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INHIBITION OF THE MYELOPEROXIDASE–H_2O_2–Cl^- SYSTEM OF NEUTROPHILS BY INDOMETHACIN AND OTHER NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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Abstract—The results presented herein demonstrate that the non-steroidal anti-inflammatory drug (NSAID) indomethacin is a strong inhibitor of the formation of HOCl by murine neutrophils (50% inhibition at 15 μM). Addition of 40 μM indomethacin to activated neutrophils caused 80% inhibition of HOCl formation throughout a 60-min time course while slightly increasing the levels of O_2^- and H_2O_2 produced. Comparable degrees of inhibition were achieved when the cells were stimulated with phorbol myristate acetate and with opsonized zymosan. Control experiments indicated that the drug did not act by scavenging HOCl. Direct inhibition of the chlorinating activity of myeloperoxidase (MPO) was confirmed using highly purified human enzyme *in vitro*. Kinetic analysis of the mechanism of inhibition showed that the drug was competitive with respect to Cl^- and uncompetitive with respect to H_2O_2, showing a K_i of 37 μM. In contrast to its inhibition of the oxidation of Cl^- by MPO, indomethacin had no effect on the peroxidative activity of the enzyme (oxidation of 4-aminoantipyrene), nor did it inhibit the activity of several other enzymes involved in H_2O_2 metabolism, including horseradish peroxidase, catalase, xanthine oxidase, and superoxide dismutase. Finally, it was found that inhibition of HOCl formation was a shared but non-uniform property of many NSAIDs; piroxicam, salicylate, sulindac, ibuprofen, and aspirin were all inhibitory but at widely different concentrations [K_i(app) values of 0.05, 0.18, 0.18, > 1, and 3 mM respectively] that correlated only partially with their therapeutic dose range. The results encourage further studies into the possibility that inhibition of HOCl formation may constitute an additional mechanism whereby NSAIDs reduce tissue distruction in chronically inflamed tissues.

Stimulation of neutrophils by inflammatory compounds (e.g. immune complexes, opsonized particles) and tumor promoters (e.g. PMA†) results in activation of an oxidative burst in which a variety of potentially destructive reactive oxygen intermediates are produced [1, 2]. This commences with the catalytic formation of the superoxide anion (O_2^-) and its dismutation product H_2O_2. In addition, the most potent oxidant produced directly by neutrophil enzymes is HOCl, formed from the myeloperoxidase (MPO; donor: H_2O_2 oxidoreductase, EC 1.11.1.7) catalyzed oxidation of Cl^- with H_2O_2 [3, 4]. HOCl reacts rapidly with amines and other oxidizable biomolecules to form a large array of relatively long-lived oxidants such as chloramines [5, 6]. MPO is a principal component of the azurophilic granules of neutrophils and has been estimated to constitute as much as 5% of the neutrophil by weight [7]. In fact, the biological generation of HOCl in inflammation appears to be a nearly unique function of neutrophils [8] and comprises a central mechanism of host defense against infection [1, 9, 10]. However, although the azurophilic granules release most of their contents intracellularly during the formation of phagolysosomes, where they act to kill injested microorganisms, a portion of the enzymes is also released into the extracellular milieu [11, 12]. Most *in vitro* assays for neutrophilic MPO measure the formation of extracellular reaction products, and free MPO has been found *in vivo* in the synovial fluid of arthritic joints [13] and in experimental inflammatory exudates [14]. Extracellularly generated HOCl is cytotoxic [15] and is thought to be a major factor in the destruction of tissues in chronic inflammatory conditions [4, 16] such as rheumatoid arthritis and emphysema [17, 18]. Hence, identification of drugs that can inhibit HOCl formation without inhibiting the overall oxidative burst may be of some utility in elucidating and possibly modulating the normal and pathological activities of neutrophils.

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) employed to treat chronic inflammatory conditions such as rheumatoid arthritis and gout [19, 20]. Like other aspirin-like drugs, it is a potent inhibitor of the cyclooxygenase step of prostaglandin synthesis [21–23] and experiments *in vivo* and *in vitro* have demonstrated that the major mode of action of the NSAIDs is the diminution of prostaglandin levels at sites of inflammation. However, numerous discrepancies arise when trying to attribute the therapeutic action of the NSAIDs solely to their ability to inhibit prostaglandin synthesis [reviewed in Refs. 24–26]. For example,

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† Abbreviations: PMA, 12-phorbol-13-myristate acetate; MPO, myeloperoxidase; NSAID, non-steroidal anti-inflammatory drug; PBS, Dulbecco's phosphate-buffered saline without Ca^{2+}/Mg^{2+}; TnCl, taurine chloramine; K_i(app), inhibitor concentration that causes 50% inhibition of measured activity.

the effective tissue and plasma levels of the drugs are usually much higher than those required to inhibit prostaglandin synthesis *in vitro*. Moreover, not all NSAIDs inhibit cyclooxygenase (e.g. salicylate). Hence, elucidation of secondary actions of the drugs *in vitro* may help in evaluating their overall efficacy *in vivo*. Using an indirect chemiluminescence assay, Pekoe *et al.* [27, 28] found evidence that the NSAIDs interfere with the MPO activity of neutrophils. Herein we demonstrate directly that indomethacin and other NSAIDs inhibit HOCl formation from inflammatory neutrophils and from purified MPO. Kinetic evidence reveals that the mode of action of indomethacin is directed specifically towards the oxidation of Cl^-.

