Biochemical Pharmacology, Vol. 38, No. 1, pp. 161–165, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00
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THE INHIBITORY EFFECT OF ACETAMINOPHEN ON THE MYELOPEROXIDASE-INDUCED ANTIMICROBIAL SYSTEM OF THE POLYMORPHONUCLEAR LEUKOCYTE

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(*Received* 1 *June* 1988; *accepted* 8 *August* 1988)

Abstract—Acetaminophen binds via its acetamido side chain to purified myeloperoxidase in a pH-dependent manner and maximum binding occurred around pH 6. The H_2O_2-dependent myeloperoxidase-catalysed polymerization products of acetaminophen had excitation maxima at 304 nm and 334 nm in acid and alkaline solutions, respectively, and an intense blue fluorescence maximum at 426 nm. Acetaminophen can compete effectively with Cl^- as myeloperoxidase substrate and thus HOCl formation is suppressed while HOCl, nevertheless present, can be scavenged by the drug. In this way the microbicidal action of the myeloperoxidase–H_2O_2–Cl^- system can be seriously limited in the presence of high concentrations of acetaminophen. To study the effect of acetaminophen on peptide bond splitting in the myeloperoxidase antimicrobial system, thyroglobulin was used as a model peptide. Peptide bond splitting was inhibited at acetaminophen concentrations below the accepted toxic range for plasma values.

Reactions catalyzed by myeloperoxidase (MPO)† appear to make major contributions to the microbicidal action of polymorphonuclear leukocytes (PMN) [1].

The oxidised product of MPO, hypochlorous acid (HOCl), which is generated at sites of inflammation, can have multiple effects. It can, for example aggravate tissue damage by inactivating α-antiproteinase and permitting uncontrolled proteinase activity [2, 3], by attacking elastin [4], by depleting ascorbic acid [5] and by activating collagenase and gelatinase [6].

Chlorination of bacteria by the cell-free MPO–H_2O_2–Cl^- system [7] and of macromolecular fractions during PMN digestion of bacteria [8] suggests that the toxic mechanisms may involve direct reaction with HOCl.

Acetaminophen is a widely used analgesic and antipyretic drug. Although therapeutic doses of acetaminophen are reported to be safe, larger doses may result in hepatic necrosis and renal damage [9, 10]. The drug is metabolized by oxidative enzymes, such as cytochrome P-450 and horseradish peroxidase [11] as well as prostaglandin H synthase [12] to reactive intermediates via 1-electron oxidation forming *N*-acetyl-*p*-benzosemiquinone imine or 2-electron oxidation forming *N*-acetyl-*p*-benzoquinone imine.

In this report we have presented evidence that high concentrations of acetaminophen not only inhibit HOCl formation, but can also scavenge available HOCl and thus paralyse the MPO–H_2O_2–Cl^- antimicrobial system of the PMN. Thyroglobulin was used as a model polypeptide to demonstrate the inhibitory effect of acetaminophen on peptide bond splitting in a cell-free MPO–H_2O_2–Cl^- system. For this purpose thyroglobulin is ideally suited, since it is also cleaved by the thyroid peroxidase–H_2O_2–I^- system during thyroid hormone synthesis [13, 14].

MATERIALS AND METHODS

Materials. Acetaminophen (4′-hydroxyacetanilide) was purchased from Sigma and sodium hypochlorite (approx. 1 N in 0.1 N NaOH) from BDH. HOCl was obtained by adjusting NaOCl to pH 6.2 with dilute H_2SO_4 and its concentration determined by the iodometric method using a molar absorption coefficient for KI_3^- of $2.6 \times 10^4\ M^{-1}\ cm^{-1}$ at 355 nm [15].

MPO was isolated from human PMNs as described previously [16] and had an RZ of 0.75. Bovine thyroglobulin was prepared by linear sucrose gradient density ultracentrifugation [17]. Acetaminophen (in 0.1 N NaOH) was coupled via its phenolic group to epoxy-activated Sepharose 6B (Pharmacia) as described by the manufacturers.

