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A COMPARATIVE STUDY ON THE EFFECTS OF INHIBITORS OF THE LIPOXYGENASE PATHWAY ON NEUTROPHIL FUNCTION

INHIBITORY EFFECTS ON NEUTROPHIL FUNCTION MAY NOT BE ATTRIBUTED TO INHIBITION OF THE LIPOXYGENASE PATHWAY

YUKIO OZAKI*, TATSUYA ŌHASHI* and YUKIE NIWA†

*First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan, and †Niwa Institute of Immunology, Tosashimizu, Kochi, Japan

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Abstract—The effects of five inhibitors of the lipoxygenase pathway were evaluated on oxygen radical production, degranulation, chemotaxis, leukotriene B_4 (LTB_4) production by neutrophils. The lipoxygenase inhibitors tested were nordihydroguaiaretic acid (NDGA), esculetin, eicosatetraynoic acid (ETYA), 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861), and 6,9-deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin I_1 (U-60,257). Neutrophils were activated by *n*-formylmethionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA), A23187, or platelet activating factor (PAF). The effects of these inhibitors on NADPH oxidase activity and phospholipase A_2 activity of isolated particulate fraction of neutrophils were also evaluated. ETYA inhibited neutrophil function induced by all the stimulators except PMA. AA-681 was unique in that it did not inhibit PAF-induced neutrophil activation. U-60,257 had virtually no effect on oxygen radical production and degranulation, but chemotaxis was moderately suppressed. NDGA effectively inhibited neutrophil function, except for chemotaxis. Esculetin inhibited only oxygen radical production, but this was due to inhibition on NADPH oxidase activity of neutrophil membrane. The inhibitory effect on neutrophil function and that of LTB_4 production were not closely correlated. It is suggested that lipoxygenase inhibitors may modify neutrophil function by the mechanism not involving the lipoxygenase pathway. It is also suggested that LTB_4 may not be a mediator in neutrophil oxygen radical production and degranulation induced by the stimulators used in the present study.

There is growing evidence suggesting that leukotrienes, derived from lipoxygenation of intracellular arachidonic acid, are closely associated with neutrophil function [1–4]. Among various kinds of leukotrienes, the products of 5-lipoxygenase appear to elicit most effectively a functional response of neutrophils; 5-hydroxyeicosatetraenoic acid and leukotriene B_4 (LTB_4) serve as chemoattractants for neutrophils [2, 4, 5], and cause degranulation of the cells [6]. LTB_4 also induce oxygen radical production by neutrophils [7]. Recent works suggest that leukotrienes act as a secondary mediator of cell activation, and in conjunction with Ca^{2+} influx, platelet activating factor, and protein kinase C, lead to a complete response of the cells [8–10]. Many agents that activate neutrophils are known to enhance production of leukotrienes in neutrophils [11, 12]. However, to what extent the products of 5-lipoxygenase contribute to activation of neutrophils by a particular stimulus has not been clearly elucidated.

Depletion of intracellular levels of these products by inhibitors of the lipoxygenase pathway has been shown to result in a diminished cellular response, and these inhibitors have been widely used to determine the role of lipoxygenase products in the functional responses of neutrophils [13–17]. In a preliminary study, we found that lipoxygenase inhibitors have differential effects on several parameters of neutrophil function. We also found that the inhibitory effects of some of these inhibitors are restricted to neutrophil activation elicited by certain stimulators but not by others. Several previous works have also referred to these findings [15, 16]. These differences may be attributed to the relative importance of lipoxygenase products in a particular parameter of neutrophil function, or they may be due to the effects of lipoxygenase inhibitors on enzymes other than lipoxygenase.

In the present study, we evaluated the effects of five inhibitors of 5-lipoxygenase on superoxide production (O_2^-), hydroxyl radical production ($\cdot OH$), degranulation, and chemotaxis, induced by four stimuli with different mechanisms of neutrophil activation. Two parameters of oxygen radical production were measured to exclude non-specific effects of lipoxygenase inhibitors on measurement of oxygen radicals.