MATERIALS AND METHODS

Materials. Dulbecco's phosphate-buffered saline (PBS) was purchased from Advanced Biotechnologies, Inc. (Columbia, MD). Indomethacin and other NSAIDs, phenol red, ferricytochrome *c* (type VI), catalase, phorbol myristate acetate (PMA), taurine, xanthine oxidase, hypoxanthine, zymosan A and 4′-aminoantipyrene were the highest quality available from Sigma (St. Louis, MO). Piroxicam was obtained from Pfizer Pharmaceuticals. Superoxide dismutase (SOD) was purchased from Boehringer-Mannheim (Indianapolis, IN). Potassium iodide (KI) and hydrogen peroxide (H_2O_2, 30% solution, preservative-free) were from Fisher Scientific (Columbia, MD). Pristane (2,6,10,14-tetramethylpentadecane) and NaOCl were from the Aldrich Chemical Co. (Milwaukee, WI). Myeloperoxidase (human, electrophoretically pure) was purchased from Biodesign, Inc. (Kennebunkport, ME) and horseradish peroxidase was from Calbiochem (San Diego, CA). Lymphocyte Separation Medium was from Organon Teknika (Durham, NC).

Solutions. Opsonized zymosan was prepared [29] using fresh mouse serum and was stored frozen at −70° in small aliquots until used. Stock solutions of other reagents were prepared and stored as follows: PBS was supplemented with 7.5 mM glucose. Ferricytochrome *c* (62 mg/mL) was prepared in distilled, deionized H_2O and stored frozen in small aliquots which were thawed and used only once. KI (2 M) was prepared in H_2O and stored dark at room temperature until used (within 1 month). Indomethacin and other NSAIDs were prepared fresh daily as 100–200 mM stock solutions in DMSO and were diluted in PBS immediately prior to use. Aspirin was prepared as a stock solution in neutralized aqueous solution. Taurine (0.15 M), phenol red (10 mg/mL) and H_2O_2 were prepared fresh daily in PBS. Catalase and purified human MPO were dissolved in H_2O and stored for up to 1 week at 4° as 1 mg/mL stock solutions.

Isolation of murine peritoneal neutrophils. Pristane-elicited neutrophils were isolated from female BALB/cAnPt mice as described previously [30]. Briefly, 2-month-old mice were given a single intraperitoneal injection of pristane (0.5 mL) and peritoneal exudates were collected at least 3 weeks later by peritoneal lavage with 8 mL of PBS. Cells were separated at 4° from the remainder of the lavage fluid by centrifugation at 2500 rpm for 15 min in a Sorvall RT6000 centrifuge, lysis of erythrocytes for 1 min in hypotonic buffer ($NH_4Cl/KHCO_3$/EDTA, 0.8%/0.1%/0.004%, pH 7.4), and washing immediately in PBS. Neutrophils were separated from total peritoneal exudate cells by density gradient centrifugation in isotonically adjusted 96% Lymphocyte Separation Medium [30]. The neutrophil cell pellets were washed twice with PBS and then resuspended in PBS containing 7.5 mM glucose and stored on ice until assayed (within 8 hr). Cell differentials were analyzed from cytocentrifuge preparations (Shandon Southern Instruments, Inc.) stained with Diff-Quick (American Scientific Products). Cell numbers and viability were determined by mixing with Trypan blue and counting live and dead cells on a hemocytometer. Neutrophils prepared in this manner were reproducibly ~ 90% homogeneous and 90–95% viable.

Analysis of respiratory burst products. All assays were carried out in duplicate at 37° in PBS containing 7.5 mM glucose. Neutrophils (6×10^5/cc) were mixed with the appropriate reagents for each assay (see below) in a final volume of 0.8 mL. The oxidative burst was stimulated by addition of either PMA (30 nM) or opsonized zymosan (0.4 mg/mL) as described by Babior and Cohen [31]. Spectrophotometric analyses were carried out on a Hewlett–Packard 8452A diode array spectrophotometer which measures the complete spectrum of the absorbance from white light within 1 sec, is sensitive down to 0.002 O.D. units, and is accurate to 0.005 O.D. units. Formation of products of the oxidative burst by resting cells was less than 10% of the maximum values obtained after stimulation.

O_2^- production was measured in a continuous assay by incubating cells in the presence of 75 μM ferricytochrome *c*, measuring the change in peak height at 550 nm after adding the stimulus, and using an extinction coefficient of 21,000 $M^{-1}\,cm^{-1}$ to calculate formation of reduced cytochrome *c* [32]. The peak height was determined by programming the spectrophotometer to subtract the absorbance at 610 nm (light scattering from cells and from zymosan) from that at 550 nm. Duplicate assays differed by less than 5% of each other.

