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Indole ring oxidation by activated leukocytes prevents the production of hypochlorous acid

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Abstract

Hypochlorous acid (HOCl) released by activated leukocytes has been implicated in the tissue damage that characterizes chronic inflammatory diseases. In this investigation, 14 indole derivatives, including metabolites such as melatonin, tryptophan and indole-3-acetic acid, were screened for their ability to inhibit the generation of this endogenous oxidant by stimulated leukocytes. The release of HOCl was measured by the production of taurine-chloramine when the leukocytes (2 x 10^6 cells/mL) were incubated at 37ºC in 10 mM phosphate-buffered saline, pH 7.4, for 30 min with 5 mM taurine and stimulated with 100 nM phorbol-12-myristate acetate. Irrespective of the group substituted in the indole ring, all the compounds tested including indole, 2-methylindole, 3-methylindole, 2,3-dimethylindole, 2,5-dimethylindole, 2-phenylindole, 5-methoxyindole, 6-methoxyindole, 5-methoxy-2-methylindole, melatonin, tryptophan, indole-3-acetic acid, 5-methoxy-2-methyl-3-indole-acetic acid, and indomethacin (10 µM) inhibited the chlorinating activity of myeloperoxidase (MPO) in the 23-72% range. The compounds 3-methylindole and indole-3-acetic acid were chosen as representative of indole derivatives in a dose-response study using purified MPO. The IC_{50} obtained were 0.10 ± 0.03 and 5.0 ± 1.0 µM (N = 13), respectively. These compounds did not affect the peroxidation activity of MPO or the production of superoxide anion by stimulated leukocytes. By following the spectral change of MPO during the enzyme turnover, the inhibition of HOCl production can be explained on the basis of the accumulation of the redox form compound-II (MPO-II), which is an inactive chlorinating species. These results show that indole derivatives are effective and selective inhibitors of MPO-chlorinating activity.

Key words
• Leukocytes
• Myeloperoxidase
• Hypochlorous acid
• Indole ring
• Melatonin
• Indole-3-acetic acid

Introduction

Indoles are among the most widely distributed heterocyclic compounds in animals and plants. Tryptophan is an essential amino acid and as such is a constituent of most proteins; it also serves as a biosynthetic precursor for a wide variety of indole-containing metabolites, which are involved in a large and essential number of physiological functions. Besides the well-established hormonal and/or neurotransmitter functions for indoles such as melatonin, indole-3-acetic acid and serotonin, evidence has been emerg-

ing over the last decade in favor of immunomodulatory, antioxidant and antiproliferative roles for these molecules or the enzymatic systems related to their metabolism. In this respect, the depletion of tryptophan in the local cellular microenvironment by the rate-limiting enzyme indoleamine 2,3-dioxygenase has been implicated in the inhibition of cytotoxic T lymphocytes, leading to the immunological tolerance necessary for successful pregnancy (1). By scavenging oxidant species produced by monocytes, serotonin protects natural killer cells from apoptosis (2). The potential physiological effects of melatonin include the ability to directly scavenge toxic free radicals, such as hydroxyl radical (OH), peroxynitrite anion ($ONOO^-$) and hypochlorous acid (HOCl), reducing macromolecular damage in all organs (3). In addition, melatonin prevents the translocation of nuclear factor-kappa B (NF-κ B) to the nucleus and its binding to DNA, thereby reducing the up-regulation of a variety of pro-inflammatory cytokines (4).

The neutrophil enzyme myeloperoxidase (MPO) plays an essential part in the innate immune system by catalyzing the production of HOCl. However, this antimicrobial oxidant has also been implicated as a very harmful agent in an increasing number of inflammatory-mediated disorders (5). Recently, a strong correlation between serum MPO levels and an increased risk of subsequent cardiovascular diseases has been demonstrated in patients with acute coronary syndrome (6). This is suggestive evidence for a role of MPO as a mediator of vascular inflammation and further points toward the significance of neutrophil activation and the generation of oxidants species, including HOCl, in the pathophysiology of vascular and other inflammatory diseases.