To determine whether the effects of these inhibitors on neutrophils may be attributed to inhibition of some enzymes other than lipoxygenase, we evaluated the effects of these lipoxygenase inhibitors on NADPH oxidase activity of isolated neutrophil membrane, which regulates the final step of oxygen radical production by neutrophils [18], and on phospholipase A_2, which is a key enzyme in arachidonic acid metabolism [19].

MATERIALS AND METHODS

Materials. Five lipoxygenase inhibitors were evaluated in the present study. Nordihydroguaiaretic acid (NDGA) and esculetin were obtained from Sigma Co., MO. Eicosatetraynoic acid (ETYA), 2-(12-hydroxydodeca-5,10-dinyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861), and 6,9-deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin I_1 (U-60,257) were generous gifts from Roche Japan, Kanagawa, Japan; Takeda Chemical, Osaka, Japan; and Upjohn Company, MI, respectively. Unless otherwise stated, an appropriate concentration of an inhibitor was added to the cell suspension 5 min before the addition of a stimulus. The stimuli were *n*-formyl-methionyl-leucyl-phenylalanine (fMLP) 1 μM, phorbol myristate acetate (PMA) 20 ng/ml, A23187 1 μM, and PAF 1 μM. In experiments dealing with oxygen radical production and degranulation induced by fMLP, A23187, and PAF, cytochalasin B (CB) at the concentration of 5 μg/ml was added to the cell suspension 5 min before the addition of a stimulus.

Preparation of human neutrophils. Human neutrophils were purified by the method described elsewhere [20]. To lyse the contaminating red blood cells, the pellet of cells was suspended in a Tris–ammonium chloride solution for 30 sec and washed twice in Krebs–Ringer's phosphate solution with 0.1% gelatin and 5 mM glucose (KRP). Neutrophils were resuspended in KRP at the concentration indicated in each measurement. The cell suspension thus prepared contained more than 95% neutrophils as determined on a Giemsa-stained smear.

Measurement of superoxide (O_2^-). O_2^- production was measured with a slight modification of the technique of Johnston and Lehmeyer [21]. In brief, 2×10^6 neutrophils were suspended in 1 ml of KRP containing 100 μM cytochrome *c*. After addition of a stimulator, the mixture was incubated at 37° for 10 min with mild shaking. After the incubation, the cell suspension was immediately placed on ice to stop the reaction, and was centrifuged at 100 *g* for 10 min. The absorbance of the supernatant thus obtained was measured at the wavelength of 550 nm. The reduction of cytochrome *c* that could be inhibited by SOD was calculated with the extinction coefficient of $2.1 \times 10^4\ M^{-1}\ cm^{-1}$. Controls consisted of cell preparations not exposed to stimuli but incubated with ferricytochrome *c* or ferricytochrome *c* plus SOD.

Measurement of ·OH. Production of ·OH was measured using the formation of ethylene from 2-keto-4-thiomethylbutyric acid (KMB), a reaction that is accelerated in the presence of ·OH [22]. Briefly, 5×10^6 neutrophils in 2 ml of KRP containing 1 mM KMB were incubated in a vial capped with sleeve-type rubber stopper at 37° for 15 min with mild shaking. After addition of a stimulator, the mixture was incubated again for another 30 min in a shaking water bath maintained at 37°. At the end of incubation, a 0.2 ml sample of the gas phase was obtained using an air-tight syringe, and the ethylene was measured by analysis in a gas chromatography device. The amount of ethylene formed was standardized by a known amount of ethylene. Since ethylene formation from KMB can be mediated by radicals other than ·OH, the specificity of the ·OH assay was checked by another assay method, utilizing the fluorimetric analysis of the production of formaldehyde from dimethylsulfoxide [23].

