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EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY AGENTS ON HUMAN NEUTROPHIL FUNCTIONS *IN VITRO* AND *IN VIVO**

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Abstract—Human blood neutrophils exposed to appropriate stimuli aggregate, degranulate and generate superoxide anion (O_2^-). These responses are anteceded by mobilization of membrane-associated calcium, monitored as a decrease in fluorescence of cells preloaded with chlortetracycline (CTC). We studied the effects, both *in vitro* and *in vivo*, of non-steroidal anti-inflammatory agents (aspirin, indomethacin, ibuprofen and piroxicam) on these neutrophil responses to three stimuli: a chemoattractant, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP); a tumor promotor, phorbol myristate acetate (PMA); and a lectin, concanavalin A (Con A). The effects of these drugs were compared with those of two polyenoic inhibitors of arachidonate metabolism: eicosatrienoic acid (ETI) and eicosatetraynoic acid (ETYA). The pattern of inhibition of neutrophil functions varied both with inhibitor and the nature of the stimulus. Thus, aspirin, piroxicam, ETYA and ETI inhibited neutrophil aggregation, degranulation, and O_2^- generation in response to FMLP, whereas ibuprofen inhibited only aggregation and degranulation and indomethacin only inhibited aggregation. None of the agents inhibited aggregation or degranulation induced by PMA or Con A: only piroxicam inhibited O_2^- generation in response to PMA or Con A. ETI and ibuprofen inhibited decrements of CTC fluorescence induced by FMLP, but whereas ETI inhibited the CTC response to PMA or Con A, ibuprofen was without effect. The agents had varying effects on binding of the stimulus ([^{3}H]FMLP, [^{3}H]Con A), but these did not correlate with neutrophil responses to the ligands. Neutrophils from subjects taking therapeutic doses of ibuprofen, indomethacin, or piroxicam showed profiles of inhibited responses to FMLP similar to those observed with these agents *in vitro*. These data suggest that, although non-steroidal anti-inflammatory agents may inhibit discrete neutrophil functions both *in vitro* and *in vivo*, their effects do not duplicate those of polyenoic inhibitors of arachidonate metabolism. Moreover, since the susceptibility of neutrophils differed not only with respect to each inhibitor, but also to the stimulus, it is unlikely that all neutrophil responses are necessarily linked by a common pathway that is blocked by inhibitors of arachidonic acid metabolism.

When human neutrophils are exposed to appropriate stimuli, they will aggregate [1, 2], generate superoxide [3], secrete lysosomal constituents [4], generate stable prostaglandins [4, 5], thromboxanes [6], and leukotrienes [7] and undergo a characteristic decrease in CTC fluorescence. Recent evidence suggests that endogenously derived products of arachidonic acid oxidation may mediate these responses of the neutrophil [7]. In 1971, Vane [8] proposed that the major action of aspirin-like drugs was the ability to inhibit the metabolism of arachidonic acid to stable prostaglandins. This theory has since evolved to include virtually all agents of the class of non-steroidal anti-inflammatory drugs [9], each of which has the capacity to inhibit acute inflammation in laboratory models and human disease. Recent work by Smolen and Weissmann [10], Lackie [11], and O'Flaherty *et al.* [12] indicate that various non-steroidal anti-inflammatory drugs modulate neutrophil responses to various stimuli. While these data are consistent with the hypothesis that products of arachidonic acid metabolism regulate a discrete step in the responses of neutrophils to inflammatory stimuli, other mechanisms of action have been suggested. Kaplan *et al.* [13] reported that indomethacin can inhibit phospholipase A_2 activity, and Gullikson *et al.* [14] have reported alterations in membrane integrity in the presence of various non-steroidal anti-inflammatory agents.

Since neutrophils are primary participants in acute inflammation, we have studied the effects, both *in vitro* and *in vivo*, of non-steroidal anti-inflammatory drugs on four neutrophil responses (aggregation, degranulation, O_2^- generation, and CTC fluorescence). To avoid errors in interpretation which might result when the effects of these agents are studied with only one stimulus, we exposed neutrophils to the "complete" secretagogue, FMLP‡ [2],

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‡ Abbreviations: FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; Con A, concanavalin A; DMSO, dimethyl sulfoxide; O_2^-, superoxide anion; LER, lysosomal enzyme release; ETI, 5,8,11-eicosatrienoic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; CTC, chlortetracycline; AA, arachidonic acid; and NSAID, non-steroidal anti-inflammatory drug.

as well as to the "incomplete" secretagogues, PMA and concanavalin A [15]. Finally, we have compared the actions of non-steroidal anti-inflammatory agents with polyenoic inhibitors of the lipoxygenase pathway (ETI) and of the lipoxygenase and cyclooxygenase pathways of arachidonate metabolism (ETYA).

MATERIALS AND METHODS

Reagents. *N*-Formyl-methionyl-leucyl-phenylalanine (Peninsula Laboratories, CA) was prepared in DMSO (0.01%) (Fisher Scientific Co., Pittsburgh, PA). Aspirin, concanavalin A, cytochrome *c*, indomethacin, and phorbol myristate acetate were obtained from the Sigma Chemical Co., St. Louis, MO. [^{3}H]FMLP and [^{3}H]concanavalin A were obtained from the New England Nuclear Corp., Boston, MA. All salts were of reagent grade and were obtained from the Fisher Scientific Co., Pittsburgh, PA. 5,8,11-Eicosatrienoic acid, 5,8,11,14-eicosatetraynoic acid and ibuprofen were gifts of Dr. B. Samuelsson, Karolinska Institutet, Stockholm, Sweden; Dr. Aaron Marcus, N.Y.V.A. Medical Center-Cornell Medical College, New York NY; and Dr. John Pike, The Upjohn Co., Kalamazoo, MI, respectively.

