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EFFECT OF ANTI-INFLAMMATORY DRUGS ON MYELOPEROXIDASE-DEPENDENT HYDROXYL RADICAL GENERATION BY HUMAN NEUTROPHILS

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Abstract—Neutrophils comprise a group of leukocytes that play a pivotal role in inflammation and vascular diseases like ischemia/reperfusion. These activated phagocytic cells are drawn to the site of injury, secreting superoxide and other oxidants derived from the formation of this free radical. This series of events frequently results in localized tissue damage. Surprisingly, free radical scavengers frequently offer only minimal relief. Why this is so may be due, in part, to our limited understanding of mechanisms that govern generation of free radicals in these settings. Although the metal ion-catalyzed Haber–Weiss reaction is considered the classical pathway for neutrophil-derived hydroxyl radical, an alternative mechanism, such as the myeloperoxidase-dependent pathway, may undoubtedly contribute to the formation of this free radical by stimulated neutrophils. In this study, we explored this possibility by investigating the role of different classes of anti-inflammatory drugs to ameliorate hydroxyl radical generation via the myeloperoxidase-dependent pathway. In this paper, we report that meclofenamic acid inhibited myeloperoxidase-dependent hydroxyl radical generation through scavenging of hypochlorous acid and not by direct inhibition of myeloperoxidase. The importance of these results with regard to the clinical efficacy of this anti-inflammatory compound remains to be determined as studies into the significance of myeloperoxidase-dependent hydroxyl radical formation in inflammatory tissue injury continue.

Key words: superoxide; hydroxyl radical; neutrophils; myeloperoxidase; anti-inflammatory drugs

There is a large body of evidence that supports the contribution of neutrophils to tissue injury in a number of pathological conditions, including myocardial ischemia and reperfusion injury [1, 2], adult respiratory distress syndrome [3], emphysema [4], carcinogenesis [5], vasculitis [6], ulcerative colitis [7], cerebral ischemia and stroke [8], arthritis [9] and renal ischemic injury [10]. Coincident with the involvement of neutrophils in these disease states is evidence implicating leukocyte-derived oxygen-centered free radicals in the injury process [11–17]. Consequently, there is increasing interest in the development of pharmacological interventions that prevent or slow the progression of neutrophil-mediated tissue injury by reducing levels of free radicals.

In respect of the highly reactive hydroxyl radical, ambiguity in the detection of endogenous hydroxyl radical generation by neutrophils and other biological sources has prevented an understanding of the mechanisms responsible for its formation. Therefore, the primary approach to pharmacological amelioration of hydroxyl radical in experimental models has been the use of free radical scavengers, such as dimethylthiourea [18], dimethyl sulfoxide [19], salicylates [20] and mannitol [21]. It is well known that these compounds and many other small molecules react readily with hydroxyl radical at second order rate constants of 10^9–$10^{10}\,M^{-1}\,sec^{-1}$. However, these agents are unlikely to be effective scavengers physiologically due to their inability to accumulate at concentrations necessary to compete with diffusion-controlled reactions of hydroxyl radical with biomolecules at sites of damage [22]. In addition, use of large concentrations of these compounds in biological systems can result in substantial cytotoxicity.

Some years ago, using the spin trapping system of 4-POBN§/ethanol, we [23] delineated a myeloperoxidase-dependent mechanism of hydroxyl radical generation by neutrophils that does not require a supplemental iron catalyst:

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \rightarrow H_2O + O_2 \quad (1)$$

$$H_2O_2 + Cl^- + MPO \rightarrow HOCl + OH^- \quad (2)$$

$$HOCl + \cdot O_2^- \rightarrow \cdot OH + O_2 + Cl^- \quad (3)$$

More recently, using HPLC techniques, Candeias *et al.* [24] confirmed our findings. Therefore, a detailed understanding of the mechanism of hydroxyl radical

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§ Abbreviations: 4-POBN, 4-pyridyl 1-oxide *N-tert*-butylnitrone; DTPA, diethylenetriaminepentaacetic acid; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; PMA, phorbol 12-myristate 13-acetate; TNB, 5-thio-2-nitrobenzoic acid; and HBSS, Hanks' balanced salt solution.

formation provides a rational basis for investigating potential pharmacological approaches to ameliorating this free radical. Accordingly, the objective of this study was to explore the potential modulation of neutrophil myeloperoxidase-dependent hydroxyl radical formation by a series of classical anti-inflammatory agents.