Measurement of degranulation. Neutrophil degranulation was accessed by measurement of the release of *n*-acetyl-*b*-D-glucosaminidase (NAG). In brief, 3×10^6 neutrophils in 0.5 ml of KRP was preincubated with 5 μg/ml CB for 5 min. Then, a stimulus was added to the cell suspension and the mixture was incubated at 37° for another 15 min. After the incubation period, the cell suspension was set on ice to stop the reaction. The resultant supernatant after centrifugation was assayed for the content of NAG. NAG was measured with NAG Test Kit Shionogi, Shionogi Pharmaceuticals, Osaka, Japan, which used *m*-cresolsulfonphthaleinyl *n*-acetyl-*b*-D-glucosaminide as the substrate [24]. NAG release was expressed as percent of total NAG available in detergent lysate of the cell suspension.

Chemotaxis. Neutrophil chemotactic responsiveness was measured by the agarose method [25]. Agarose plates were prepared by mixing 2.5 ml of 2.4% agarose solution with 2.5 ml of 2 × RPMI medium supplemented with 10% heat-inactivated fetal calf serum. Where indicated, an appropriate concentration of an inhibitor was added to the agarose mixture. Three wells with a diameter of 3 mm were cut on a straight axis 7 mm from each other. To the center well, 10 μl of 1×10^8/ml neutrophils, preincubated for 5 min with the same inhibitor contained in the agarose mixture, was added. To the outer well, 10 μl of 10^{-7} M fMLP was added as a chemo-attractant. To the inner well, 10 μl of the RPMI medium was added as a control. The plates were incubated at 37° in a humidified atmosphere containing 5% CO_2 in air for 2 hr. The distance traveled by the ten fastest-moving neutrophils toward the outer well (chemotaxis, C.R.) was measured with a microprojector.

Production of Leukotriene B_4 (LTB_4). The effects of lipoxygenase inhibitors on LTB_4 production by neutrophils were evaluated by a RIA assay of LTB_4. In brief, 5×10^6 neutrophils in KRP were preincubated with an appropriate concentration of an inhibitor at 37° for 5 min. Then, A23187 at the final concentration of 1 μM was added to the cell suspension, and the mixture was incubated at 37° for another 10 min. The cell suspension was then set in ice to stop the reaction. The resultant supernatant after centrifugation was assayed for the content of LTB_4. LTB_4 was assayed with the Leukotriene B_4 [^{3}H] assay reagents system provided by Amersham International, U.K.

Effects of lipoxygenase inhibitors on the NADPH oxidase activity of isolated particulate fraction of neutrophils. The NADPH oxidase activity of the particulate fraction of neutrophils was assayed by NADPH-dependent O_2^- production. The particulate fraction of neutrophils was prepared according to the method described by Bender *et al.* [26]. Briefly, 3×10^7 neutrophils in KRP were stimulated with 10 μg/ml of PMA for 3 min in the presence of KCN. The cells were washed twice and resuspended in

0.34 M sucrose. The suspension was then sonicated, and centrifuged at 400 *g* for 10 min to remove intact cells. The resultant supernatant was centrifuged at 27,000 *g* for 30 min, and the pellet was resuspended in 0.34 M sucrose. Protein determinations were done by the method of Lowry *et al.*, using bovine serum albumin as a standard [27].

NADPH-dependent O_2^- production in isolated particulate fractions was measured as superoxide dismutase inhibitable reduction of cytochrome *c* [26]. The assay mixture consisted of 0.05 M potassium phosphate buffer, pH 7.0, 0.2 mM NADPH, 0.08 mM cytochrome *c*, 0.2 mg/ml particulate fraction, and an appropriate concentration of one of the lipoxygenase inhibitors in a total volume of 1.5 ml. Controls contained 50 μg/ml superoxide dismutase in addition to the mixture mentioned above. The reaction was performed at room temperature, and the initial slopes for the first 4 min were used for calculations.