The deleterious properties of HOCl have led to a constant search for inhibitors of this endogenous oxidizing enzyme. Compounds such as taurine, methionine, phenolic antioxidants, carbocysteine lysine salt, and melatonin have been described to be potent scavengers of HOCl, and *in vivo* and *in vitro* studies have shown their value in a very large number of experimental models (7-9). Besides acting directly as scavengers, many substances are also able to suppress the production of HOCl by inhibiting the MPO-chlorinating activity. This is the case for melatonin itself, for which an IC_{50} of 10 μM has been reported for chlorinating activity measured via the monochlorodimedone assay (10). This finding points to the promising possibility of indoles as inhibitors of HOCl generation.

These considerations led us to investigate the potential of endogenous and non-endogenous indole derivatives as inhibitors of MPO. Here we document that, irrespective of the substituted group in the indole ring, this class of compounds acted as efficient and selective inhibitors of MPO-chlorinating activity.

Material and Methods

Chemicals

Hydrogen peroxide (30%, w/w) was purchased from Peroxidos do Brazil (São Paulo, SP, Brazil). Myeloperoxidase (EC 1.11.1.7) was purchased from Planta Natural Products (Vienna, Austria). Fetal bovine serum (FBS) was obtained from Cultilab (Campinas, SP, Brazil). Phorbol-12-myristate-13-acetate (PMA), 3,3',5,5'-tetramethylbenzidine (TMB), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), taurine, indole, 2-methylindole, 3-methylindole, 2,3-dimethylindole, 2,5-dimethylindole, 2-phenylindole, 5-methoxyindole, 6-methoxyindole, 5-methoxy-2-methyl-3-indole, indole-3-acetic acid, 2-methyl-5-methoxy-3-indole-acetic acid, indomethacin, tryptophan, melatonin, ascorbic acid, Ficoll-Hypaque 1077 and 1113, RPMI-1640, 2-mercaptoethanol, and streptomycin were purchased from Sigma-

Aldrich Chemical Co. (St. Louis, MO, USA). All the reagents used for buffer preparation were of analytical grade. Hydrogen peroxide was prepared by diluting a 30% stock solution and its concentration was calculated from its absorption at 240 nm ($\varepsilon = 43.6$ M^{-1} cm^{-1}) (11). HOCl was prepared by diluting a concentrated commercial bleach solution and calculating its concentration from its absorption at 292 nm ($\varepsilon = 350$ M^{-1} cm^{-1}) (11). Indole stock solutions were prepared by dissolving the compound in ethyl alcohol. The final concentration of alcohol in the assays was 1% or less. These solutions were prepared daily. PMA was dissolved in DMSO so that its final concentration in neutrophil reactions was 1% (v/v). An MPO stock solution was prepared by dissolving the lyophilized protein in water.

Isolation of human leukocytes

Total leukocytes were isolated from blood of healthy donors by dextran sedimentation (12). After isolation, leukocytes were washed twice and kept in 10 mM phosphate-buffered saline Dulbecco's buffer (PBS-D) supplemented with 1 mg/mL glucose.

Effect of indole derivatives on hypochlorous acid production by leukocytes

Leukocytes (2 x 10^6 cells/mL) were pre-incubated at 37°C in PBS-D supplemented with 1 mg/mL glucose, 5 mM taurine and the indole derivatives or ascorbic acid for 10 min. The cells were then stimulated by the addition of 0.1 µM PMA. After 30 min the reaction was stopped by adding 20 µg/mL catalase. The leukocytes were pelleted by centrifugation and the supernatant was stored on ice. Formation of HOCl was measured by assaying accumulated taurine chloramine. In this assay, the HOCl produced is trapped by taurine, leading to the accumulation of the stable taurine-chloramine compound. The amount of taurine-chloramine was measured by the oxidation of TMB (see below). The production of HOCl by the control with the indole derivatives omitted was used to calculate percent inhibition (13).

Effect of indole derivatives on HOCl production by MPO/H_2O_2/Cl^-

MPO (0.72 µg) was pre-incubated in PBS-D at 37ºC with 5 mM taurine in the presence or absence of the indole derivatives or ascorbic acid. The reaction was started by adding 25 µL of a stock solution of hydrogen peroxide (1 mM). The total reaction volume was 500 µL. After 20 min the reaction was stopped by adding 10 µL of a stock solution of catalase (1 mg/mL). HOCl formation was measured by assaying accumulated taurine chloramine (see below).