HOCl production was determined by incubating 1×10^6 neutrophils/mL in buffer containing 15 mM taurine and measuring formation of extracellular taurine chloramine (TnCl) as described by Weiss *et al.* [33]. Assays were terminated by addition of 10 μg/mL catalase and removal of cells by centrifugation. The difference spectrum obtained from the addition of 20 mM KI gave a characteristic peak of absorbance at 350 nm only in the presence of TnCl (I_3^-). The peak height was directly proportional to the amount of OCl^- (extinction coefficient of 22,900 $M^{-1}\,cm^{-1}$) and was unaffected by the presence of NSAIDs that absorb in this spectral range. Duplicate assays differed by less than 5% of each other.

H_2O_2 was assayed by the phenol red method of Pick and Keisari [34] with the modification described previously [30]. The amount of H_2O_2 (reduced phenol red) was determined from the increase in

absorbance at 610 nm obtained following addition of 20 mM NaOH to the cell-free supernatants. Absolute values were derived from standard curves obtained with the reagent H_2O_2 (1–20 μM).

Enzyme assays. All assays were carried out at 37°. MPO was assayed for catalysis of HOCl formation using reagent H_2O_2 (2–14 μM), 5 mM taurine, a highly purified preparation of human MPO (0.5–1 μg/mL) and different concentrations of indomethacin in 20 mM sodium phosphate buffer (pH 7.3 ± 0.1) containing 137 mM NaCl unless otherwise indicated. The reagents were preincubated for 5 min at 37° prior to addition of the enzyme, and the assay was terminated after 1–2 min by addition of 10 μg/mL catalase. Formation of TnCl was assayed as described for the cellular assays. MPO was also assayed for peroxidative activity by following the oxidation of 4-aminoantipyrene [35].

Catalase (0.2 μg/mL) was assayed spectrophotometrically by following the decrease in absorbance of H_2O_2 (10 mM) at 240 nm [36]. Horseradish peroxidase was assayed by measuring the oxidation of phenol red in the presence of H_2O_2 [34]. Xanthine oxidase (2 μg/mL) -catalyzed formation of O_2^- from hypoxanthine (150 μM) and O_2 was assayed in 50 mM potassium phosphate buffer, pH 7.0, using reduction of ferricytochrome *c* as the indicator. Superoxide dismutase (SOD) activity was quantified by adding different amounts (0.2 to 50 μg/mL) of SOD to the xanthine oxidase/hypoxanthine assay and measuring the diminution in O_2^- formation in a linear portion of the reaction compared to control values [37]. The effect of indomethacin (50 and 250 μM) on SOD activity was determined by assaying a concentration of SOD (0.39 μg/mL) that gave half-maximal inhibition of O_2^- formation.

RESULTS

Inhibition of HOCl formation by neutrophils. The rapid and stoichiometric chlorination of taurine by HOCl provides a reliable method for measuring the production of HOCl from stimulated neutrophils [33, 38, 39]. Employing this assay, we found that murine neutrophils produced a prolonged flux of HOCl in response to 30 nM PMA (Fig. 1); typically, 10^6 neutrophils generated HOCl over roughly a 2-hr period and reproducibly yielded an average of 10.0 ± 0.2 nmol HOCl in 30 min. However, when 40 μM indomethacin was included in the incubations, HOCl formation was inhibited by 80% throughout the course of the assay. Inhibition by indomethacin was concentration dependent (Fig. 2) with a K_i(app) (concentration causing 50% inhibition) of 15 μM. Because HOCl formation is a secondary reaction in the oxidative burst of neutrophils, the effect of indomethacin on O_2^- and H_2O_2 generation from the cells was also tested. It was found (Table 1) that 50 μM indomethacin, a concentration that severely inhibited HOCl formation, actually caused a small (18%) but reproducible increase in O_2^- production and a somewhat smaller increase in H_2O_2 production by the cells (data not shown). Similar results were achieved regardless of whether the cells were activated with PMA as a soluble stimulus of the oxidative burst or with opsonized zymosan as a particulate stimulus for the cells (Table 1).

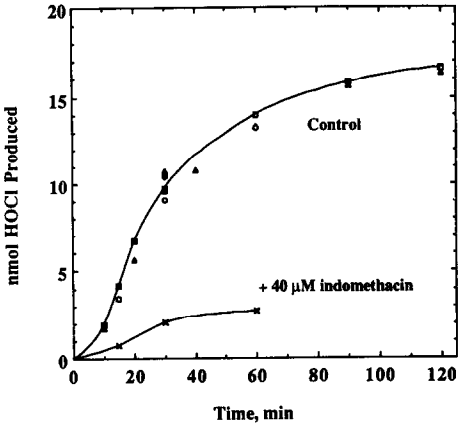

Fig. 1. Effect of indomethacin on the time-course of formation of HOCl by murine neutrophils. Neutrophils (1 × 10^6/mL) were stimulated to undergo an oxidative burst with 30 nM PMA, and production of HOCl was determined by measuring formation of TnCl as described in Materials and Methods. The results represent data obtained from seven separate experiments and are calculated as nanomoles of HOCl produced per 10^6 cells. Indomethacin (×—×) was tested at a concentration of 40 μM.

