of peak 2 had a maximum at 317 nm and a $[M+H^+]$ ion with a *m*/*z* of 176 mass units, which would represent a doubly charged dimer of

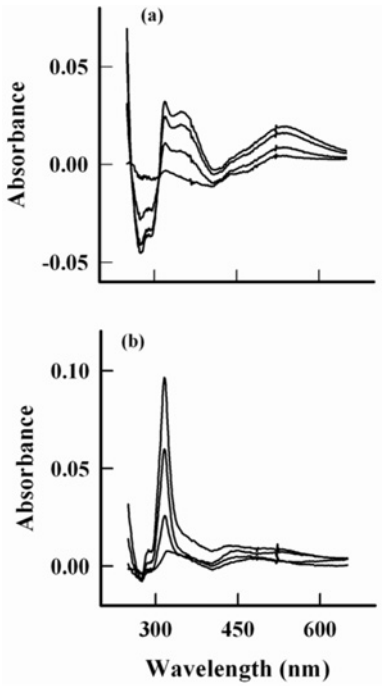


Figure 4 Effect of superoxide on myeloperoxidase-dependent oxidation of serotonin

The difference spectrum of serotonin (100 μM) was monitored after adding xanthine oxidase to 100 nM myeloperoxidase in 50 mM phosphate buffer (pH 7.4) containing 100 μM DTPA and 10 mM acetaldehyde. The rate of superoxide production was 10 $\mu M \cdot min^{-1}$. Reactions were carried out either in the absence (**a**) or presence (**b**) of 20 μg/ml SOD. Scans were recorded 15, 60, 120, 180 and 240 s after adding xanthine oxidase.

serotonin (results not shown). The absorbance spectrum of peak 3 had maxima at 355 and 546 nm (results not shown). This product had a $[M + H^+]$ ion with a m/z of 191 mass units (Figure 5b). These features are consistent with it being tryptamine dione, as characterized previously by Dryhurst and co-workers [20,30]. When it was fragmented in the mass spectrometer, it gave major product ions with m/z ratios of 162 and 173 mass units (Figure 5b). Authentic tryptamine-4,5-dione co-eluted with the product in peak 3 and had the same mass and fragmentation pattern. The product in the presence of glutathione (peak 4) had absorbance maxima at 340 and 545 nm (results not shown) and a $[M+H^+]$ ion with a m/z of 496 mass units (Figure 5c). When authentic tryptamine-4,5-dione was reacted with GSH, it co-eluted with peak 4, had the same absorbance spectrum and a $[M + H^+]$ ion with the same m/z. On this basis, we propose that the product in peak 3 is tryptamine-4,5-dione and that it reacts with GSH to form a conjugate, identified previously by Dryhurst and co-workers as 7-*S*-(glutathionyl)-tryptamine-4,5-dione [20,30].

To understand the mechanism of oxidation of serotonin by myeloperoxidase plus the xanthine oxidase system, we determined the affects of various inhibitors on the loss of serotonin and formation of its dimer and tryptamine-4,5-dione (Table 1). Oxidation of serotonin to the dimer and dione required myeloperoxidase and hydrogen peroxide because omission of this enzyme or addition of catalase and sodium azide inhibited the reaction. SOD had contrasting effects on the reaction. It enhanced formation of the dimer, but inhibited formation of tryptamine-4,5-dione. Glutathione completely prevented production of tryptamine-4,5-dione, but it did not prevent oxidation of serotonin or formation the dimer.