Hypochlorous acid assay

The accumulated taurine chloramine was assayed by adding 400 µL of the leukocyte supernatant or 400 µL of the MPO reaction system to 100 µL of a solution containing 14 mM TMB dissolved in 50% dimethylformamide, 100 µM potassium iodide and 400 mM acetic acid. Under these conditions taurine chloramine oxidizes TMB to a blue product with an absorbance maximum at 655 nm. A standard curve was generated by adding pure HOCl to PBS-D containing taurine. Absorbance was measured with a Hitachi (Tokyo, Japan) U-3000 spectrophotometer (13).

Effect of indole derivatives on superoxide production by stimulated leukocytes

Superoxide was measured via superoxide dismutase-inhibitable reduction of ferricytochrome *c*. Leukocytes (3 x 10^6 cells/mL) were pre-incubated for 10 min at 37ºC in PBS-D supplemented with 1 mg/mL glucose plus 20 µg/mL catalase, indole derivatives and 100 µM ferricytochrome *c*. Cells were stimulated by adding 0.1 µM PMA and

Table 1. Indole derivatives inhibit the release of hypochlorous acid by phorbol-12-myristate-13-acetate-activated leukocytes.

Indole derivative	Inhibition of HOCl production (%)
Indole	
10 µM	59 ± 5
50 µM	82 ± 3
2-Methylindole	
10 µM	55 ± 2
50 µM	77 ± 6
3-Methylindole	
10 µM	48 ± 3
50 µM	73 ± 4
2,3-Dimethylindole	
10 µM	62 ± 2
50 µM	75 ± 3
2,5-Dimethylindole	
10 µM	53 ± 7
50 µM	77 ± 6
2-Phenylindole	
10 µM	66 ± 8
50 µM	79 ± 7
5-Methoxyindole	
10 µM	51 ± 4
50 µM	86 ± 5
6-Methoxyindole	
10 µM	72 ± 7
50 µM	85 ± 5
5-Methoxy-2-methylindole	
10 µM	53 ± 2
50 µM	72 ± 8
Melatonin	
10 µM	45 ± 4
50 µM	72 ± 3
Tryptophan	
10 µM	25 ± 4
50 µM	41 ± 3
Indole-3-acetic acid	
10 µM	33 ± 4
50 µM	55 ± 7
5-Methoxy-2-methyl-3-indole-acetic acid	
10 µM	51 ± 3
50 µM	64 ± 5
Indomethacin	
10 µM	23 ± 7
50 µM	45 ± 6
Ascorbic acid	
10 µM	5 ± 2
50 µM	13 ± 4

Data are reported as means ± SD for experiments carried out in triplicate using a pool of leukocytes from 3 healthy blood donors. The production of HOCl by the control was 38 ± 3 mM (N = 9) (2 x 10^6 cells/mL incubated for 30 min). The antioxidant ascorbic acid was included for comparison.

the change in absorbance was measured at 550 nm over the second minute of the reaction. The rate of change in absorbance was converted to rate of release of superoxide anion ($\varepsilon_{550\ nm}$ = 21 mM^{-1} cm^{-1}). The absorbance measurements were made with a Hitachi U-3000 spectrophotometer (14).

Hydrogen peroxide-mediated irreversible MPO inactivation and the protective effect of indole derivatives

MPO (150 nM) was incubated in 100 mM phosphate buffer, pH 7.4, alone or with 50 µM hydrogen peroxide in the presence or absence of the indole derivatives. The amount of peroxidase activity remaining after 30 min was determined by measuring TMB oxidation as follows: the incubated enzyme was diluted 10-fold in 0.5 mL of 50 mM acetate buffer, pH 5.4, containing 1.4 mM TMB. Reactions were started by adding 300 µM hydrogen peroxide and incubated at 37ºC. After 5 min the reactions were stopped by adding 20 µg/mL catalase and 2.25 mL of ice-cold 200 mM acetic acid. Peroxidase activity was measured by recording the increase in absorbance at 655 nm. The absorbance measurements were made with a Hitachi U-3000 spectrophotometer (15).