MATERIALS AND METHODS

Reagents. DTPA, hydrogen peroxide solution (30%), ferricytochrome *c* (type VI), NADH, pyruvic acid, Triton X-100, 4-aminoantipyrine, phenol, taurine, DTNB, catalase, xanthine, PMA, ferrous sulfate and the following anti-inflammatory agents: acetylsalicylic acid, indomethacin, ibuprofen, naproxen sodium, ketoprofen, flurbiprofen, piroxicam, etodolac, tolmetin sodium, meclofenamic acid and dexamethasone were obtained from the Sigma Chemical Co. (St. Louis, MO). Stock solutions of anti-inflammatory drugs were prepared daily in 100% ethanol except for dexamethasone 21-phosphate, which was dissolved in buffer. 4-POBN and sodium hypochlorite solution (5%) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Xanthine oxidase and superoxide dismutase were obtained from Boehringer-Mannheim (Indianapolis, IN). Purified human neutrophil myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) was purchased from Calbiochem (La Jolla, CA). Pure ethyl alcohol, U.S.P. was obtained from Warner-Graham (Cockeysville, MD). HBSS without calcium, magnesium or phenol red was purchased from Gibco Laboratories (Grand Island, NY). The buffer system used in this study was Chelex-treated HBSS supplemented with 0.1 mM DTPA, pH 7.4.

Neutrophil isolation. Human neutrophils were isolated from heparinized venous blood of normal human volunteers using dextran sedimentation and Hypaque-Ficoll gradient centrifugation [25]. Cells were suspended in Chelex-treated HBSS, pH 7.4, and maintained on ice until used.

ESR/spin trapping of hydroxyl radical in the presence of anti-inflammatory drugs. Reaction mixtures consisted of neutrophils (2×10^6/mL), 4-POBN (10 mM), PMA (100 ng/mL in ethanol, 170 mM final concentration), drug (0.1 mM) or vehicle (170 mM ethanol) and sufficient Chelex-treated HBSS with 0.1 mM DTPA for a final volume of 0.5 mL. Reaction mixtures were transferred to a quartz ESR flat cell (Wilmad Glass Co., Buena, NJ) open to air, and placed in the cavity of an ESR spectrometer (Varian Associates, model E-109, Palo Alto, CA); spectra were recorded at 25°. Hydroxyl radical was detected as the α-hydroxyethyl radical adduct of 4-POBN (4-POBN-CH(CH_3)OH), as previously described [23, 26]. Instrument settings, unless noted differently, were microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude 1.0 G; sweep time, 12.5 G/min; and response time, 1 sec. The effect of anti-inflammatory agents on hydroxyl radical generation was determined by comparing low-field ESR peak height relative to a vehicle control 20 min after addition of PMA. Statistically significant inhibition was determined using a one-tailed Student's *t*-test.

Superoxide detection. Generation of superoxide by neutrophils and cell-free systems was measured spectrophotometrically at 550 nm and 25° as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* using an extinction coefficient of 21 $mM^{-1} cm^{-1}$ [27]. Xanthine (0.4 mM) in the presence of xanthine oxidase was used as a model superoxide-generating system to test for superoxide scavenging.