Preparation of cell suspensions. Heparinized (10 units/ml) venous blood was obtained from healthy donors. Purified suspensions of neutrophils were isolated by means of Hypaque/Ficoll gradients [16], followed by dextran sedimentation and the hypotonic lysis of remaining erythrocytes [17]. The neutrophils were then suspended in a buffered salt solution consisting of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4, 1.5 mM KH_2PO_4, 1 mM $MgCl_2$, and 0.6 mM $CaCl_2$, pH 7.4 (PiCM).

Neutrophil aggregation, enzyme release, O_2^- generation, CTC fluorescence, responses of neutrophils, and the binding of [^{3}H]*FMLP to neutrophils.* Neutrophil aggregation was studied as previously described [1]. The generation of O_2^- and the release of neutrophil granule-associated enzymes (lysozyme, beta-glucuronidase) were measured as previously described [1]. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was measured by the method of Wacker *et al.* [18] and was employed as an indicator of cell viability. In none of the experiments described below (stimulated or unstimulated neutrophils) was the percentage of total LDH release > 3%. Inhibitors, when employed, were preincubated with neutrophils for 5 min at 37°. Enzyme release is expressed as the percentage of total enzyme activity released from neutrophils exposed to 0.2% Triton X-100. CTC fluorescence was performed by the method of Naccache *et al.* [19] and modified by Edelson *et al.* [20]. [^{3}H]FMLP and [^{3}H]Con A binding was performed as previously described [20].

RESULTS

Activation of human neutrophils by FMLP and the effects on non-steroidal anti-inflammatory agents.

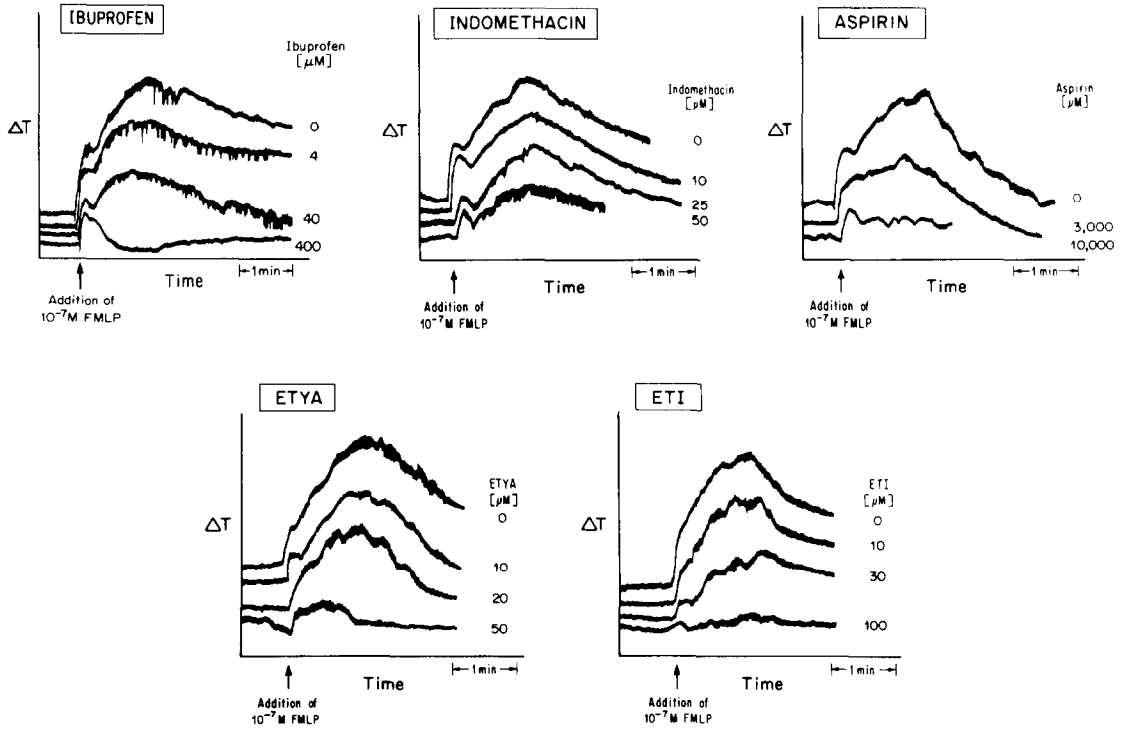


Fig. 1. Dose-dependent effects of ibuprofen, indomethacin, aspirin, ETYA and ETI on neutrophil aggregation. Neutrophils (30×10^6/ml) were preincubated at 37° for 2–4 min prior to stimulation with FMLP (10^{-7} M).