Effect of indoles on leukocyte viability using the MTT conversion assay

Cell viability was determined using an MTT conversion assay. Leukocytes (6 x 10^6 cells/mL) were suspended in RPMI-1640 containing 5% heat-inactivated FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin and 50 M 2-mercaptoethanol. The suspension (100 µL) was added to each well of a 96-well tissue culture plate and the cells were incubated at 37ºC in a humidified atmosphere containing 5% CO_2. After 1 h, the wells were washed and adhering cells exposed to various concentrations of 3-methylindole or indole-3-acetic acid in RPMI

medium for 1 h. The test was accompanied by a positive viability control (medium + cells). Finally, the MTT assay was performed and absorbance at 540 and 620 nm was determined with a microplate reader (Spectra III Classic, Tecan Austria; Ges.m.b.H, Salzburg, Austria) (16).

Results

The release of HOCl by PMA-activated leukocytes, which were isolated from the blood of healthy donors, was quantified and the effect caused by the addition of 14 different indoles, including melatonin, tryptophan and indole-3-acetic acid, was determined. The average production of HOCl by the control in the absence of indoles was 38 µM. Table 1 shows the inhibition obtained when leukocytes were incubated with 10 or 50 µM indole derivatives.

The property of the indole derivatives as effective inhibitors of the chlorinating activity of MPO was also confirmed by using the purified enzyme. Figure 1 shows the dose-response curve for 3-methylindole and indole-3-acetic acid, which were taken to be representative of the indole moiety. Data for ascorbic acid are shown for comparison.

As indoles are readily oxidized by pure HOCl (17,18), we performed experiments to determine whether the HOCl-scavenging property of these substances could account for the inhibition. For this purpose, pure HOCl (40 µM) was added to the reaction mixture of PBS, taurine and the indoles. Next, the taurine-chloramine produced was measured as above. The lack of an apparent effect shows that, at the concentrations tested here, the indole compounds were not able to compete with taurine for HOCl and also did not react with taurine-chloramine (data not shown). As a consequence, the strong inhibition obtained in the leukocytes and cell-free assays indicates a direct effect on the generation of HOCl.

To determine whether the inhibition of the MPO-catalyzed production of HOCl by indoles was linked to the antioxidant properties of these molecules, the effect of the antioxidant ascorbic acid was compared. As depicted in Table 1 and Figure 1, this compound did not significantly inhibit the chlorinating activity of MPO. However, the classic antioxidant activities of ascorbic acid as a free radical scavenger (DPPH) (19) was higher compared to 3-methylindole and indole-3-acetic acid (Figure 2). This result confirms the relatively poor antioxidant capacities of 3-methylindole and indole-3-acetic acid, which is not important regarding the inhibition of the chlorinating activity of MPO.

The potential of the indoles to be strong inhibitors of MPO-chlorinating activity was not apparent when the peroxidation activity of MPO was determined. In this assay the peroxidation activity was evaluated by the oxidation of TMB in a chloride-free buffer. The presence of 3-methylindole or indole-3-

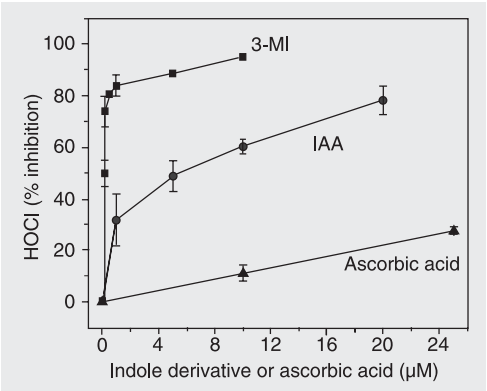

Figure 1. Inhibition of myeloperoxidase (MPO)-generated hypochlorous acid (HOCl) by 3-methylindole (3-MI), indole-3-acetic acid (IAA) and ascorbic acid. The reaction was carried out in 10 mM PBS buffer containing 10 nM MPO, 50 mM H_2O_2, and 5 mM taurine, pH 7.4, at 37ºC. Data are reported as means ± SD of experiments carried out in triplicate.