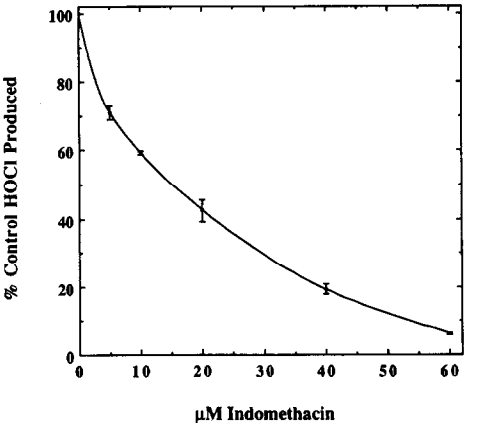

Fig. 2. Concentration–response curve for inhibition by indomethacin of neutrophil HOCl formation. HOCl formation from PMA-stimulated neutrophils (6 × 10^5 to 1 × 10^6/mL) was measured after a 30-min assay in the presence of different concentrations of indomethacin as described in Materials and Methods. The data are calculated as the percent control HOCl produced per 10^6 cells in the absence of drug (10 ± 0.2 nmol) and represent three separate experiments (mean ± SEM).

Inhibition of neutrophil HOCl formation by other NSAIDs. To determine whether the action of indomethacin on HOCl production from neutrophils was specific for this drug alone, similar experiments were carried out with a number of other NSAIDs. As shown in Fig. 3, all of the drugs tested were

Table 1. Effect of indomethacin on HOCl and O_2^- production by neutrophils

Stimulus	Indomethacin concentration	HOCl Production (nmol/10^6 cells/ 30 min)	% control	O_2^- production (nmol/10^6 cells/ 15 min)	% control
PMA (30 nM)		10.0 ± 0.2 (N = 7)	100	71.6 ± 5.9 (N = 4)	100
PMA (30 nM)	50 μM	1.2 ± 0.9 (N = 3)	12	84.7 ± 9.6 (N = 4)	118
Opsonized zymosan (0.4 mg/mL)		8.8 ± 0.1 (N = 2)	100	26.5 ± 3.4 (N = 2)	100
Opsonized zymosan (0.4 mg/mL)	50 μM	1.5 ± 0.1 (N = 2)	17	28.0 ± 2.0 (N = 2)	106

Levels of production of HOCl and O_2^- by murine neutrophils (1×10^6 cells/cc) were assayed in the presence and absence of indomethacin as described in Materials and Methods. Values are means ± SEM when N > 2 and the average ± range when N = 2.

Table 2. Comparison of apparent inhibition constants for murine neutrophil HOCl formation and peak plasma concentrations achieved from therapeutic doses of NSAIDs in humans

Drug	K_i(app) (M)	K_i(app) (μg/mL)	Peak plasma concentration (μg/mL)	K_i(app) Prostaglandin synthase (μg/mL)	References
Indomethacin	15×10^{-6}	5.4	0.5–2	0.06–2	[20,40,41]
Piroxicam	50×10^{-6}	16.6	5–8	0.26	[19,41,42]
Sodium-salicylate	180×10^{-6}	28.8	150–300	Ineffective	[19]
Sulindac	180×10^{-6}	64.1	4	Ineffective*	[19,43,44]
Aspirin	3×10^{-3}	540	250–350†	6.6–20	[19,20,45]
Ibuprofen	$>1 \times 10^{-3}$	>206	15–40	0.8	[19,40,43,45]

The data on inhibition of HOCl production by murine neutrophils are taken from Fig. 3. Peak plasma levels achieved in humans treated with anti-inflammatory doses of the different drugs are derived from the references given in the table.

* Converted to active metabolite (sulindac sulfide) *in vivo*.

† Given as the concentration of salicylate achieved following metabolism of aspirin *in vivo*.

capable of inhibiting HOCl formation but at widely varying concentrations. Piroxicam inhibited with a K_i(app) of 50 μM while ibuprofen and aspirin (acetylsalicylic acid) were only inhibitory at concentrations in excess of 200 μM, with 50% inhibition occurring at approximately 1–3 mM. On the other hand, salicylate, which is the major circulating metabolite of aspirin *in vivo*, was at least one order of magnitude more effective than aspirin in inhibiting HOCl formation, having a K_i(app) similar to that of sulindac (~ 180 μM). Comparison of the K_i(app) for each drug with the peak plasma concentrations reported for therapeutic doses of the drugs *in vivo* is given in Table 2. None of these drugs was found to inhibit O_2^- production by the cells (data not shown).