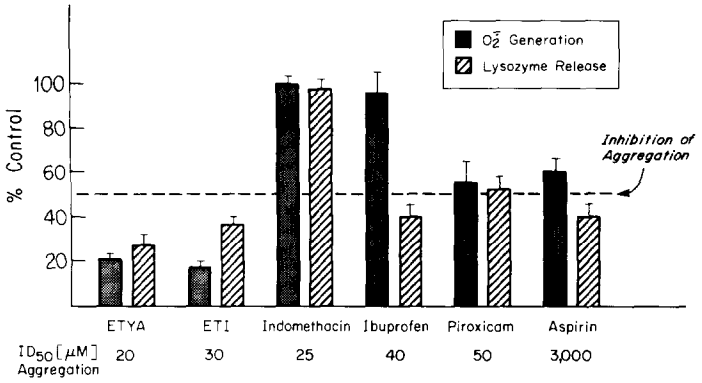
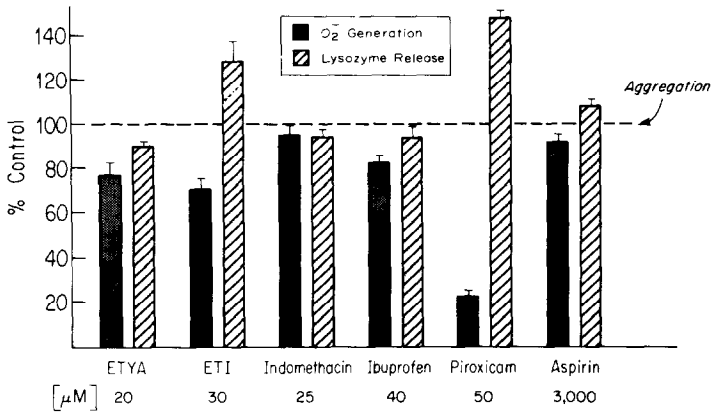


Fig. 2. Effects of inhibitors of FMLP-induced neutrophil aggregation on superoxide generation and lysozyme release. Neutrophils (4–6 × 10^6/ml) were preincubated at 37° with the inhibitor at the IC_{50} (for aggregation) for 2–4 min prior to stimulation with FMLP (10^{-7} M). All values are expressed as percent control (PMN + no inhibitor for appropriate time). Superoxide generation is expressed in nmoles of cytochrome *c* reduced/10^6 neutrophils/5 min. Lysozyme release is expressed as percent total enzyme, as determined by Triton X-100. Control values for FMLP-induced lysozyme release were 9.9 ± 0.6%/5 min; O_2^- control values were 12.7 ± 0.67 nmoles cytochrome *c* reduced/10^6 neutrophils/5 min. All values are expressed as percent control rates of reactions. All reactions were conducted at 37° for 5 min (N = 5).



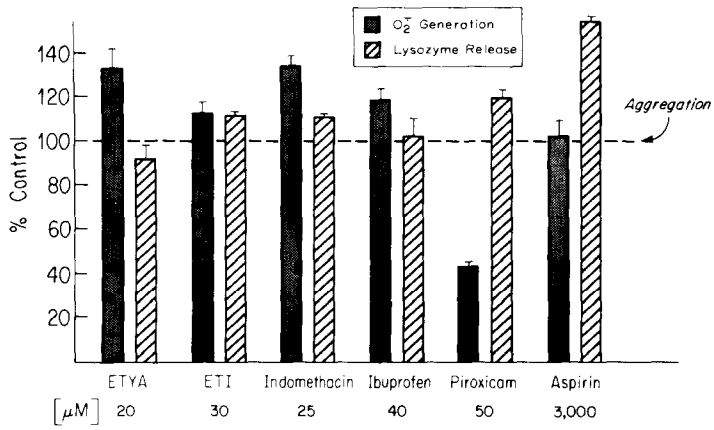


Fig. 3. Effects of non-steroidal anti-inflammatory agents and putative inhibitors of arachidonic acid metabolism on activation of neutrophils by Con A or PMA. Neutrophils (4–6 × 10^6/ml) were preincubated at 37° for 2–4 min at the IC_{50} of aggregation with the various agents and were stimulated with either Con A (30 μg/ml) (A) or PMA (50 ng/ml) (B). The protocol was identical to that of Fig. 2 (N = 5). Control values for Con A-induced lysozyme release were 10.3 ± 0.3%/5 min; control values for PMA-induced lysozyme release were 17.6 ± 2.3%/5 min. Control values for Con A-induced O_2^- generation were 9.7 ± 0.9 nmoles cytochrome *c* reduced/10^6 neutrophils/5 min. Control values for PMA-induced O_2^- generation were 9.9 ± 1.0 nmoles cytochrome *c* reduced/10^6 neutrophils/5 min.

When human neutrophils are exposed to the chemotactic peptide FMLP (10^{-7} M), they aggregate, generate O_2^-, and release lysozyme with no concomitant release of beta-glucuronidase [1]. Illustrated in Fig. 1 is a typical aggregation curve of neutrophils exposed to 10^{-7} M FMLP. A wave of aggregation occurred after addition of stimulus and was followed by disaggregation, as indicated by the downslope of the curve. This aggregation–disaggregation response to FMLP is in agreement with previously published results [1].

Pretreatment of neutrophils with ETYA, ETI, ibuprofen, indomethacin, or aspirin for 5 min at 37° (Fig. 1) inhibited this response in a dose-dependent fashion. The data for piroxicam are consistent with this observation, as previously published by this laboratory [20].

Ibuprofen and indomethacin are potent cyclooxygenase inhibitors [8, 21], while aspirin is a much weaker cyclooxygenase inhibitor [8]. As shown in Fig. 1, the concentrations required to inhibit aggregation by 50% (IC_{50}) appear to parallel the abilities of these agents to inhibit cyclooxygenase. ETYA and ETI, which are arachidonic acid analogues, inhibited both pathways of arachidonic acid metabolism, i.e. the cyclooxygenase and lipoxygenase pathways. ETYA and ETI inhibited neutrophil aggregation by approximately 50% at micromolar concentrations. Thus, the order of potencies that inhibited neutrophil aggregation induced by FMLP was ETYA > indomethacin > ETI > ibuprofen > piroxicam >> aspirin. To determine if these agents solely affected neutrophil aggregation at these concentrations or were capable of affecting all neutrophil responses to stimulation, their effects on two other neutrophil functions, i.e. O_2^- generation and lysozyme release, were examined.