Detection of hypochlorous acid from neutrophils and model systems. Production of hypochlorous acid by neutrophils and purified myeloperoxidase systems was determined by measuring the formation of taurine chloramine, resulting from the reaction of hypochlorous acid and the reagent taurine [28, 29]. Taurine chloramine was determined via the ability of this species to oxidize TNB to DTNB, which was detected spectrophotometrically at 412 nm and 25°. Neutrophils (2×10^6/mL) stimulated with PMA (100 ng/mL) or a hypochlorous acid-generating system (400 mU purified human myeloperoxidase and 75 μM hydrogen peroxide) were incubated in the presence of 15 mM taurine. The reaction was terminated by the addition of catalase (300 U/mL) 20 min after neutrophil stimulation (after 5 min of incubation in the purified enzyme system), followed by the addition of TNB (0.5 mM) and centrifugation to remove neutrophils. TNB (reduced DTNB) was prepared by raising the pH of a solution of 10 mM DTNB in buffer to 10 and readjusting to pH 7.4 [30]. Absorbance was recorded at 412 nm against a non-stimulated neutrophil or myeloperoxidase-free reference. Reagent hypochlorous acid (NaOCl) scavenging was determined using an order of addition scheme [31].

Determination of peroxidative activity of myeloperoxidase. Peroxidase activity of purified myeloperoxidase was assayed by measuring the oxidation of 4-aminoantipyrine (1 mM) spectrophotometrically at 510 nm and 25° over time in the presence of hydrogen peroxide (0.85 mM) [32]. A stock solution of 4-aminoantipyrine (2.5 mM) was prepared by dissolving 810 mg phenol in 40 mL deionized, distilled water followed by the addition of 25 mg 4-aminoantipyrine. The initial rate of the reaction was represented as the slope of the linear portion of the ΔA_{510}/min curve.

Lactate dehydrogenase release assay for assessment of neutrophil viability. Neutrophil viability in the presence and absence of an anti-inflammatory compound was determined by following the oxidation of NADH spectrophotometrically at 340 nm during the lactate dehydrogenase conversion of pyruvate to lactate [33]. The reaction mixture consisted of cells, pyruvate (1 mM), NADH (0.22 mM) and drug or vehicle (170 mM ethanol). Rates were determined from the limiting slope of the ΔA_{340}/min curve. Viability for each treatment was represented as a percentage of the activity in the presence of 0.1% Triton X-100.

RESULTS AND DISCUSSION

The effects of a series of anti-inflammatory

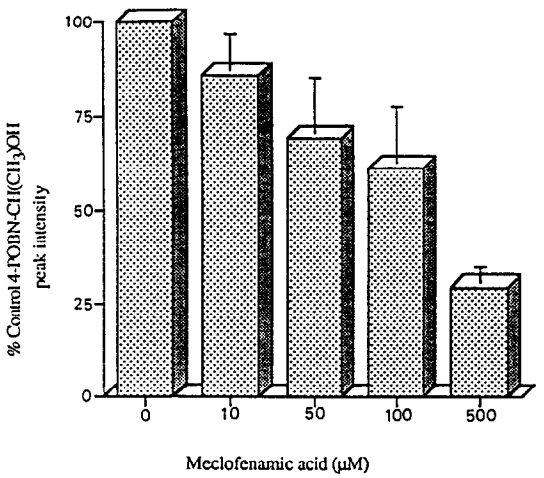

Fig. 1. Concentration–response curve for meclofenamic acid inhibition of myeloperoxidase-dependent hydroxyl radical generation by human neutrophils. Response is defined as percent of control low-field peak intensity of 4-POBN-$CH(CH_3)OH$ 20 min after stimulation of neutrophils (10×10^6/mL) with PMA (100 ng/mL) at 25°. Data are means ± SD of three separate experiments.