Inhibition of purified MPO in vitro. The conclusion that indomethacin inhibited cellular HOCl formation by inhibiting the MPO–H_2O_2–Cl^- system and not by inhibiting an associated pathway was verified by assaying a highly purified preparation of human MPO *in vitro*. The studies were carried out in phosphate-buffered saline at neutral pH in order to simulate approximately the conditions required for the neutrophil experiments. Under the standard assay conditions employed herein (0.5 to 1.0 μg/mL of enzyme, 137 mM Cl^-, 5 mM taurine, pH 7.3, 37°), the assay was linear for a period of up to 1.5 min before auto-inactivation was observed [46]. The enzyme showed a K_m for H_2O_2 of approximately 3 μM and a V_{max} for HOCl formation of about 17 μmol/min/mg enzyme. Concentrations of H_2O_2 in excess of 14 μM produced substrate inhibition at this pH and hence were not employed. The low levels of H_2O_2 required for maximal activity were very similar to the values predicted and observed by others for MPO chlorinating activity at pH 7.3 [47–50].

Figure 4A shows that when the enzyme was assayed for 1 min in the presence of a constant concentration of Cl^- (120 mM) and different concentrations of H_2O_2 (2.5 to 14 μM) and indomethacin (0 to 80 μM), the drug inhibited HOCl formation by lowering both the K_m and V_{max} of MPO activity with respect to H_2O_2. The double-reciprocal plot shown is typical of the pattern expected for an

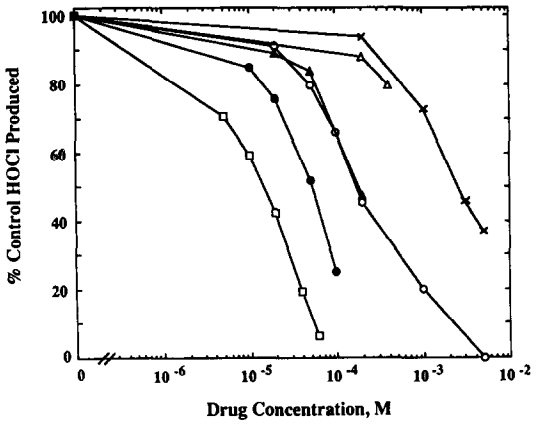

Fig. 3. Inhibition by different NSAIDs of neutrophil HOCl production. Production of HOCl from PMA-stimulated neutrophils (1×10^6/mL) was measured in a 30-min assay in the presence of the indicated concentrations of the following drugs: (□—□) indomethacin; (●—●) piroxicam; (○—○) salicylate; (▲—▲) sulindac; (△—△) ibuprofen; and (×—×) aspirin. The data are calculated as the percent of HOCl produced in the absence of any drug (10.2 ± 0.3 nmol/10^6 cells/30 min).

uncompetitive inhibitor [51]. The secondary plot of the y-intercepts achieved at different concentrations of indomethacin produced a straight line with a K_i intercept at 37 μM (Fig. 4B). Similar studies were carried out by varying the concentration of Cl^- (0 to 360 mM) and using a single concentration of H_2O_2 (14 μM). The ionic strength in these assays was roughly equalized by adjusting the total salt concentrations to 360 mM with sodium phosphate buffer (pH 7.3). The double-reciprocal plot for HOCl formation at different concentrations of indomethacin (Fig. 5A) showed that the drug was competitive with respect to Cl^- and the secondary plot of the slopes (Fig. 5B) revealed a K_i of 37 μM.

In contrast to the inhibitory effect of indomethacin on the oxidation of Cl^- by MPO, the drug had no effect on the peroxidase activity of the enzyme when tested in the standard 4-aminoantipyrine assay, nor did it inhibit the activity of several other oxidative enzymes involved in H_2O_2 formation or metabolism, including horseradish peroxidase, catalase, superoxide dismutase, and xanthine oxidase (data not shown; see Materials and Methods).

Potential scavenging of HOCl by indomethacin. It has been reported that indomethacin and other nonsteroidal anti-inflammatory drugs can scavenge OCl^- [52], a powerful oxidant that can react with numerous compounds. The possibility that indomethacin scavenged HOCl in these experiments was examined by performing standard curves for reagent NaOCl in the presence and absence of the drug. It was found (Fig. 6) that when indomethacin (50 μM) and NaOCl (1 μM to 1 mM) were mixed together in the absence of taurine, significant scavenging of the NaOCl did indeed take place, as evidenced by loss of the optical density peak at 350 nm. However, in the presence of the standard concentrations of taurine employed in these experiments (5 and 15 mM), indomethacin levels as high as 250 μM were ineffective in competing with the amino acid for OCl^-. As taurine was always present in considerable excess in the neutrophil and MPO assays, it is highly unlikely that the inhibition of HOCl formation observed with indomethacin derived from scavenging of OCl^-. Similarly, the data shown in Fig. 6 rule out the possibility that indomethacin acted by scavenging I_3^- which was stable in the presence of at least 100 μM drug throughout the measurement period. Comparable control experiments were conducted for the other drugs shown in Fig. 3 at one to two