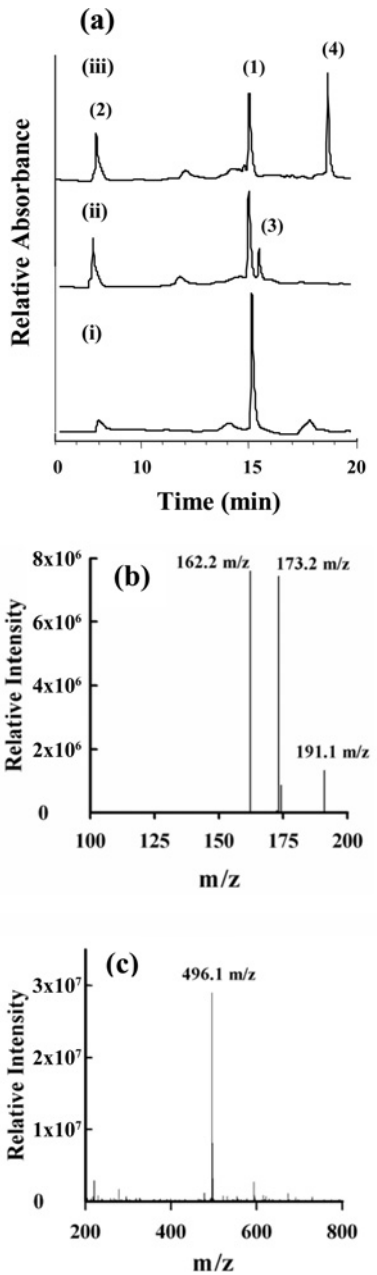


Figure 5 Identification of the oxidation products of serotonin

(**a**) Serotonin was oxidized by myeloperoxidase and its products were separated by HPLC. Reactions were started by adding xanthine oxidase to 10 mM acetaldehyde, 10 nM myeloperoxidase and 100 μM serotonin in 50 mM phosphate buffer (pH 7.4). The flux of superoxide was 10 $\mu M \cdot min^{-1}$. After 5 min, 50 μl of the reaction mixtures were injected directly into the HPLC and products were identified by their UV absorbance at 254 nm. (i) Serotonin only, (ii) complete reaction system, and (iii) complete reaction system + 100 μM GSH. (**b**) MS/MS spectrum of peak 3. The parent ion had a m/z ratio for $[M + H]^+$ of 191 and was fragmented with 35 % collision energy. (**c**) Mass spectrum of the peak 4.

Reactions of serotonin with compound III of myeloperoxidase

We determined the ability of serotonin to reduce compound III because myeloperoxidase is converted into predominantly compound III by stimulated neutrophils [31]. When serotonin was added to pre-formed compound III, it readily reduced the enzyme to ferric myeloperoxidase. In contrast, over the same period, ascorbate, which reduces compound III slowly ($k = 4.0 \times 10^2$ $M^{-1} \cdot s^{-1}$) [32], had little affect on the spectrum of compound

Table 1 Effects of scavengers and inhibitors of myeloperoxidase on oxidation of serotonin

Serotonin (100 μM) was incubated with 10 nM myeloperoxidase (MPO) in 50 mM phosphate buffer (pH 7.4) containing 10 mM acetaldehyde and 100 μM DTPA. Reactions were started by adding sufficient xanthine oxidase to generate 10 $\mu M \cdot min^{-1}$ superoxide. Reactions were assayed after 10 min by separating serotonin, dihydroxybitryptamine and tryptamine-4,5-dione by HPLC. When used, catalase and SOD were present at 20 μg/ml, and the concentrations of sodium azide and glutathione were 100 μM. The data are means ± ranges for duplicate experiments. Each condition for the different variables was compared with the complete system using Student's *t* test. $^*P < 0.05$.

Reaction system	Loss of serotonin (μM)	Formation of serotonin dimer (% of complete system)	Formation of typtamine-4,5-dione (% of complete system)
Complete system	43 ± 7	100 ± 0	100 ± 0
−MPO	6 ± 2*	7.4 ± 0.5*	0 ± 0*
+Catalase	17 ± 6	12 ± 4*	17 ± 3*
+Sodium azide	26 ± 4	6.8 ± 0.7*	6 ± 4*
+GSH	36 ± 5	97 ± 18	0 ± 0*
+SOD	82 ± 14	218 ± 19*	26 ± 8*

Table 2 Effects of scavengers and inhibitors of myeloperoxidase on the oxidation of serotonin by human neutrophils

Neutrophils were pre-incubated for 10 min at 37 °C in PBS with 100 μM serotonin. Methionine (1 mM) was present to scavenge hypochlorous acid. They were then stimulated with PMA. After 20 min, reactions were stopped by adding 20 μg/ml catalase and pelleting the neutrophils. The supernatants were kept on ice until they were analysed by HPLC for serotonin, dihydroxybitryptamine and tryptamine-4,5-dione. When used, catalase and SOD were present at 20 μg/ml, and the concentrations of sodium azide and glutathione were 100 μM. Results are means ± ranges for duplicates and are representative of results for cells from three blood donors. Each condition for the different variables was compared with the complete system using Student's *t* test. $^*P < 0.05$.