agents, including acetylsalicylic acid, indomethacin, ibuprofen, naproxen sodium, ketoprofen, flurbiprofen, piroxicam, etodolac, tolmetin sodium, meclofenamic acid and dexamethasone on myeloperoxidase-dependent hydroxyl radical generation by human neutrophils were examined. First, however, we explored whether any of these anti-inflammatory agents would directly react with our spin trapping system. Even at concentrations as high as 500 μM, none of the compounds listed above interfered with 4-POBN/ethanol spin trapping of hydroxyl radical from the model hydroxyl radical-generating systems, xanthine/xanthine oxidase (10 μM superoxide/min)/ferrous sulfate (0.1 mM) and hydrogen peroxide (88 nM)/ferrous sulfate (0.1 mM). Similar results were obtained when investigating the effects of these agents (even at 500 μM) on the reduction of cytochrome *c* by the model superoxide-generating system, xanthine/xanthine oxidase, at either low (5 μM superoxide/min) or high (10 μM superoxide/min) rates. Indomethacin, however, did exhibit partial inhibition at high concentrations (500 μM), but this was likely due to inhibition of xanthine oxidase and not to the scavenging of superoxide. These results indicate the inability of these anti-inflammatory agents to react with superoxide directly or interfere with the spin trapping detection system by hydroxyl radical scavenging.

When neutrophils (10×10^6/mL) were stimulated with PMA (100 ng/mL) in the presence of 4-POBN (10 mM), ethanol (170 mM) and our model anti-inflammatory agents, only meclofenamic acid exhibited appreciable inhibition of 4-POBN-$CH(CH_3)OH$. To investigate this observation further, a concentration–response curve for meclofenamic acid inhibition of neutrophil hydroxyl radical generation was determined (Fig. 1). From these data, it appears that at 100 μM, inhibition of 4-POBN-$CH(CH_3)OH$ was greater than 60% (61.1 ± 16.5% of control low-field peak intensity after 20 min; N = 3; $P < 0.05$). Inhibition was not due to cytotoxicity, because meclofenamic acid did not enhance release of lactate dehydrogenase from neutrophils at a concentration as high as 500 μM. The general inability of a broad range of non-steroidal anti-inflammatory drugs to inhibit neutrophil hydroxyl radical formation, as detected by 4-POBN/ethanol spin trapping, indicates that the source of this free radical is independent of cyclooxygenase activity. The inability of dexamethasone to inhibit neutrophil hydroxyl radical production is consistent with previous studies [34, 35], which found that the drug does not depress the neutrophil oxidative burst, even after prolonged pretreatment with isolated cells.

Some studies [29, 30, 36] have demonstrated that certain anti-inflammatory agents are capable of inhibiting myeloperoxidase. This property, however, is concentration dependent and not common to all of the non-steroidal anti-inflammatory drugs. For example, diclofenac [36], piroxicam and indomethacin [29, 30] inhibit the formation of hypochlorous acid by purified myeloperoxidase at micromolar concentrations, whereas aspirin and ibuprofen require millimolar levels for inhibition [29, 30]. Interestingly, compounds that are reasonably potent inhibitors of purified myeloperoxidase fail to inhibit myeloperoxidase-dependent formation of hydroxyl radical by neutrophils. A likely reason for this paradox stems from the work of Kettle *et al.* [37], who observed that the ability of diclofenac to inhibit myeloperoxidase in PMA-stimulated neutrophils was much less compared with inhibition of the purified enzyme. They concluded that superoxide serves as an antagonist of diclofenac inhibition of myeloperoxidase by reducing the drug-inactivated form of the enzyme back to the active state [37]. Furthermore, there is strong evidence that superoxide closely regulates hypochlorous acid production by neutrophils through modulation of myeloperoxidase activity [38, 39]. It follows that agents that inhibit superoxide production are more likely to be effective inhibitors of myeloperoxidase-dependent hydroxyl radical generation. Of the compounds examined in this study, only piroxicam has been consistently reported to partially inhibit neutrophil superoxide generation [40, 41]. However, this effect appears not to be associated with cyclooxygenase inhibition [42]. Many non-steroidal anti-inflammatory drugs have demonstrated the capacity to enhance superoxide generation by stimulated neutrophils [41]. Consequently, inhibition of myeloperoxidase by these agents would not necessarily result in inhibition of myeloperoxidase-dependent hydroxyl radical formation.