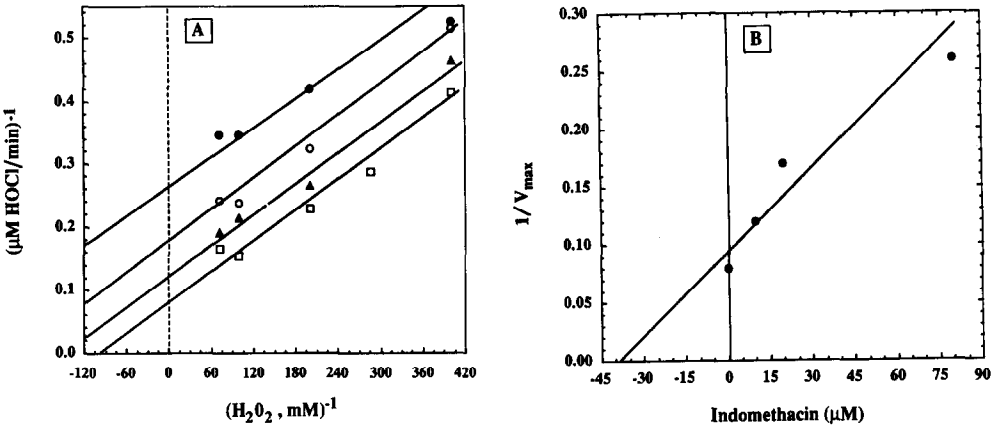

Fig. 4. (A) Lineweaver–Burk plot for inhibition by indomethacin of HOCl formation from purified MPO *in vitro*: Effect of varying the H_2O_2 concentration. Purified human MPO (0.5 μg/mL) was assayed for production of HOCl in the presence of a nearly constant concentration of NaCl (120 mM) and various concentrations of H_2O_2 (2.5 to 14 μM) and of indomethacin as follows: none (□—□); 10 μM (▲—▲); 20 μM (○—○); and 80 μM (●—●). Data are the averages of three separate experiments. (B) Dixon plot for the data shown in panel A. The y-intercepts (V_{max} values) are plotted for each concentration of indomethacin shown in panel A.

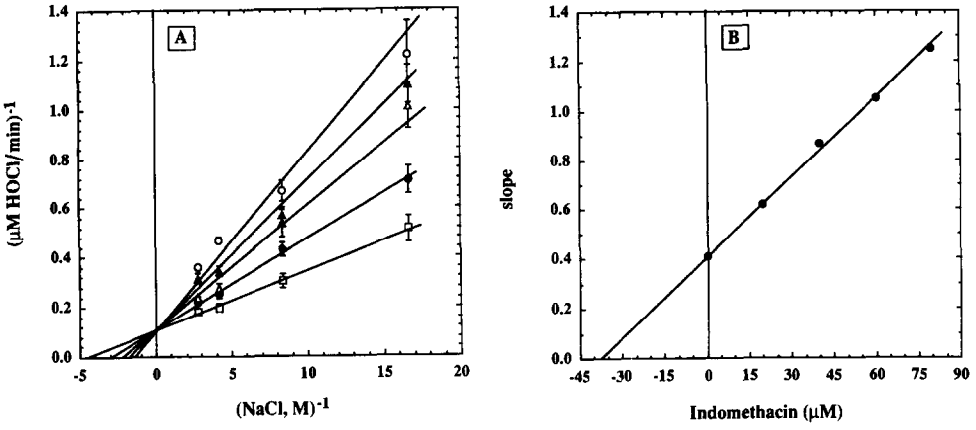

Fig. 5. (A) Lineweaver–Burk plot for inhibition by indomethacin of HOCl formation from purified MPO *in vitro*: effect of varying the Cl^- concentration. Purified human MPO was assayed for formation of HOCl in the presence of 14 μM H_2O_2 and various concentrations of Cl^- (40–360 mM) and indomethacin as follows: none (□—□); 20 μM (●—●); 40 μM (△—△); 60 μM (▲—▲); 80 μM (○—○) as described in the text. Data are the means ± SEM obtained from three separate experiments. (B) Slope replot for the data shown in panel A. The slopes are plotted for each concentration of indomethacin.

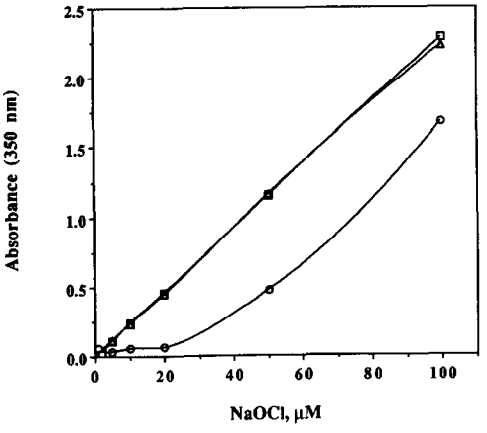

Fig. 6. Assay for TnCl formation from reagent NaOCl in the presence and absence of indomethacin. TnCl was assayed as for the MPO assays except that different concentrations of reagent NaOCl were employed to generate standard curves. The order of addition of reactants was as follows: (a) (□—□) taurine → NaOCl (no indomethacin, control); (b) (△—△) indomethacin (50 or 250 μM) → taurine → NaOCl and (c) (○—○) indomethacin (50 μM) → NaOCl → taurine. Taurine was tested at 5 and 15 mM. The results shown in lines (a) and (b) are representative of the conditions found in the enzymatic and cellular HOCl assays.

times the K_i(app) concentrations without detectible scavenging of OCl^- or I_3^-. DMSO used as solvent for most of the drugs did not interfere with the assay at the highest concentration employed (0.4%, 56 mM) (data not shown).