As shown in Fig. 2, preincubation of neutrophils with either ETYA (20 μM), ETI (30 μM), piroxicam (50 μM), or aspirin (3 mM) for 5 min at 37° resulted in inhibition of FMLP-induced O_2^- generation and lysozyme release ($N = 5$, $P < 0.001$). ETYA, ETI, and aspirin, at the concentrations employed, did not appear to affect cellular integrity, as measured by extracellular release of LDH. Thus, it appears that ETYA, ETI, piroxicam, and aspirin were capable of inhibiting a step crucial in the activation of the neutrophil. Indomethacin, however, at a concentration (25 μM) that inhibited aggregation, had no effect on either O_2^- generation or lysozyme release. Previous reports have indicated that indomethacin can inhibit O_2^- generation and degranulation by neutrophils stimulated by FMLP; however, these studies were conducted in the presence of cytochalasin B [10]. Ibuprofen, also at a concentration (40 μM) which inhibited neutrophil aggregation, inhibited only lysozyme release while having no effect on O_2^- generation. Thus, while each agent employed

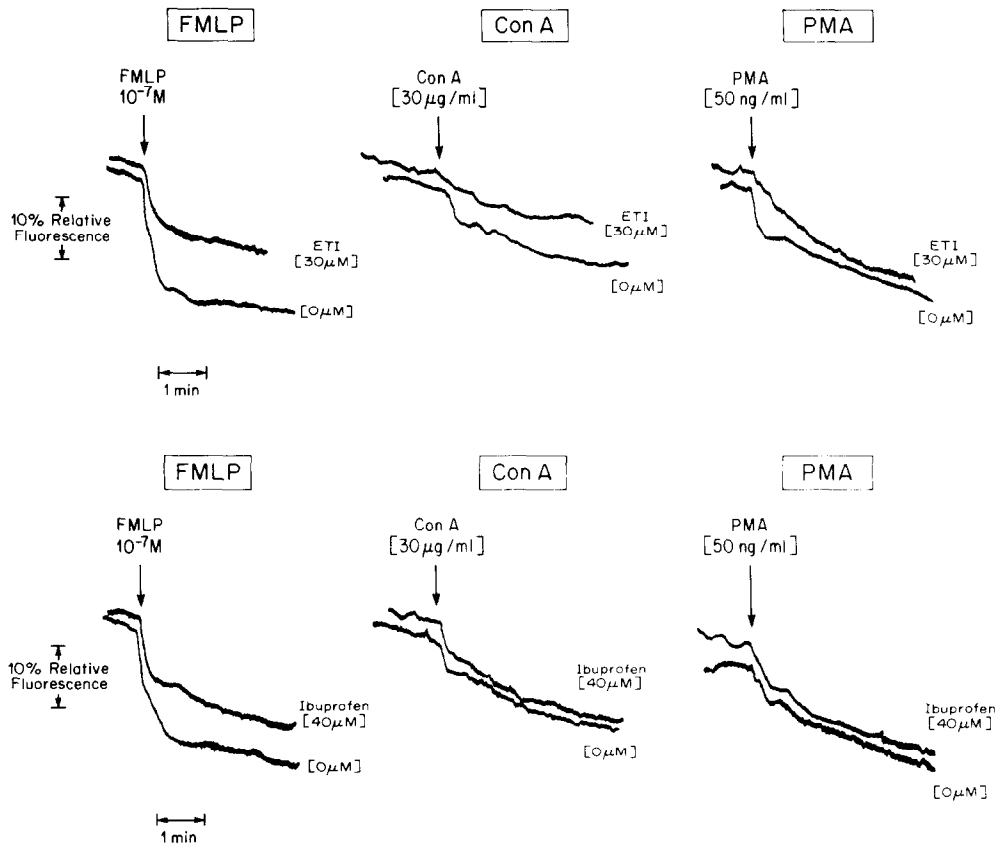


Fig. 4. Effects of ETI and ibuprofen on CTC fluorescence. Neutrophils (20×10^6/ml) were preloaded with CTC (50 μM) prior to preincubation at 37° for 5 min with either inhibitor. FMLP (10^{-7} M), Con A (30 μg/μl) or PMA (50 ng/ml) was employed as stimulus.

may affect arachidonic acid metabolism, their differing patterns of inhibition indicate that these agents may have multiple sites of activity in the neutrophil.

Activation of neutrophils by concanavalin A or phorbol myristate acetate and the effects of non-steroidal anti-inflammatory agents. The effects of ETYA, ETI, ibuprofen, indomethacin, piroxicam and aspirin, at their IC_{50} values for FMLP-induced neutrophil aggregation, were examined on neutrophil O_2^- generation and lysozyme release induced by the plant lectin concanavalin A (Con A, 30 μg/ml) and the tumor promoter phorbol myristate acetate (PMA). As shown in Fig. 3A, no significant effect was exerted by any of these agents on neutrophil O_2^- generation and lysozyme release induced by Con A or PMA (50 ng/ml), with the exception of piroxicam which inhibited superoxide generation but not lysozyme release [20] (Fig. 3B). Therefore, it appears that the activities of these inhibitors of arachidonic acid metabolism were stimulus specific.