In some experiments, the NADPH oxidase activity of the particulate fraction was assessed by NADPH-dependent ·OH production, using the formation of ethylene from KMB. The assay mixture in a total volume of 1.5 ml consisted of 0.05 M potassium phosphate buffer, pH 7.0, 0.2 mM NADPH, 2 mM KMB, 0.1 μM $FeSO_4$ 5 μg/ml horseradish peroxidase, 0.2 mg/ml of particulate fraction, and an appropriate concentration of one of the inhibitors.

Assays of phospholipase A_2. Effects of the lipoxygenase inhibitors on phospholipase A_2 activity of isolated microsomal fractions of human neutrophils were evaluated by the method described previously [28]. In brief, neutrophils in 0.25 M sucrose were disrupted by sonication at 24 W for 10 sec on ice. The crude sonicates were centrifuged at 14,000 *g* for 10 min at 4°, and the resultant supernatant was further centrifuged at 104,000 *g* for 60 min at 4°. Protein determinations were done by the method of Lowry *et al.*, using bovine serum albumin as a standard. The assay mixture consisted of 0.5 μCi *b* - [1 - ^{14}C] - arachidonyl - L - *a* - phosphatidylcholine (Amersham, 59.3 mCi/nmol), 200 μg microsomal fraction, 2 mM $CaCl_2$, and an appropriate concentration of one of the lipoxygenase inhibitors in 0.5 M Tris–HCl, pH 8.55. After the mixture had been incubated at 37° for 10 min, 1.5 ml of chloroform–methanol (2:1, v/v) at 4° was added and vortexed. The chloroform layer was evaporated, and applied to a thin-layer chromatography. The spot corresponding to arachidonic acid was scraped off and the radioactivity was counted in a Packard liquid scintillation counter. The data were expressed as percent radioactivity recovered/mg protein/min.