Methods. To study the interaction of acetaminophen with MPO, about 1.3 μM of enzyme in 1 ml 10 mM buffer at various pH values was loaded on 30 μl acetaminophen-coupled Sepharose 6B. This MPO concentration was sufficient to saturate the substituted gel at any pH studied. After washing the gel with 5 ml of the appropriate buffer, bound enzyme was eluted with 0.5 M NaCl in buffer.

Peroxidase activity was measured by the guaiacol assay. One unit of activity was defined as that amount of enzyme effecting a change of 1 absorbance unit per minute at 470 nm. Protein concentration was measured by the microbiuret method of Goa as described by Brewer *et al.* [18].

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† Abbreviations used: MPO, myeloperoxidase; PMN, polymorphonuclear leukocytes; SDS, sodium dodecyl sulphate.

Spectrophotometric measurements were made with a Cary 219 recording spectrophotometer and fluorescence monitored on a Perkin Elmer MPF-44 A fluorescence spectrophotometer.

SDS–polyacrylamide gel electrophoresis was performed according to Laemmli [19]. A linear gradient 4–10% gel was used and a constant current of 40 mA was applied.

Polymerization products formed in the H_2O_2-dependent MPO-catalysed oxidation of acetaminophen were analyzed by thin layer chromatography on silica plates in an ammoniated formaldehyde dimethyl acetal system. The products, which could be located by their fluorescence under ultraviolet light, were removed and extracted in absolute ethanol.

RESULTS

The binding of acetaminophen to MPO is pH-dependent. Figure 1 demonstrates that the capacity of acetaminophen-coupled Sepharose 6B for MPO was highest near pH 6. At this pH, about 5 mg MPO/ml substituted gel was retained and could be eluted with 1 ml 0.5 M NaCl.

One electron oxidation of acetaminophen leads to the formation of phenoxyl radicals, which are precursors of biphenols and other products. The fluorescence characteristics of polymers formed during MPO-catalyzed oxidation of acetaminophen are shown in Fig. 2. At acid pH (pH 6) the products had excitation and emission maxima at 304 nm and 426 nm, respectively. At alkaline pH, the excitation maximum shifted to 334 nm (results not shown). The fluorescence characteristics of the acetaminophen biphenol, isolated by thin layer chromatography, were indistinguishable from that of the total reaction mixture.

The extent of polymer formation was also studied in the presence of chloride (Fig. 3). At physiological NaCl concentrations (0.154 M) the fluorescence intensity of polymers formed from 80 μM acetaminophen was about 60% of what it was in the absence of chloride.

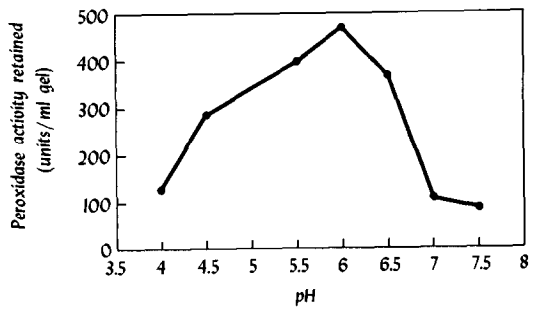

Fig. 1. The effect of pH on binding of acetaminophen to MPO. Aliquots of enzyme (1.3 μM) in 10 mM buffer (acetate pH 4–pH 5; phosphate pH 5.5–pH 7.5) were applied to a column containing 30 μl acetaminophen-coupled Sepharose 6B previously equilibrated in the appropriate buffer. The column was washed with 5 ml buffer and bound MPO eluted with 1 ml buffer containing 0.5 M NaCl. Peroxidase activity, determined by the guaiacol assay, is expressed as units/ml gel.

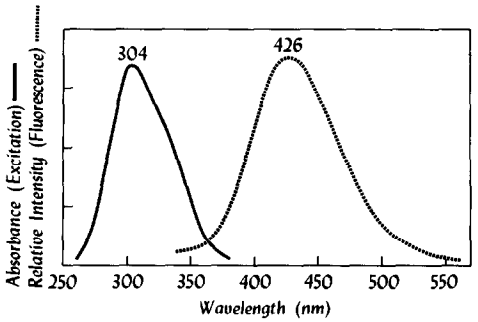

Fig. 2. Excitation and fluorescence spectra of polymers formed during MPO-catalyzed oxidation of acetaminophen. The cuvette contained 67 nM MPO and 80 μM acetaminophen in 50 mM phosphate buffer (pH 6.0). The reaction was initiated by the addition of H_2O_2 (250 μM final concentration) to the cuvette.