Reaction system	Loss of serotonin (μM)	Formation of serotonin dimer (% of complete system)	Formation of typtamine-4,5-dione (% of complete system)
Complete system	39 ± 5	100 ± 0	100 ± 0
−Neutrophils	0 ± 0*	0 ± 0*	0 ± 0*
−PMA	5 ± 3*	6 ± 2*	0 ± 0*
+Catalase	9 ± 4*	20 ± 5*	0 ± 0*
+Sodium azide	17 ± 8	36 ± 6*	0 ± 0*
+GSH	25 ± 8	54 ± 6*	0 ± 0*
+SOD	63 ± 8	125 ± 15	104 ± 8

III (results not shown). These results demonstrate that serotonin is a substrate for compound III.

Oxidation of serotonin by stimulated human neutrophils

We also determined the ability of isolated neutrophils to use myeloperoxidase to promote oxidation of serotonin. Serotonin was incubated with neutrophils which were subsequently stimulated with PMA. Loss of serotonin and formation of its dimer as well as tryptamine-4,5-dione were measured by HPLC (Table 2). Oxidation of serotonin and formation of both products required the presence of stimulated neutrophils and was inhibited by sodium azide and catalase. GSH, which scavenges radicals and hypochlorous acid, prevented formation of dimer by approx. 40 %. In contrast, it completely blocked production of tryptamine-4,5-dione. Methionine, which also scavenges hypochlorous acid, had no effect on the loss of serotonin or formation of the products. SOD did not affect formation of tryptamine-4,5-dione over the 30 min incubation period. However, at a shorter incubation time of 5 min, it inhibited formation of tryptamine-4,5-dione by 54 %. From these results, we conclude that, under physiological conditions, neutrophils oxidized serotonin to its dimer and tryptamine-4,5-dione using myeloperoxidase, hydrogen peroxide and superoxide. Oxidation did not involve hypochlorous acid because it occurred in the presence of methionine. Hence, it must have occurred by direct reaction of serotonin with the redox forms of myeloperoxidase to produce radical intermediates.

To assess the potential for myeloperoxidase to oxidize serotonin *in vivo*, we added serotonin (20 μM) and isolated neutrophils to plasma. The cells were then stimulated with PMA in the presence of cytochalasin B to promote degranulation and release of myeloperoxidase. The oxidation of serotonin and formation of its dimer were followed using LC–MS with MRM (Figure 6a). After approx. 10 min, serotonin dimer was detected in plasma and it accumulated over 1 h of incubation (Figure 6b). Similar kinetics for the loss of serotonin were also observed (results not shown). Formation of serotonin dimer required stimulation of the cells and was inhibited by DPI, which blocks activity of the NADPH oxidase (Figure 6c). Dimer formation was also retarded by the haem poison sodium azide as well as catalase and thiocyanate, which is a substrate for myeloperoxidase. However, SOD had no effect. Collectively, these results demonstrate that activated neutrophils oxidize serotonin in plasma in reactions that involve the NADPH oxidase and myeloperoxidase.

DISCUSSION

In the present study, we have shown that serotonin is a preferred substrate for myeloperoxidase. It is readily oxidized in the presence of physiological concentrations of other major substrates of the enzyme. Furthermore, we have demonstrated that human neutrophils oxidize serotonin in plasma using hydrogen peroxide and myeloperoxidase. Consequently, serotonin and myeloperoxidase are likely to influence each other's activities during inflammation when they are released by platelets and neutrophils respectively. This interaction could either lower the concentration of serotonin and limit its physiological effects or shift oxidant production from hypochlorous acid to serotonin radicals. The fate of these radicals may have a profound impact on inflammation, particularly if they give rise to toxic tryptamine-4,5-dione.