Effects of ETI and ibuprofen on CTC fluorescence. Illustrated in Fig. 4 is a normal CTC fluorescence response elicited by FMLP (10^{-7} M) from CTC-loaded neutrophils. When neutrophils were preincubated for 5 min at 37° with either ETI (30 μM) or ibuprofen (40 μM), prior to the addition of FMLP, the CTC fluorescence response was inhibited markedly. Thus, when FMLP was used as a stimulus, the CTC fluorescence response and the secretion of lysozyme were inhibited in a parallel fashion. However, ETI was able to inhibit the binding of [^{3}H]FMLP. The effects of piroxicam on CTC fluorescence have been reported previously [20].

Also shown in Fig. 4 is the response of neutrophils to either Con A or PMA with respect to CTC fluorescence. When neutrophils were preincubated with ETI (30 μM), the response to either Con A or PMA was reduced markedly. Furthermore, this inhibition of the CTC response occurred under conditions where ETI had no effect on the release of lysozyme. In contrast, ibuprofen had no effect on the CTC fluorescence response induced by either Con A or PMA under conditions where ibuprofen had no effect on lysozyme release. Thus, it appears that when either ConA or PMA was used as stimulus, ETI selectively inhibited the CTC fluorescence response while allowing secretion to proceed normally.

Table 1. Effects of NSAIDs on [^{3}H]FMLP binding*

Agent	[^{3}H]FMLP (% inhibition)
ETI (30 μM)	57
Piroxicam† (50 μM)	57.5
ETYA (20 μM)	19
Indomethacin (25 μM)	0
Aspirin (3000 μM)	0
Ibuprofen (40 μM)	0

* Neutrophils were incubated at 37° and at 0° with 12.5 Ci/mmole FMLP. Incubation was terminated by the addition of 2.0 ml of ice-cold buffer filtered on a Millipore cillotate filter (0.5 μM pore size) and washed twice with ice-cold buffer. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled FMLP. Results are expressed as cpm/mg protein.

† Previously reported by Edelson *et al.* [20].

Binding of [^{3}H]FMLP to neutrophils and modulation of binding by non-steroidal anti-inflammatory agents. To elucidate the mechanism of the stimulus specificity exhibited above, the binding of [^{3}H]FMLP to neutrophils in the presence of these agents was examined. As shown in Table 1, only ETI, and to a lesser extent ETYA, affected [^{3}H]FMLP binding. However, while ETI (30 μM) inhibited binding by approximately 57%, ETI inhibited O_2^- generation and lysozyme release by 84.6% and 64.3% respectively (compare Fig. 1 and Table 1). ETYA (20 μM) inhibited [^{3}H]FMLP binding by only 19, and yet inhibited O_2^- generation and lysozyme release by 79.4 and 73.7% respectively. Indomethacin and aspirin had no effect on [^{3}H]FMLP binding while ibuprofen even enhanced binding somewhat, at concentrations which inhibited neutrophil responses to FMLP. To further elucidate the effects of ETYA and ETI on [^{3}H]FMLP binding and to dissociate the question of direct correlation of inhibition of binding to inhibition of neutrophil function, we examined the effects of these inhibitors on [^{3}H]Con A binding. It was observed that, at the IC_{50} level of inhibition of aggregation found for FMLP-stimulated neutrophils, neither inhibitor was effective on Con A-stimulated neutrophils. In addition, at levels 200 times the IC_{50} of aggregation for FMLP-stimulated neutrophils, neither ETYA nor ETI had an inhibitory effect on Con A-stimulated neutrophils. Thus, there appeared to be no direct correlation between the abilities of these agents to affect receptor–ligand interactions and their effects on subsequent cellular responses.

Effects of ibuprofen, indomethacin and piroxicam on neutrophil function in vivo. To examine whether non-steroidal anti-inflammatory agents could affect neutrophil function *in vivo* when used at normal pharmacologic doses, normal human subjects were given ibuprofen (2400 mg/day), indomethacin (100 mg/day) or piroxicam (20 mg/day) for 3 days, at which time their neutrophils were isolated and stimulated with FMLP (10^{-7} M). The neutrophil responses after drug therapy were compared to their matched responses before drug therapy and are shown in Fig. 5. Neutrophils from subjects taking ibuprofen were inhibited in their ability to aggregate and release lysozyme, while O_2^- generation proceeded unimpaired. This pattern of inhibition was identical to that seen *in vitro* (compare to Fig. 2). Similarly, neutrophils from subjects taking indomethacin were inhibited in their ability to aggregate, while O_2^- generation and lysozyme release were not significantly different from their pre-therapy responses. Cells isolated from patients taking piroxicam were inhibited with respect to ability to aggregation, generation of O_2^-, and release of lysozyme. Thus, it appears that non-steroidal anti-inflammatory agents affect neutrophil function *in vivo* and that a good correlation was observed between functions that were inhibited *in vivo* and *in vitro*.