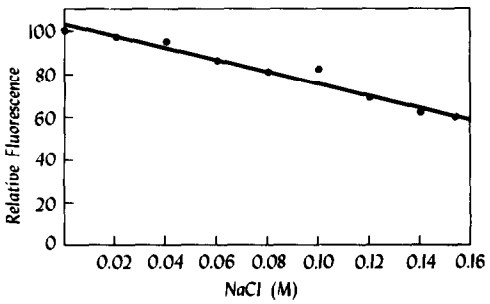

Fig. 3. MPO-catalysed acetaminophen polymer formation in the presence of chloride. The cuvette contained 67 nM MPO and 80 μM acetaminophen in 50 mM phosphate buffer (pH 6.0). Fluorescence intensity (excitation and emission wavelengths 334 nm and 426 nm, respectively) of the polymers formed, were determined 2 min after the addition of H_2O_2 (250 μM final concentration) to the cuvette.

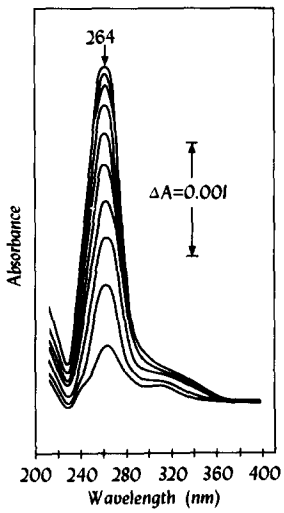

Fig. 4. Reaction of HOCl with acetaminophen. For the difference spectrum, the sample and reference cuvettes contained 90 μM acetaminophen in 50 mM phosphate buffer (pH 6.0). Reaction was initiated by the addition of HOCl (50 μM final concentration) to the sample cuvette and the reaction monitored at 2 min intervals.