The reactions of serotonin with myeloperoxidase are shown in Scheme 1. The ferric enzyme reacts with hydrogen peroxide to form compound I, which has a formal oxidation state of 5+ [2,26]. This redox intermediate of the enzyme has a high two-electron reduction potential of 1.16 V, which allows it to oxidize chloride to hypochlorous acid [33]. Under physiological conditions, chloride and thiocyanate are equally preferred substrates. Compound I is also one of the strongest one-electron oxidants *in vivo* ($E_0' \approx 1.35$ V) and acts on myriad substrates to produce free radicals [34]. In this reaction, the enzyme is converted into compound II, in which the haem has a formal oxidation state of 4+. Dunford and Hsuanyu [11] were the first to show that serotonin is an exceptional substrate for myeloperoxidase. They determined its rate constants for reduction of compound I and compound II, which are 2×10^7 and 5×10^6 $M^{-1} \cdot s^{-1}$ respectively. We have demonstrated that oxidation of serotonin by myeloperoxidase is optimal at neutral to alkaline pH. At 100 μM, it competed effectively with a host of other substrates for oxidation by myeloperoxidase. At these concentrations, serotonin is kinetically favoured to react with compound I and, as we found, to out-compete other substrates of myeloperoxidase. The exception was chloride, which decreased the oxidation

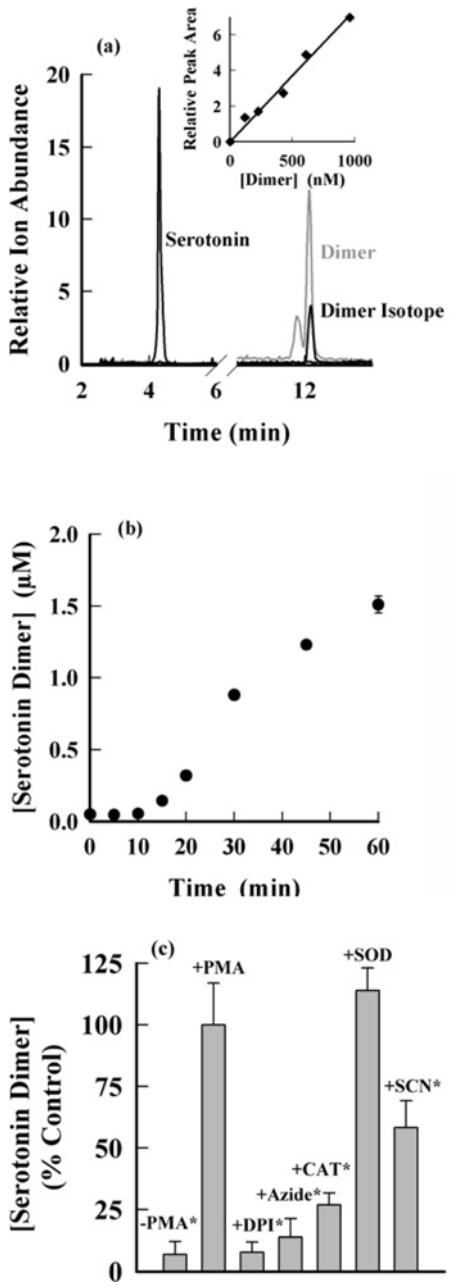
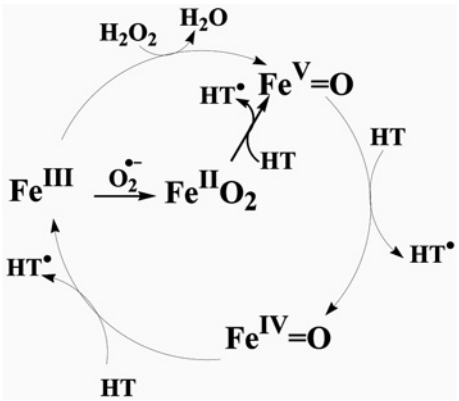