Structural requirements for inhibition by indomethacin. In preliminary efforts to identify the molecular features of indomethacin responsible for its inhibitory activity, a number of structurally related compounds were tested for an effect on neutrophil- and MPO-catalyzed HOCl formation (Table 3). The data obtained thus far revealed that *N*-deschlorobenzoxy-indomethacin (5-methoxy-2-methyl-3-indoleacetic acid) was roughly equipotent to indomethacin in inhibiting HOCl formation and that 3-indoleacetic acid was particularly effective in inhibiting MPO. In contrast, 4-chlorobenzyl alcohol and 3-(4-chlorobenzoyl)-propionic acid were completely ineffective at levels up to 40–50 μM.

DISCUSSION

The results presented herein demonstrate that indomethacin inhibits the chlorinating activity of neutrophils and that the primary mode of action is through direct inhibition of MPO enzyme activity. Kinetic analysis of the mechanism of inhibition of purified MPO revealed that the drug was competitive with respect to Cl^-. Consistent with this finding, the drug did not inhibit the peroxidative activity of the enzyme when 4-aminoantipyrine was employed as the substrate instead of Cl^- nor did it inhibit the activity of the non-chlorinating peroxidase from horseradish. The specificity of the inhibition for oxidation of Cl^- likely explains why investigations on the peroxidative activity of MPO (e.g. by the guiacol assay) have failed to reflect inhibition by the drug. These results substantiate earlier conclusions of Pekoe *et al.* [27, 28] and Horan *et al.* [53] who employed luminol-enhanced chemiluminescence as a measure of MPO activity.

Inhibition of HOCl formation from PMA-stimulated neutrophils was evident at micromolar concentrations of indomethacin and had a K_i(app) of 15 μM. This level of inhibition was somewhat more potent than inhibition of MPO enzyme activity *in vitro* (K_i = 37 μM), suggesting that the drug may

Table 3. Preliminary results of testing analogs and metabolites of indomethacin for inhibition of HOCl formation from PMA-stimulated neutrophils and from purified MPO

Compound	Assay	Concentration (μM)	% Inhibition
Indomethacin	Neutrophils	10	41 ± 1
	Neutrophils	40	81 ± 2
	MPO	20	33 ± 2
N-Deschlorobenzoxy indomethacin	Neutrophils	10	36
	Neutrophils	40	77
	MPO	20	60
3-Indoleacetic acid	MPO	20	70
4-Chlorobenzyl alcohol	Neutrophils	10	0
	Neutrophils	40	4
3-(4-Chlorobenzoyl) propionic acid	Neutrophils	50	2

Assays were carried out under the standard conditions described in Materials and Methods.

have acted by inhibiting more than one step required for HOCl formation during the neutrophil oxidative burst. Numerous studies have been carried out to examine the effects of NSAIDs on neutrophil function [26, 54]. For example, O_2^- production has been reported to be either increased (up to 2-fold) [55–57] or unaffected [58–60] by indomethacin and decreased by piroxicam [54], depending partly on the stimulus employed [54, 57, 59, 61]. We found that concentrations of indomethacin and piroxicam (not shown) that almost eliminated HOCl formation by cells increased slightly the amounts of O_2^- and H_2O_2 measured outside the cells (Table 1), thus indicating that the drug did not act by lowering the availability of H_2O_2 required for the oxidation of chloride. Moreover, indomethacin was almost equipotent in inhibiting HOCl formation when the cells were stimulated by PMA and opsonized zymosan even though these two agents activate the oxidative burst by significantly different pathways [62, 63]. We have yet to test the possibility that indomethacin may have also inhibited degranulation and release of MPO from the neutrophils. However, other studies have shown that this should not have been the case at the relatively low concentrations of drug employed herein [64–66].

An alternative explanation for why indomethacin was more potent in inhibiting HOCl formation from cells than from purified enzyme may have derived from changes in pH and H_2O_2 that occur in the vicinity of activated neutrophils; for example, the enzyme assays were carried out starting with a bolus of H_2O_2 whereas neutrophils generate a flux of this substrate. Because MPO activity varies dramatically with the concentrations of these two variables [47–50, 67, 68], such differences could have had an adequate effect on MPO activity to influence inhibitability by indomethacin. It may also be relevant that the kinetic studies on MPO activity were carried out on enzyme purified from human cells whereas our cellular experiments were carried out with murine neutrophils. To our knowledge, no mechanistic studies have been published on the chlorinating activity of purified murine MPO. The enzymes from both systems are expected to be similar, but the possibility should be considered that although both are inhibitable by indomethacin, the enzyme kinetics may differ somewhat. In preliminary experiments, we have found that indomethacin inhibits HOCl formation from human peripheral blood neutrophils but that somewhat higher concentrations are required (Shacter E and Hahn S, unpublished results).