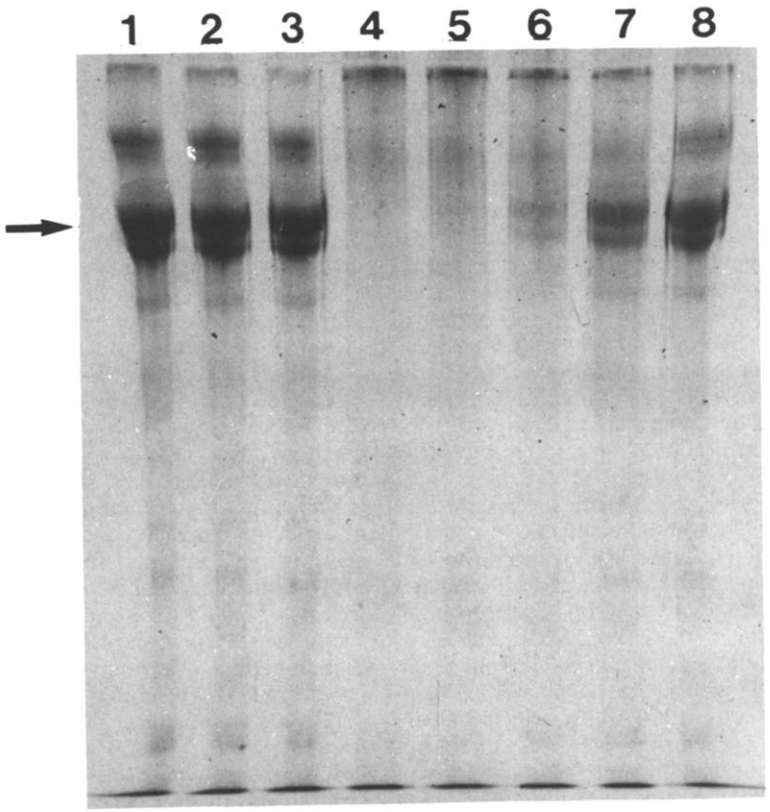

Fig. 5. SDS–polyacrylamide gel electrophoresis (4% to 10% linear gradient gel) demonstrating the influence of acetaminophen on thyroglobulin (TG) fragmentation in a MPO–H_2O_2–Cl^- system. Degradation of TG (1.5 μM) was achieved by incubation with 70 nM MPO in 50 mM phosphate buffer containing 0.154 M NaCl. The reaction was initiated by the addition of H_2O_2 (500 μM final concentration) and proceeded for 10 min at 37°. Before electrophoresis, the samples were treated with SDS (final concentration 1%) reduced by boiling for 10 min in 5% 2-mercaptoethanol and 50 μl aliquots were applied to the gel. A constant current of 40 mA was applied for approximately 5 hr. The gel was stained with 0.02% Coomassie Brilliant Blue R-250 and destained with acetic acid/methanol/water (1:2:7, v/v). The following mixtures were analysed: Lanes 1:TG; 2: TG + MPO; 3: TG + MPO + 3.3 mM acetaminophen + H_2O_2; 4: TG + MPO + NaCl + H_2O_2; lanes 5 to 8 contained TG and 0.3 mM, 0.7 mM, 1.3 mM and 3.3 mM acetaminophen, respectively, in the MPO–Cl^-–H_2O_2 system. The TG half-molecule indicated by the arrow, has a molecular mass of 330 kDa [17].

The direct effect of 50 μM HOCl on 90 μM acetaminophen at pH 6 is shown in the difference scan of Fig. 4. Repetitive overlay scans were taken at 2-min intervals. Two minutes after starting the reaction, a difference peak with maximum at about 264 nm was present. This peak reached its maximum after about 20 min.

Since HOCl is rapidly consumed by bacterial components and this results in fragmentation of peptides [7], we have used thyroglobulin as a model polypeptide to study bond splitting. In the SDS–polyacrylamide gel shown in Fig. 5, it is evident that acetaminophen oxidation intermediates and/or products had no effect on thyroglobulin structure (Fig. 5, lane 3). In the absence of acetaminophen, the MPO–H_2O_2–Cl^- system caused complete fragmentation of thyroglobulin (Fig. 5, lane 4). Addition of acetaminophen to concentrations of 0.3 mM, 0.7 mM, 1.3 mM and 3.3 mM to the HOCl-generating system (Fig. 5, lanes 5–8, respectively) leads to the increasing conservation of thyroglobulin structure. At a concentration of 3.3 mM acetaminophen, thyroglobulin was largely protected.

DISCUSSION

MPO is able to chlorinate a wide range of substrates via the formation of hypochlorous acid. However, activities with different substrates are strongly dependent on the concentrations of H_2O_2 and halide as well as the pH of the medium [20]. Weiss and coworkers [21] concluded from their studies with neutrophils that about 40% of the detected H_2O_2, was utilized by the MPO system to generate HOCl.

Since HOCl has a damaging effect on bacterial membranes, any substance interfering with HOCl production and/or scavenging available HOCl, will have a detrimental effect on the microbicidal system of the PMN. In this study it is shown that MPO has an affinity for acetaminophen (Fig. 1). The affinity gel was prepared by coupling acetaminophen via its phenolic moiety to epoxy-activated Sepharose 6B. Interaction of MPO with acetaminophen must therefore involve the free acetamido side chain of the immobilised drug. The fact that acetaminophen binding was pH-dependent and that retained MPO could be released by increasing the ionic strength (i.e.

addition of NaCl) of the eluting buffer, suggests an ion-exchange phenomenon.

Davis and Osawa [22], in their studies on the binding of thyroid hormones to haemo-proteins, concluded that the amino-group of the thyronine side chain was essential for binding and that the free propionates of the haem prosthetic group of the protein was the most likely site of interaction. Something similar may be the case with the MPO–acetaminophen interaction. Immobilised acetaminophen binds via its acetamido group also to hematin (van der Walt and van Zyl, unpublished observation). At pH values between 4.5 and 6.5, where most of the binding to MPO occurred, the amido group of acetaminophen should be sufficiently protonated to allow ionic interaction with the negatively charged propionate groups of the MPO haem.