Figure 6 Oxidation of serotonin in plasma by stimulated neutrophils

Neutrophils (5×10^6 cells/ml) were suspended in a solution of 75% plasma and 25% PBS containing 20 μM serotonin at 37°C. Cells were stimulated with PMA (1 μg/ml) and cytochalasin B (10 μg/ml) and the conversion of serotonin into its dimer was monitored by LC–MS. (**a**) A chromatogram showing serotonin (black) and co-elution of dimer (grey) produced by stimulated neutrophils and the synthesized stable isotope of the serotonin dimer (black). The inset shows the linear response of the peak area to the concentration of dimer. (**b**) The time course for conversion of serotonin into its dimer. Results are means ± ranges for duplicate experiments. (**c**) Effect of inhibitors on the production of serotonin dimer after 30 min of stimulation in the presence of 10 μM DPI ($n = 4$), 1 mM sodium azide ($n = 4$), 100 μg/ml catalase (CAT; $n = 3$), 20 μg/ml SOD ($n = 4$) or 1 mM thiocyanate (SCN; $n = 4$). Results are means + S.D. for n experiments performed on two different donors and are representative of cells obtained from three individuals. Comparisons with the stimulated control ($n = 6$) including that for unstimulated cells ($n = 8$) were made using Student's t test. $^*P < 0.05$.

of serotonin at its physiological concentrations. Extracellular concentrations of serotonin are normally at sub-micromolar levels. However, they can reach 100 μM transiently in the immediate vicinity of platelets when they are stimulated [35].



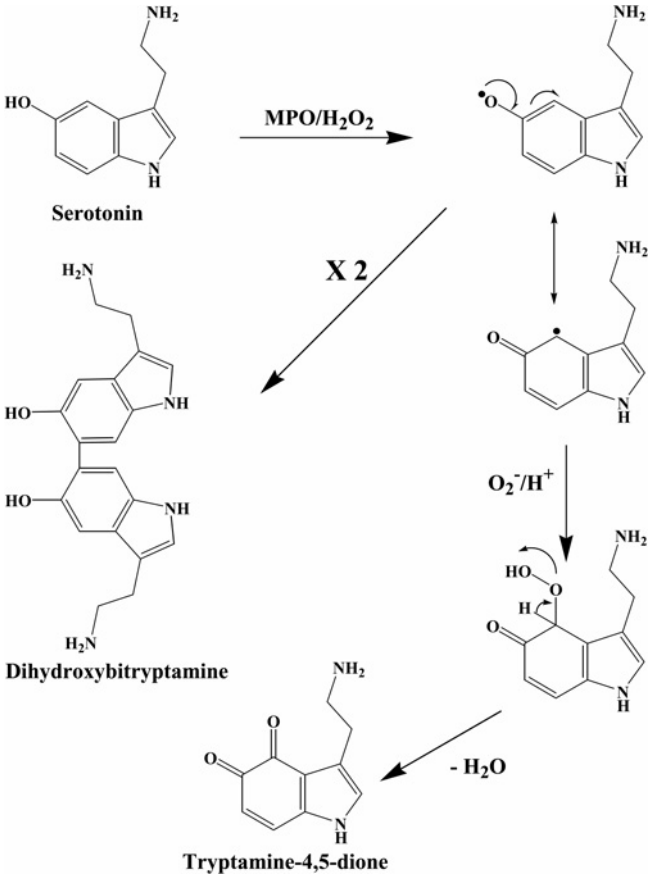
Scheme 1 Reaction of serotonin with the redox intermediates of myeloperoxidase

The redox intermediates of myeloperoxidase are represented by the oxidation states of the haem iron prosthetic group where Fe^{III}, $Fe^{V}=O$, $Fe^{IV}=O$ and $Fe^{II}O_2$ are native myeloperoxidase, compound I, compound II and compound III respectively. HT is 5-hydroxytryptamine or serotonin.