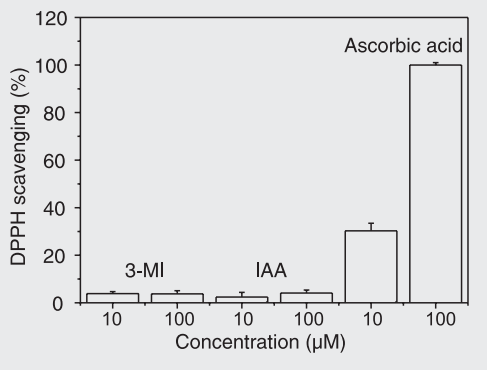

Figure 2. Antioxidant scavenging activity of 3-methylindole (3-MI), indole-3-acetic acid (IAA) and ascorbic acid for 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. Indole derivatives or ascorbic acid were incubated at room temperature in an ethanol solution containing 60 µM DPPH. Absorbance at 517 nm was measured after 30 min and compared with the control, from which test compounds were omitted (19). Data are reported as means ± SD of experiments carried out in triplicate.

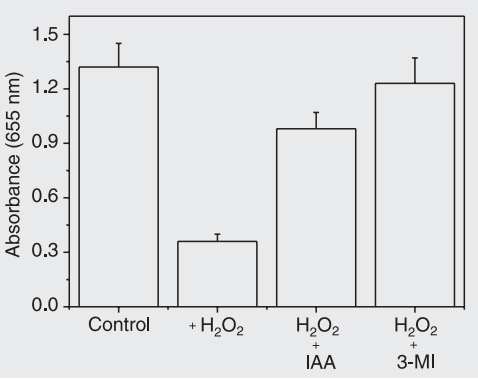

Figure 3. Protective effect of indole derivatives on the irreversible inactivation of myeloperoxidase (MPO) by hydrogen peroxide (H_2O_2). MPO (150 nM) was incubated for 30 min alone (control) or + 50 µM hydrogen peroxide, + 50 µM hydrogen peroxide + 10 µM 3-methylindole (3-MI), and + 50 µM hydrogen peroxide + indole-3-acetic acid (IAA) in 100 mM phosphate buffer, pH 7.4, at 37ºC. The residual MPO peroxidation activity was measured by the oxidation of 3,3',5,5'-tetramethylbenzidine. Data are reported as means ± SD of experiments carried out in triplicate.

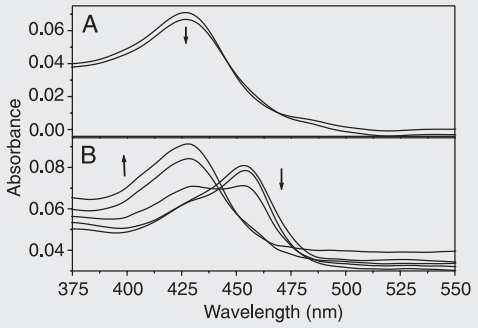

Figure 4. Effect of 3-methylindole on the absorption spectrum of myeloperoxidase (MPO). MPO (1 µM) was incubated with 5 mM taurine in phosphate-buffered saline in the absence (A) or presence (B) of 100 µM 3-methylindole. The reaction was started by adding 50 µM hydrogen peroxide. The spectra were taken at 60 and 300 s (A) or 60, 180, 240, 300, and 600 s (B).

Table 2. Inhibition by indole-3-acetic acid (IAA) and 3-methylindole (3-MI) of the peroxidation of MPO and generation of superoxide anion by phorbol-12-myristate-13-acetate-stimulated leukocytes.

	Peroxidation activity (ΔAbs/min)	Generation of superoxide anion (µM/min)
Control	0.32 ± 0.04	8.2 ± 1.1
+ IAA		
10 µM	0.28 ± 0.06	8.3 ± 0.4
50 µM	0.24 ± 0.06	8.9 ± 1.0
+ 3-MI		
10 µM	0.32 ± 0.04	9.1 ± 0.9
50 µM	0.30 ± 0.05	8.0 ± 1.4