In trying to determine the structural features of indomethacin responsible for its inhibitory action on MPO, we found that, contrary to our initial expectation, the chloride moiety of the molecule was not required for inhibition; *N*-deschlorobenzoxy-indomethacin, a major metabolite of indomethacin *in vivo*, was equally as effective as indomethacin in inhibiting HOCl formation from neutrophils and from purified MPO whereas chlorobenzyl alcohol and chlorobenzoylpropionic acid, employed as analogs of the chlorobenzoxy group of indomethacin, had no measurable effect on enzyme activity at concentrations up to 50 μM. Furthermore, none of the other inhibitory NSAIDs tested herein contains a Cl^- group in the molecule. Instead, a common characteristic of the drugs is their acidity and moderate lipophilicity [19, 69]. In accordance with this observation, the minimal inhibitory moiety that we have found thus far is indoleacetic acid, which was even more effective than indomethacin in its inhibitory activity on purified MPO (Table 3).

A number of reports indicate that NSAIDs can scavenge HOCl [52] and our studies using reagent NaOCl in the taurine chloramine assay are in agreement with this conclusion (Fig. 6). Yet it is also clear that in the presence of oxidizable amino acids (e.g. taurine) and proteins (e.g. α_1-antitrypsin), the NSAIDs are ineffective scavengers of HOCl

unless extremely high concentrations of drugs are employed [52].

The most potent action of the NSAIDs identified to date remains their inhibition of prostaglandin synthase [21–23] (see Table 2) and this is likely their main site of action *in vivo* [19, 20]. Nonetheless, it is clear that individual NSAIDs can also inhibit other enzymes and pathways associated with inflammation [26, 54, 63], including phospholipase A_2 [70], 12- and 15-lipoxygenases [71, 72], glutathione *S*-transferases [73], and MPO ([27, 28, 53], and herein). Whereas the extent to which these inhibitory activities may occur *in vivo* has not been established, it is plausible to assume that individual differences between the capacity of each drug to influence the different pathways may contribute to the divergence in the specific therapeutic effectiveness of the NSAIDs. Moreover, secondary actions of the NSAIDs may account for some of the problems in attributing attentuation of inflammation *in vivo* solely to inhibition of prostaglandin synthesis [24–26]. The data presented herein and elsewhere [27, 28, 53] support the possibility that in addition to their well established role in inhibiting prostaglandin synthesis, the NSAIDs may retard progression of the inflammatory condition by diminishing HOCl formation. All of the available data suggest that this did not occur via an effect on prostaglandin synthesis by the neutrophils: concentrations of indomethacin that were sufficient to inhibit cyclooxygenase activity (i.e. 1–2 μM) were ineffective in inhibiting HOCl formation. Furthermore, the drug inhibited activity of purified MPO directly. The NSAIDs differed widely in their effectiveness, but comparison of the concentrations of drugs required to inhibit HOCl formation from neutrophils *in vitro* to the peak plasma concentrations achieved from therapeutic doses of the drugs *in vivo* (Table 2) suggests that this activity may occur to some extent *in vivo*, especially in chronic inflammatory tissues in which the concentrations of acidic NSAIDs are elevated significantly [24, 74]. Thorough pharmacokinetic studies will be required to examine this possibility. Nonetheless, it is interesting that deschlorobenzoxyindomethacin is considered to be an *inactive* metabolite of indomethacin [75] yet it was comparable to the intact drug in its capacity to inhibit HOCl formation by neutrophils. It may also be important to note that many antiarthritic drugs that are not inhibitors of prostaglandin synthesis have been postulated to act, at least in part, by inhibiting myeloperoxidase or by scavenging HOCl [18, 52, 76]. Hence, they may share a common mechanism with the aspirin-like NSAIDs.

Finally, a key problem in elucidating the mechanisms involved in the bactericidal, cytotoxic, and possible mutagenic activity of neutrophils is the determination of which of the many reactive oxygen intermediates secreted by the cells is responsible for these complex processes. Most investigations employ inhibitors or scavengers of the reactive species to accomplish this goal; for example, superoxide dismutase and catalase are employed to remove O_2^- and H_2O_2, respectively. However, few specific inhibitors are available to study the involvement of MPO. The most commonly employed agent is sodium azide, which also inhibits other cellular peroxidases such as catalase. While employment of such drugs as indomethacin and indoleacetic acid to inhibit MPO activity clearly would not be specific either, it might help sort out the contributions of enzymes that employ H_2O_2 as a substrate. For example, our finding that neither indomethacin nor azide inhibits induction of DNA strand breaks in tumor cells by activated neutrophils [30] provided combined evidence that HOCl was not involved in formation of single-strand breaks, consistent with information obtained by other methods [77]. Nonetheless, because the NSAIDs are not monospecific agents, their utility in identifying the involvement of individual metabolic pathways will be dependent upon selection of concentrations or analogs (e.g. indoleacetic acid) that can discriminate between their different activities.

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