DISCUSSION

These experiments have illustrated that, when human peripheral neutrophils were pretreated with

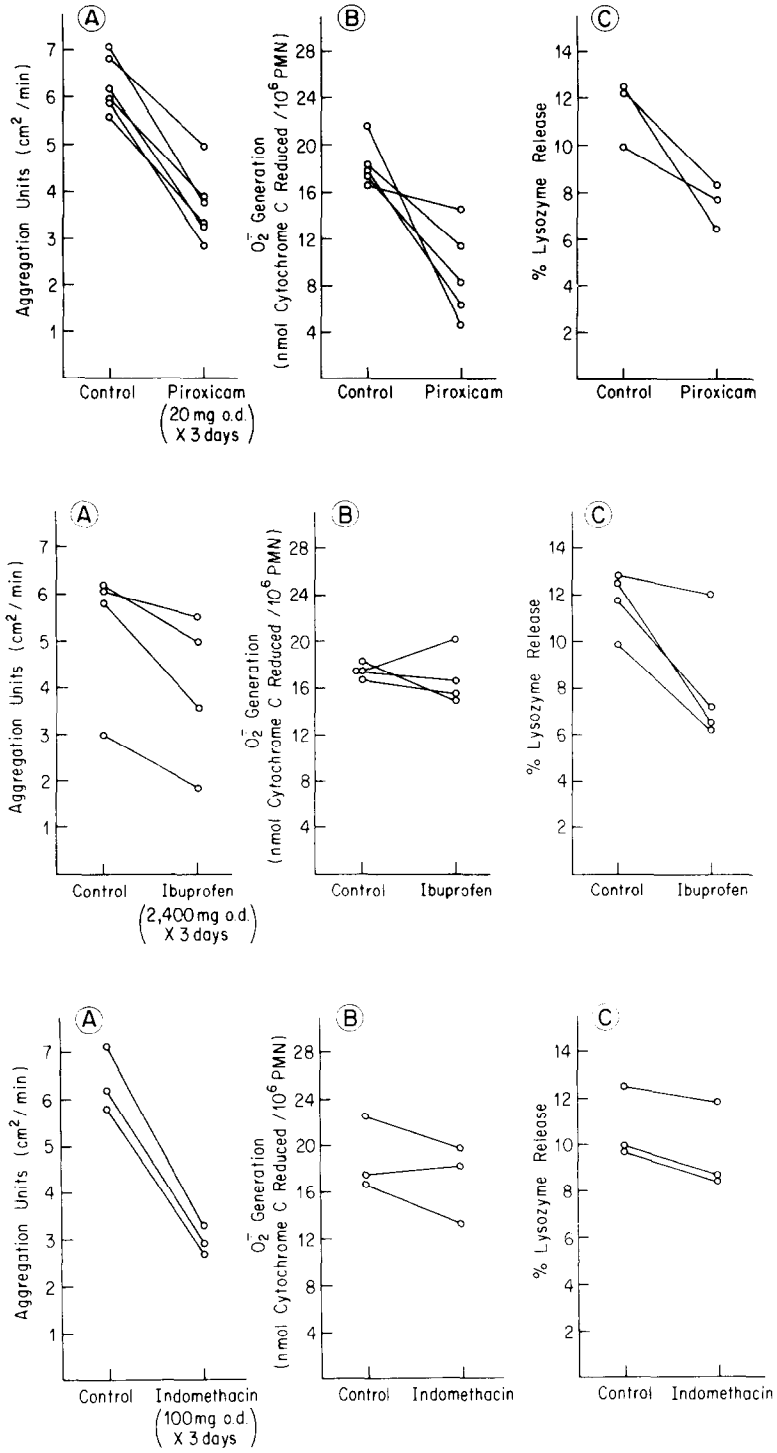


Fig. 5. Effects of ibuprofen, indomethacin, and piroxicam on neutrophil function *in vivo*. Neutrophils from healthy human donors taking the prescribed drug were prepared according to Materials and Methods. Studies of aggregation (A), superoxide generation (B), and lysozyme release (C) were also performed as described in Materials and Methods.

putative inhibitors of arachidonate metabolism, their responses (aggregation, superoxide generation, lysosomal enzyme release and changes in CTC fluorescence) differed depending upon the stimulus employed. Furthermore, when ibuprofen, indomethacin or piroxicam was administered p.o. for 3 days, their inhibitor effects on neutrophil functions *in vivo* followed patterns similar to those observed *in vitro*.

These results are in accordance with previous works indicating that NSAIDs inhibit lysosomal enzyme release from human neutrophils exposed to C5a and FMLP [22], guinea pig neutrophils exposed to serum-treated zymosan [23], rabbit neutrophils exposed to calcium ions [24], and from human neutrophils treated with FMLP, STZ, and A23187 [10]. The rank of potencies (ETYA > indomethacin > ETI > ibuprofen > aspirin) agrees with previously published data [25, 26] which illustrated a correlation with the ability of the agent to suppress the inflammatory process in which granule-associated enzymes have been localized to the site of tissue injury. Furthermore, Smolen and Weissmann [10] reported that lysosomal enzyme release in response to the soluble chemotactic peptide FMLP is greatly inhibited by indomethacin or ETYA and that these agents are less effective when Con A or the calcium ionophore A23187 is employed as a stimulus. The data presented above [10] are not entirely consistent with this report; the discrepancies can be explained by the use in previous studies of the fungal metabolite cytochalasin B and the use of a 4-fold higher concentration of indomethacin. The inconsistency with respect to the interpretation of data using cytochalasin B-treated neutrophils versus untreated neutrophils has been reported previously [1, 12]; these studies reported differences in the degranulation response [12] as well as in the kinetics of aggregation [1].

In an attempt to examine whether anti-inflammatory drugs could affect calcium metabolism in the neutrophils, their effects were examined on the CTC response of CTC-preloaded neutrophils. The use of CTC as a probe of membrane associated calcium was first suggested by Caswell and Hutchinson [27], as CTC preferentially partitions into hydrophobic regions of lipid bilayers and generates a characteristic fluorescence emission spectra when complexed with calcium. Indeed, changes in the fluorescence of cells preloaded with CTC, upon exposure to appropriate stimuli, have been recorded in a variety of cell types [19, 28]. In each secretory cell type studied, secretion anteceded, or accompanied, rapid decrements in CTC fluorescence. Indeed, Feinstein [28] reported that the CTC response of preloaded platelets is a necessary antecedent of platelet aggregation and secretion, never being observed in the absence of a CTC response, and that inhibition of the CTC response is invariably accompanied by inhibition of secretion.