Data are reported as the means ± SD of experiments carried out in triplicate. The enzyme activity was assayed by adding 0.72 µg myeloperoxidase, 1.4 mM 3,3',5,5'-tetramethylbenzidine (dissolved in dimethylformamide) and the indole derivatives to 80 mM phosphate buffer, pH 5.4, at 25ºC (final reaction volume 500 µL). The reaction was started by adding 200 µM H_2O_2. The activity was measured by the absorbance (Abs) change at 655 nm during the first minutes. The generation of superoxide anion was measured by ferricytochrome *c* reduction (see Methods). There was no significant statistical difference between controls and tests (Student *t*-test).

acetic acid (10 or 50 µM) did not have a significant effect on MPO activity (Table 2). Moreover, 3-methylindole and indole-3-acetic acid showed a protective effect on hydrogen peroxide-mediated irreversible inactivation of MPO (Figure 3). In this experiment the enzyme was incubated with hydrogen peroxide in the presence or absence of indoles and the residual peroxidation activity was measured after 30 min and compared to control (15).

To understand the mechanism by which indoles are able to suppress the generation of HOCl, we studied the changes in the absorption spectrum of MPO during the enzyme turnover in the presence or absence of 3-methylindole. In these experiments, MPO (1 µM) was added to 50 µM hydrogen peroxide and 5 mM taurine in PBS buffer. As shown in Figure 4, under steady-state conditions the native MPO (peak at 430 nm) was the form seen spectroscopically when chloride anion was the only substrate. This is actually expected since the redox intermediate MPO-I (compound-I) reacts rapidly with chloride generating HOCl (20). However, the addition of a competitor substrate such as 3-methylindole to the reaction mixture promoted the formation of the redox intermediate MPO-II (compound II; peak at 456 nm), which is unable to catalyze the production of HOCl (20).

Another important parameter when the oxidative function of leukocytes exposed to xenobiotics is evaluated is the ability of these substances to inhibit the generation of or to scavenge superoxide anion. To determine this, the effect of indoles on the reduction of ferricytochrome *c* by PMA-activated leukocytes was measured. The results in Table 2 show that 3-methylindole and indole-3-acetic acid did not interfere with the release of superoxide. Finally, the strong inhibition of the generation of HOCl by stimulated leukocytes cannot be the result of a cytotoxic effect, as demonstrated by the MTT viability assay (data not shown).

Discussion

Neutrophil-released HOCl has been proposed to be the main agent responsible for the antimicrobial action of polymorphonuclear cells. In addition, it is also an important component in terms of the tissue damage that characterizes inflammatory-mediated disorders. For instances, HOCl reacts with unsaturated fatty acids and cholesterol, generating chlorohydrins (21), it promotes the oxidation of proteins through the chlorination of tyrosine and tryptophan residues (22), and mediates the production of protein carbonyls via the breakdown of chloramines to aldehydes (11).

Our results demonstrated that the indole ring is an effective inhibitor of the generation of HOCl and this effect is due to the inhibition of the chlorinating activity of MPO. Indeed, despite the relatively poor selectivity for substrates, which, in fact, is a feature of peroxidases, MPO is unique among these heme-containing enzymes for its ability to catalyze the oxidation of chloride anion to HOCl (23). As a classic peroxidase, MPO catalyzes the one-electron oxidation of many substrates, including polyphenols, aromatic anilines and also indole derivatives such as melatonin, serotonin and tryptophan (24-26). The mechanism underlying these reactions, known as the peroxidation activity of MPO, takes place through the redox intermediate compounds I and II (MPO-I and MPO-II, respectively, reactions 1-3). On the other hand, the oxidation of chloride anion is a two-electron transfer reaction and, as such, only MPO-I is able to promote its oxidation (reactions 1 and 4). This reaction is known as chlorinating activity and, as stated before, is an exclusive property of MPO (23).

$$MPO + H_2O_2 \rightarrow MPO\text{-}I + H_2O \quad \text{(Reaction 1)}$$

$$MPO\text{-}I + SH \rightarrow MPO\text{-}II + {}^{\cdot}S \quad \text{(Reaction 2)}$$

$$MPO\text{-}II + SH \rightarrow MPO + {}^{\cdot}S + H_2O \quad \text{(Reaction 3)}$$

$$MPO\text{-}I + Cl^- + H^+ \rightarrow MPO + HOCl \quad \text{(Reaction 4)}$$

where SH is a peroxidase substrate.