At lower concentrations, we found that serotonin competed poorly with chloride. Rather, it enhanced production of hypochlorous acid by both purified myeloperoxidase and neutrophils. With the isolated enzyme, serotonin would have boosted production of hypochlorous acid by reducing compound II back to the active ferric enzyme, as occurs with superoxide, urate and tyrosine [36]. Enhanced turnover of compound II fails to totally account for the ability of serotonin to increase production of hypochlorous acid by neutrophils. This is because superoxide reacts with myeloperoxidase to convert it into oxymyeloperoxidase or compound III (see Scheme 1) [37], which occurs in stimulated neutrophils [31,38]. Consequently, the rate of production of hypochlorous acid by neutrophils is dependent on the turnover of compound III. We found that serotonin reduced compound III to the ferric enzyme, presumably via intermediate formation of compound I and compound II (Scheme 1). Hence, serotonin is likely to boost production of hypochlorous acid by neutrophils by preventing accumulation of both compound II and compound III.

The reactions of serotonin with compound II and compound III are likely to be the main routes by which serotonin radicals are produced during inflammation. Both of these redox intermediates of myeloperoxidase will be formed *in vivo*. Compound II formation is promoted by numerous physiological substrates and compound III is the predominant form of myeloperoxidase when neutrophils are stimulated [31]. Serotonin should compete effectively for oxidation by compound II because most other substrates for compound II have rate constants 100-fold less than that of serotonin [26].

Serotonin radicals have been shown by Dryhurst and co-workers to undergo a variety of reactions: they can dimerize to form dihydroxybitryptamine [39], disproportionate to produce a quinone imine [40], or couple with reactive thiols [41]. The latter reaction produces superoxide and hydrogen peroxide, which can drive an oxygenase activity of myeloperoxidase toward thiols (see Scheme 2) [10]. The ability of SOD to temporarily inhibit formation of tryptamine-4,5-dione suggests that superoxide reacts with serotonin radical by an addition reaction to produce an unstable hydroperoxide, which would break down to form this toxic species (Scheme 2). The analogous reaction has been shown to occur for tryptophan [42], melatonin [43] and tyrosine



Scheme 2 Oxidation of serotonin by myeloperoxidase (MPO), hydrogen peroxide and superoxide

[29]. Tryptamine-4,5-dione must also form by a superoxide-independent route because of the transient effect of SOD. This probably occurs by conversion of serotonin into an intermediate quinone imine that hydrolyses and is subsequently oxidized as described earlier by Dryhurst [16,44].

Production of tryptamine-4,5-dione during inflammation is likely to have detrimental sequelae. It is known to be neurotoxic [45] and readily forms conjugates with GSH that undergo further oxidation to produce additional toxic species [41,46]. It also reacts with protein thiols and has been shown to inactivate α-oxoglutarate dehydrogenase, pyruvate dehydrogenase [20] and tryptophan hydroxylase [47] by forming adducts with these enzymes.

The interactions of serotonin with myeloperoxidase are likely to be most relevant to cardiovascular disease. Platelets, neutrophils and monocytes have been implicated in the progression of atherosclerosis, as well as plaque rupture and thrombosis [48]. Early work by Clark and Klebanoff [49,50] revealed that neutrophils promote the release of serotonin and other constituents from platelets by a myeloperoxidase-dependent process. Hence, it is likely that, during formation of platelet–leucocyte aggregates at sites of thrombus formation, myeloperoxidase discharged from leucocytes will oxidize serotonin. Both myeloperoxidase [9] and serotonin [12,51] are associated with poor outcome in cardiovascular disease. Whether they collude in this and other diseases awaits the detection of tryptamine-4,5-dione adducts with proteins and glutathione or the presence of dihydroxytryptamine at sites of inflammation.

AUTHOR CONTRIBUTION

Valdecir Ximenes undertook the majority of the experimental work and contributed to the design and writing the paper. Ghassan Maghzal and Rufus Turner undertook investigations using MS. Yoji Kato synthesized the sable isotope for serotonin dimer and developed the HILIC (hydrophilic interaction liquid chromatography) method for its measurement. Christine Winterbourne undertook experiments on reaction of superoxide with serotonin radicals and helped to write the paper. Anthony Kettle undertook experimental work and was responsible for designing the projects and writing the paper.

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