We next undertook experiments to understand the basis for meclofenamic acid inhibition of myeloperoxidase-catalyzed formation of hydroxyl radical, particularly since it is independent of scavenging this free radical. Clearly, inhibition is not due to a decrease in superoxide production by neutrophils. As indicated above, meclofenamic acid did not demonstrate the ability to scavenge

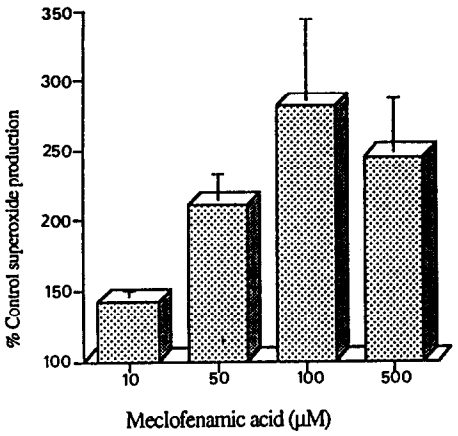

Fig. 2. Meclofenamic acid enhancement of superoxide. Response is defined as percent of control superoxide formation by 3×10^6/mL neutrophils stimulated with PMA (100 ng/mL). Meclofenamic acid (0.5 mM) in the absence of PMA did not stimulate superoxide production. Control flux of superoxide was 10 μM/min. Data are means ± SEM of duplicate determinations in two separate experiments.

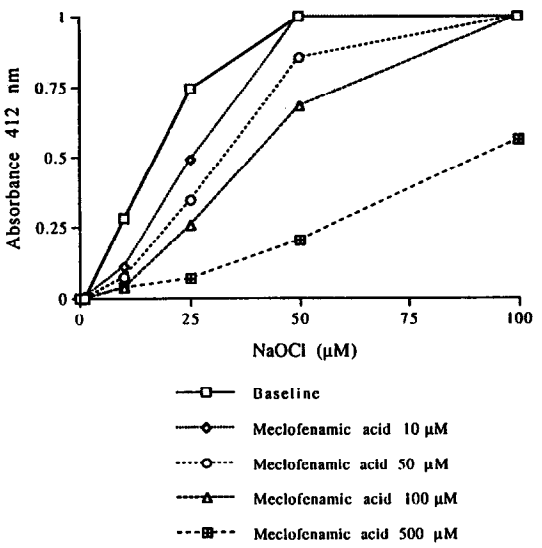

Fig. 3. Representative graph of three independent experiments depicting the ability of meclofenamic acid to scavenge hypochlorous acid in a concentration-dependent manner. The baseline curve represents the oxidation of TNB by solutions of taurine (15 mM) and increasing concentrations of sodium hypochlorite (NaOCl). The remaining curves represent the effect of addition of meclofenamic acid (10–500 μM) to NaOCl solutions before the addition of taurine.

superoxide. Moreover, meclofenamic acid actually enhanced superoxide generation by stimulated neutrophils in a concentration-dependent manner (Fig. 2), which is a property shared with a number of non-steroidal anti-inflammatory drugs [41]. Meclofenamic acid, in the absence of PMA, was not capable of stimulating neutrophil superoxide formation. The mechanism for this increase is poorly understood. It has been reported that benoxaprofen enhances neutrophil superoxide generation through modulation of protein kinase C [43], a key component in the signal transduction pathway for neutrophil activation. However, in the study cited above [41], anti-inflammatory agents that enhanced superoxide production did not alter protein kinase C activity.