Many substances, including several antioxidants and non-steroidal anti-inflammatory drugs, are able to scavenge and also suppress the generation of HOCl. In the latter case, competition between these substances and chloride anion for MPO-I has been suggested to be responsible for this effect. Additionally, an accumulation of MPO-II is observed, since this intermediate cannot oxidize chloride anion, and the reduction of MPO-II to the MPO native form is the rate-limiting step for peroxidation reactions (27,28). Recently, a stopped-flow kinetic study demonstrated that melatonin and tryptophan react very fast with MPO-I (bimolecular rate constants, $k = 6.1 \times 10^6$ and 4.5×10^5 M^{-1} s^{-1}, respectively), but are poor substrate for MPO-II ($k = 9.6 \times 10^2$ and 6.9×10^0 M^{-1} s^{-1}, respectively) (26). This high reactivity with MPO-I and, conversely, the low reactivity with MPO-II suggest that these indoles may also inhibit the chlorinating activity by accumulating the latter intermediate. Indeed, this property was observed for melatonin itself, for which an IC_{50} of 10 μM was reported for the chlorinating activity measured via the monochlorodimedone assay (10). Here, although we have not performed stopped-flow kinetic studies to measure reaction rates between the indoles and MPO intermediates, our results point in the same direction since all non-endogenous indoles tested were also effective inhibitors of the chlorinating activity. Indeed, we also verified that 3-methylindole was able to lead the enzyme to its inactive chlorinating specie during the oxidation.

The quite similar inhibition pattern obtained for most of the derivatives studied

here is an indication that the indole ring itself is mainly responsible for this effect. However, the substituted groups on the ring also contribute to a lesser extent, as depicted in the dose-response curve that shows a higher inhibition obtained with 3-methylindole than with indole-3-acetic acid. This difference could be attributed to the oxidizability of these molecules, which is obviously linked to reactivity with MPO (27). Supporting this notion, 3-methylindole has a lower reduction potential than indole-3-acetic acid (E^o = 0.65 and 0.92 V, respectively) (29,30) and therefore presents a higher reactivity with MPO.

The absence of an effect when the classic peroxidation activity of MPO was evaluated reinforces the role of indoles as selective inhibitors of the chlorinating activity. In fact, assuming that the inhibition of the chlorinating activity is caused by accumulation of MPO-II, this effect should not be observed when the peroxidation activity is measured, since its accumulation is avoided by the chromogenic substrates used in these assays (TMB in our studies). In agreement with this idea and as would be expected, considering its high affinity for MPO-II, the reductant ascorbic acid was also not an inhibitor of the chlorinating activity (31). The selectivity of the indoles as exclusive inhibitors of chlorinating activity was additionally demonstrated by the fact that these compounds did not inhibit the NADPH oxidase superoxide anion generated by stimulated leukocytes.

Here we also determined whether the inhibition of the chlorinating activity of MPO could be explained by irreversible inactivation of the enzyme. In fact, some substances such as hydrazides are well known for their irreversible inactivation of MPO. This class of compounds promotes the destruction of the heme prosthetic group of the enzyme by reducing the ferrous MPO-hydrogen peroxide complex. 4-Aminobenzoic acid hydrazide, for instance, has been reported to inhibit HOCl production by the purified enzyme, having an IC_{50} value of 0.3 μM (32). The preservation of MPO activity after incubation with 3-methylindole or indole-3-acetic acid shows that this was not the case for these substances. It should also be mentioned that, at the concentration used in our experiments, the indoles were not toxic to the cells. This is important, since 1 mM indole-3-acetic acid has been reported to be toxic to neutrophils (33).

Lastly, considering the well-characterized deleterious effects of HOCl on several biomolecules, the indole moiety should be considered to be a promising candidate in the search for new, reversible and selective MPO inhibitors. Furthermore, these findings should be added to the growing discoveries about the useful properties of indole-related molecules. This is the case for indole-3-acetic acid itself which, in combination with horseradish peroxidase, has been proposed as an enzyme/prodrug combination for cancer gene therapy (34), demonstrating the relevance of indoles in peroxidase-mediated reactions.

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