It has been reported that the intraphagosomal pH of stimulated PMNs is initially alkaline, but that a gradual acidification occurs reaching a value around pH 6 after 1 or 2 hr [23, 24]. At this pH, a protonated polypeptide may interact with MPO haem to bring it in closer contact with the site of HOCl generation. In view of the above reasoning, acetaminophen may effectively compete with a bacterial membrane polypeptide for binding to MPO. HOCl, which is freely diffusable from the enzyme [25] will then preferentially react with acetaminophen rather than with the invading micro-organism. Indeed, HOCl reacts with acetaminophen as is evident from the difference spectrum in Fig. 4. Furthermore, a concentration of HOCl (50 μM) was chosen, which may be physiologically relevant [26]. Wasil *et al.* [27] have recently reported the ability of acetaminophen to inhibit the inactivation of α_1-antiprotease by HOCl. They, however, concluded that the reaction rate between acetaminophen and HOCl was too slow under physiological conditions to protect important biological targets against HOCl.

The formation of 2,2′-biphenols due to dimerization and enolization of tyrosine phenoxyl radicals was proposed decades ago from investigations on the peroxidase-induced oxidation of tyrosine [28]. The most characteristic features of 2,2′-biphenols are their strong absorption at 316 nm and 280 nm in basic and acid media, respectively [28], and the intense fluorescence near 410 nm [29]. Although several polymers of acetaminophen have been isolated following reaction with horseradish peroxidase and H_2O_2, the dimer (4′,4′″-dihydroxy-3′,3′″-biacetanilide) as well as higher polymers seem to be the major products [30]. In the horseradish system, disproportionation of the semiquinone imine can also take place to form *N*-acetyl-*p*-benzoquinone imine, but polymerization seems to be the major pathway [11]. To the best of our knowledge, however, studies on the oxidative metabolism of acetaminophen do not include fluorescence data. With the fluorescence characteristics of the polymers (Fig. 3) at hand we could study substrate competition between chloride and acetaminophen. When 80 μM acetaminophen was oxidised in the MPO–H_2O_2 system in the presence and absence of 0.154 M NaCl, the fluorescence yield with Cl^- was still 60% of that without Cl^-. Thus, even at a Cl^- to acetaminophen molar ratio of nearly 2000, acetaminophen seems to be preferentially oxidised.

From the SDS–PAGE data, the protective action of high concentrations of acetaminophen on thyroglobulin fragmentation in the MPO–H_2O_2–Cl^- system is evident. A clear inhibition of thyroglobulin breakdown is noted at acetaminophen concentrations as low as 0.7 mM. For serum values below 1.3 mM, 4 hr after acetaminophen ingestion, antidote is not recommended [31].

Post-translational modifications of thyroglobulin, the principle protein synthesized in the thyroid gland, include iodination and coupling of iodotyrosine derivatives to form thyroid hormones and cleavage of peptide bonds to produce several hormone-rich low molecular weight fragments [32]. *In vitro* studies confirmed its sensitivity to oxidative [13, 14] or proteolytic degradation [33, 34].

The proposed mechanism for the protective action of acetaminophen against oxidative peptide bond splitting, may be summarized as follows:

(1) Acetaminophen can compete effectively with Cl^- as MPO substrate and thus HOCl formation is suppressed. Oxidation products or intermediates of acetaminophen generated in the absence of Cl^- did not degrade thyroglobulin.

(2) Acetaminophen can further scavenge HOCl formed and may compete with amido or amino groups of a polypeptide for the same binding site (presumably the free propionate side groups of MPO haem). This may result in a more effective scavenging effect, since acetaminophen will then be closer to the site of HOCl generation than the polypeptide.

It has been proposed that HOCl can diminish inflammation by killing bacteria and fungi [35], inactivating neutrophil chemo attractants and attacking other neutrophil-derived products [36]. Conversely, by inhibiting HOCl formation and/or scavenging generated HOCl, the bactericidal action of the PMN can be seriously restricted and inflammation aggravated. This may in particular apply to renal papillary necrosis induced by acetaminophen [37], which is known to accumulate in the medulla [38]. It has also been proposed that analgesics may increase the susceptibility to urinary tract infection, or that infection may predispose to drug toxicity [39].

Acknowledgements—We are indebted to the South African Medical Research Council and the University of Stellenbosch for financial support.

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