We reported recently that piroxicam, a new non-steroidal anti-inflammatory agent and putative inhibitor of arachidonic acid metabolism, is capable of dissociating the CTC response from secretion in human neutrophils [20]. While this suggests that the CTC response may not be an absolute requirement for secretion, the possibility exists that piroxicam may interact with the assay in some novel manner and so alter the CTC response. In our present report, we have demonstrated that another putative inhibitor of arachidonic acid metabolism, ETI, which is structurally unrelated to piroxicam, can also dissociate the CTC response from secretion induced by either Con A or PMA. The only characteristic shared by both ETI and piroxicam is the ability to inhibit the binding of [^{3}H]FMLP to neutrophils. It is unclear whether their effects on [^{3}H]FMLP binding and their abilities to dissociate secretion from the CTC response are causally related. Recently, Yuli, *et al.* [29] reported that "membrane-fluidizers" may nonspecifically alter the affinity of the FMLP receptor for its ligand. It is, therefore, possible that both piroxicam and ETI share effects on membrane architecture which affect the function of the FMLP receptor. Since the fluorescence of CTC is also dependent on membrane events, those agents may interfere with the capacity of this probe to report on the mobilization of membrane-associated calcium. In contrast, ibuprofen, which is also a putative cyclooxygenase inhibitor, inhibited the CTC response only when secretion was inhibited. Furthermore, ibuprofen had no effect on the binding of [^{3}H]FMLP to neutrophils. Therefore, it appears that inhibitors of arachidonic acid metabolism can affect neutrophil function through mechanisms not attributable to their effects on either the cyclooxygenase or lipoxygenase pathway.

That non-steroidal anti-inflammatory agents have effects other than the inhibition of prostaglandin biosynthesis is well documented, and these effects depend on the doses employed. Kantor and Hampton [30] have reported that indomethacin inhibits cyclic AMP-dependent protein kinase in rabbit ileal mucosa and Kaplan *et al.* [13] have reported the inhibitory activity of this agent on rabbit phospholipase A_2 (PLA_2) activity. Our data demonstrate that indomethacin had a minor effect on lysosomal enzyme release despite the interpretation offered by Smolen and Weissmann [10] that indomethacin inhibition of LER may be due to diminished phospholipase activity. However, the inhibition by indomethacin of neutrophil aggregation is consistent with the hypothesis that indomethacin inhibits PLA_2 activity. In rabbit polymorphonuclear leukocytes, it has been shown that AA released by the action of PLA_2 is sufficient for degranulation and aggregation [11]. However, previous work by this laboratory has demonstrated that LER and aggregation are parallel, but independent, processes in human cells. AA is metabolized not only to stable prostaglandins and thomboxanes via cyclooxygenase but also to mono-, di- and trihydroxy-fatty acids via 5-lipoxygenase enzyme [31–33]. The effect seen with ETYA could be attributed to the putative inhibitory activity of the agents on the lipoxygenase pathway. These HPETE and HETE compounds have been shown to be chemotactic for alveolar macrophages and human neutrophils [25, 34, 35], suggesting a possible role for these compounds in the activation of the neutrophil in an inflammatory response. The effect seen with ETYA may be attributable to an effect of the agents on lipoxygenase pathways and the result-

ant AA metabolites, since it cannot be explained by an interference with or inhibition of [^{3}H]FMLP binding (see Results). We have demonstrated that the concentration of ETYA employed inhibited [^{3}H]FMLP binding by 19% while all other neutrophil functions were inhibited by greater than 50%. Clearly, this agent exerted actions other than those attributable to direct inhibition of binding. Conversely, ETI, another putative lipoxygenase inhibitor, inhibited binding by 57% while inhibiting O_2^- and LER by 84.6 and 63% respectively. These observations correlate with the inhibition of FMLP binding and the inhibition of neutrophil activation.

Neither ETYA nor ETI is a specific inhibitor of the 5-lipoxygenase pathway. This raises the question as to what effects any specific inhibitor(s) may have on neutrophil function. Indeed, Egan *et al.* [36] reported recently the development of a class of agents which appear to specifically inhibit 5-lipoxygenase in cell-free systems. However, these authors question the selectivity of even these inhibitors when used in intact cells. More recently, Serhan *et al.* [37] reported that colchicine may specifically block the leukotriene B synthetase. In addition, Hoffstein *et al.* [38] have reported previously that colchicine is capable of inhibiting lysosomal enzyme release and microtubule assembly in intact cells. Certainly, the development of more specific probes of the 5-lipoxygenase pathway will greatly aid our ability to characterize its role in neutrophil function(s).

One problem with reports indicating that non-steroidal anti-inflammatory agents can inhibit neutrophil function *in vitro* is the question of whether or not they can similarly inhibit neutrophil function *in vivo*. In this study we report that NSAIDs can affect the responses of neutrophils from subjects given these drugs in therapeutic doses. It has been reported that the addition of protein to isolated neutrophils interferes with the inhibitory activity of indomethacin [10, 26], presumably by nonspecific absorption. These *in vivo* studies suggest that the effects seen with indomethacin, ibuprofen or piroxicam *in vivo* were irreversible since any free drug that was present was eliminated during the isolation procedure. Thus, if one accepts the hypothesis that neutrophils play a critical role in the inflammatory response, then it appears that non-steroidal anti-inflammatory agents may, at least in part, affect the inflammatory response through their action on neutrophils. It is clear, however, that the current hypothesis, which suggests that NSAIDs retard neutrophil reactions entirely by inhibiting cyclooxygenase, is not supported by these data, and that other mechanisms of action of these agents on the neutrophil remain to be elucidated.