Next, the ability of meclofenamic acid to interfere with the critical involvement of the myeloperoxidase/hypochlorous acid system in neutrophil hydroxyl radical generation (Reactions [2] and [3]) was examined. Initial experiments were designed to determine if meclofenamic acid is capable of inhibiting hypochlorous acid generation by stimulated neutrophils. Assay of hypochlorous acid was based on the ability of taurine chloramine, resulting from the reaction of hypochlorous acid and taurine, to oxidize TNB to DTNB [28]. Incubation of meclofenamic acid (10–500 μM) with PMA (100 ng/mL)-stimulated neutrophils (2×10^6/mL) in the presence of 15 mM taurine resulted in marked inhibition only at the 500 μM concentration (56.1% of control hypochlorous acid production after 20 min; N = 4). Inclusion of superoxide dismutase (15 U/mL) did not alter significantly the effect of meclofenamic acid on neutrophil hypochlorous acid production. This result is in contrast to the ability of superoxide dismutase to enhance the inhibitory effect of diclofenac on hypochlorous acid formation [36], which was attributed to enhanced myeloperoxidase inhibition by diclofenac in the absence of superoxide antagonism. Therefore, the failure of superoxide dismutase to unmask meclofenamic acid inhibition of hypochlorous generation suggested that the effect of this compound on hypochlorous acid formation, and subsequently hydroxyl radical generation, was not mediated through inhibition of myeloperoxidase.

This possibility was tested by examining the effect of meclofenamic acid on hypochlorous acid generation by purified myeloperoxidase. Purified human myeloperoxidase (400 mU) was added to a solution of hydrogen peroxide (75 μM), taurine (15 mM) and various concentrations of meclofenamic acid (10–500 μM). Superoxide was not required in this model system, which eliminated complications associated with superoxide modulation of myeloperoxidase. In this case, meclofenamic acid failed to inhibit hypochlorous acid production by purified myeloperoxidase at any of the concentrations tested. Similarly, in the same concentration range, meclofenamic acid was unable to inhibit the oxidation of 4-aminoantipyrine by purified myeloperoxidase (400 mU). Thus, based on results from assays for both chlorination and peroxidative functions, meclofenamic acid appeared to have no inhibitory effect on the activity of myeloperoxidase.

Finally, the possibility of direct hypochlorous scavenging by meclofenamic acid was considered. Addition of meclofenamic acid (10–500 μM) to a reaction mixture of sodium hypochlorite (NaOCl) in various concentrations (1–100 μM) and taurine

(15 mM) did not result in inhibition of taurine chloramine-mediated oxidation of TNB. However, if the order of addition were changed, such that the drug was allowed to first react with NaOCl before addition of taurine, inhibition occurred in a manner that was dependent on both NaOCl and meclofenamic acid concentrations (Fig. 3), thus implicating hypochlorous acid scavenging by the drug. Interestingly, the compound is capable of scavenging hypochlorous acid at lower concentrations than that required for inhibition of myeloperoxidase-dependent hydroxyl radical generation. This is explained by the ability of these same concentrations to enhance neutrophil superoxide generation. Increased superoxide production results in enhanced formation of hypochlorous acid, which would effectively decrease the scavenging potency of meclofenamic acid in the whole-cell system. The scavenging activity also explains the inability of meclofenamic acid, even at concentrations as high as 500 μM, to decrease hypochlorous acid production in the purified enzyme system compared with whole-cell activation. The large bolus of hydrogen peroxide present in the enzyme system likely facilitates a corresponding bolus of hypochlorous acid upon addition of the purified enzyme, which prevents favorable competition of meclofenamic acid with excess taurine. Taken together, these findings suggest that meclofenamic acid inhibits neutrophil myeloperoxidase-dependent hydroxyl radical generation through scavenging of hypochlorous acid and not by direct inhibition of myeloperoxidase.

Overall, the results of this study indicate that meclofenamic acid, contrary to other non-steroidal anti-inflammatory drugs and dexamethasone, attenuates myeloperoxidase-dependent generation of hydroxyl radical by stimulated human neutrophils. This antagonism occurs despite the meclofenamic acid-enhanced production of superoxide by these stimulated phagocytic cells. The importance of this finding in the clinical efficacy of this class of anti-inflammatory compounds is uncertain and is dependent upon results from further experiments designed to delineate the significance of myeloperoxidase-dependent hydroxyl radical formation in inflammatory tissue injury.

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