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REFERENCES

1. H. B. Kaplan, H. S. Edelson, R. Friedman and G. Weissmann, *Biochim. biophys. Acta* **721**, 55 (1982).
2. P. R. Craddock, D. E. Hammerschmidt, A. P. Dalmasso, J. G. White and H. S. Jacob, *J. clin. Invest.* **60**, 260 (1977).
3. I. M. Goldstein, D. Roos, H. B. Kaplan and G. Weissmann, *J. clin. Invest.* **56**, 1155 (1975).
4. I. M. Goldstein, S. Hoffstein, J. Gallin and G. Weissman, *Proc. natn. Acad. Sci. U.S.A.* **70**, 2916 (1973).
5. R. B. Zurier and D. M. Sayadoff, *Inflammation* **1**, 93 (1975).
6. I. M. Goldstein, C. L. Halmsten, H. Kindahl, H. B. Kaplan, O. Radmark, B. Samuelsson and G. Weissmann, *J. exp. Med.* **148**, 787 (1978).
7. B. Samuelsson and S. Hammarstrom, *Prostaglandins* **19**, 645 (1980).
8. J. R. Vane, *Nature New Biol.* **231**, 232 (1971).
9. S. H. Ferreira and J. R. Vane, *A. Rev. Pharmac.* **14**, 691 (1974).
10. J. E. Smolen and G. Weissmann, *Biochem. Pharmac.* **29**, 533 (1980).
11. J. M. Lackie, *Inflammation* **2**, 1 (1977).
12. J. T. O'Flaherty, H. J. Showell, P. A. Ward and E. L. Becker, *Am. J. Path.* **96**, 799 (1979).
13. L. Kaplan, J. Weiss and P. Elsbach, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2955 (1978).
14. G. W. Gullikson, M. Sender and P. Bars, *J. Pharmac. exp. Ther.* **220**, 236 (1982).
15. I. M. Goldstein, S. T. Hoffstein and G. Weissmann, *J. Cell Biol.* **66**, 647 (1975).
16. A. Boyum, *Scand. J. clin. Lab. Invest.* **21**, (Suppl. 97), 77 (1968).
17. I. M. Goldstein, M. Brai, G. Osler and G. Weissmann, *J. Immun.* **111**, 33 (1973).
18. W. E. C. Wacker, D. D. Ulmer and B. C. Vallee, *New Engl. J. Med.* **255**, 449 (1956).
19. P. H. Naccache, M. Volpi, H. J. Showell, E. L. Becker and R. I. Sha'afi, *Science* **203**, 461 (1979).
20. H. S. Edelson, H. B. Kaplan, H. M. Korchak, J. E. Smolen and G. Weissmann, *Biochem. biophys. Res. Commun.* **104**, 247 (1982).
21. S. S. Adams and J. W. Butler, *Clin. rheum. Dis.* **5**, 359 (1979).
22. R. O. Webster and P. M. Henson, *Inflammation* **3**, 129 (1978).
23. R. J. Smith, *J. Pharmac. exp. Ther.* **207**, 618 (1978).
24. R. J. Flower, *Pharmac. Rev.* **26**, 33 (1974).
25. J. A. Tainer, S. R. Turner and W. S. Lynn, *Am. J. Path.* **81**, 401 (1975).
26. A. J. Anderson, *Pharmac. Res. Commun.* **3**, 13 (1971).
27. A. H. Caswell and J. D. Hutchinson, *Biochem. biophys. Res. commun.* **42**, 43 (1971).
28. B. B. Feinstein, *Biochem. biophys. Res. Commun.* **93**, 593 (1980).
29. I. Yuli, A. Tomonage, C. Koo, L. Takoi and R. Snyderman, *Clin. Res.* **30**, 3617 (1982).
30. H. S. Kantor and M. Hampton, *Nature, Lond.* **276**, 841 (1978).
31. M. Hamberg and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **71**, 3400 (1974).
32. E. J. Goetzl and F. F. Sun, *J. exp. Med.* **150**, 406 (1979).
33. P. Borgeat, M. Hamberg and B. Samuelsson, *J. biol. Chem.* **251**, 7816 (1977).
34. S. R. Turner, J. A. Tainer and W. S. Lynn, *Nature, Lond.* **257**, 680 (1976).
35. E. J. Goetzl, J. M. Woods and R. R. Gorman, *J. clin. Invest.* **59**, 179 (1977).
36. R. W. Egan, A. N. Tischler, E. M. Baptista, E. A. Ham, D. D. Soderman and P. H. Gale, in: *Advances in Prostaglandin, Thromboxane and Leukotriene Research* Eds. B. Samuelsson, R. Paoletti and P. Ramwell), Vol. 11, pp. 151–7. Raven Press, New York (1983).
37. C. N. Serhan, U. Lundberg, S. Abramson, B. Samuelsson and G. Weissman, *Clin. Res.* **31**, 521A (1983).
38. S. Hoffstein, I. M. Goldstein and G. Weissmann, *J. Cell Biol.* **73**, 242 (1977).