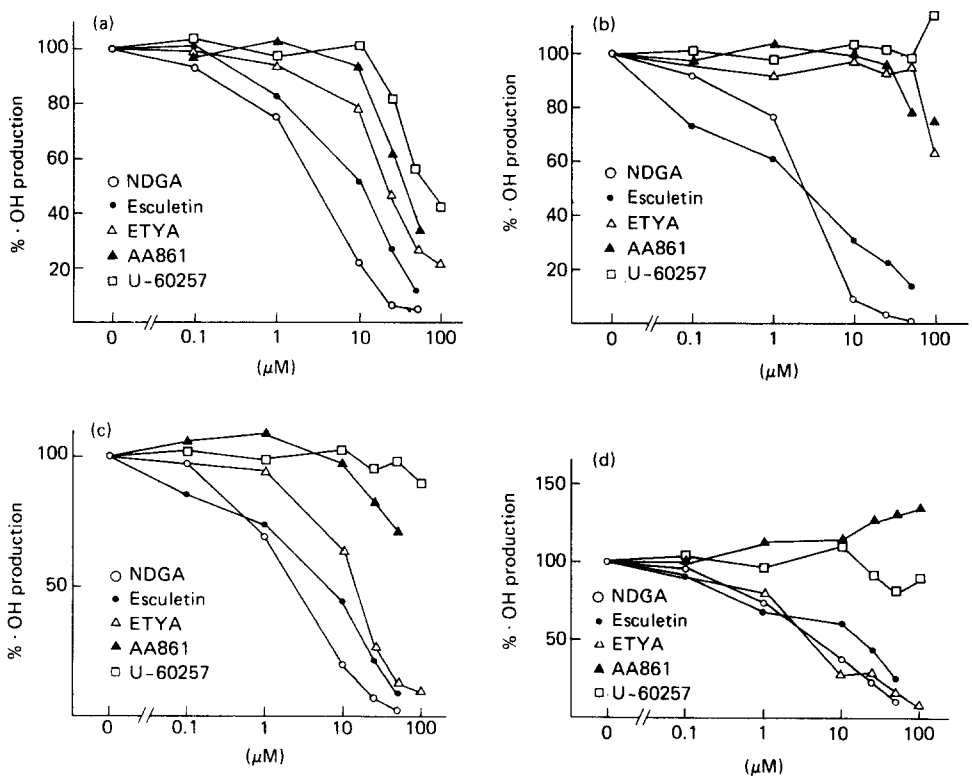

Fig. 1. Effects lipoxygenase inhibitors on ·OH production by neutrophils. Purified neutrophils (3×10^6) in 2 ml of KRP containing 1 mM KMB were preincubated for 5 min at 37° with CB at the concentration of 5 μg/ml (CB was omitted when the stimulator was PMA) and the indicated concentrations of the inhibitors. After addition of the stimulator, the mixture was incubated further for 30 min with mild shaking. A 0.2 ml sample of the gas phase was obtained and the content of ethylene gas was measured in a gas chromatography device. The results were compiled from three experiments and the data are expressed as percentages of the activity of control cells. (a) fMLP (1 μM) was used as the stimulator; (b) PMA (20 ng/ml); (c) A23187 (1 μM); (d) PAF (1 μM).

RESULTS

Effects of the lipoxygenase inhibitors on ·OH *production*

(a) fMLP-induced ·OH *production.* The average ·OH production induced by fMLP was 18.63 ± 4.3 pmole/3 × 10^6/30 min. All the lipoxygenase inhibitors tested inhibited ·OH production induced by fMLP dose-dependently, potency being in the order of NDGA > esculetin > ETYA > AA861 > U-60, 257 (Fig. 1a).

(b) PMA-induced ·OH *production.* The average ·OH production induced by PMA was 60.8 ± 21.9 pmole/3 × 10^6/30 min. NDGA and esculetin inhibited ·OH production dose-dependently (Fig. 1b). AA861 and U-60,257 had no or only minimal inhibitory effects on PMA-induced ·OH production. ETYA had no inhibitory effect at the concentration of less than 50 μM. At 100 μM, ETYA reduced ·OH production by 38%. AA861 at a concentration higher than 50 μM inhibited ·OH production only by 22.1–28.7%. U-60257 had no inhibitory effect at any concentration tested.

(c) A23187-induced ·OH *production.* The average ·OH production induced by A23187 was 11.6 ± 4.9 pmole/3 × 10^6/30 min. NDGA, esculetin and ETYA inhibited ·OH production dose-dependently, potency decreasing in that order (Fig. 1c). AA861 and U-60,257 were not inhibitory at any concentration tested.

(d) PAF-induced ·OH *production.* The average ·OH production induced by PAF was 6.8 ± 3.6/ 3 × 10^6/30 min. NDGA, esculetin, and ETYA inhibited ·OH production dose-dependently (Fig. 1d). U-60,257 had a weak inhibitory effect on ·OH production, the highest rate of reduction being 17.1% at 50 μM. AA861 did not inhibit ·OH production at any concentration tested.

Effects of the lipoxygenase inhibitors on the study O_2^- *production*

The effects of lipoxygenase inhibitors on O_2^- production were essentially similar to those of ·OH production, except for two points. First, NDGA was omitted from the study on the inhibitory effects of the lipoxygenase inhibitors on O_2^-, since NDGA itself had a reducing activity on cytochrome *c* used for the measurement of O_2^-. Second, the inhibitory effect of AA861 was greater with O_2^- than with ·OH. The potency of AA861 was nearly that of ETYA.

Effects of the lipoxygenase inhibitors on degranulation

(a) fMLP-induced release of NAG. Neutrophils released 36.5% of the total cellular NAG upon stimulation by fMLP. NDGA, ETYA, and AA861 dose-dependently inhibited degranulation, potency being in the order of NDGA > ETYA > AA861 > U-60,257 (Fig. 2a). Although NDGA and ETYA almost completely inhibited degranulation, the effect

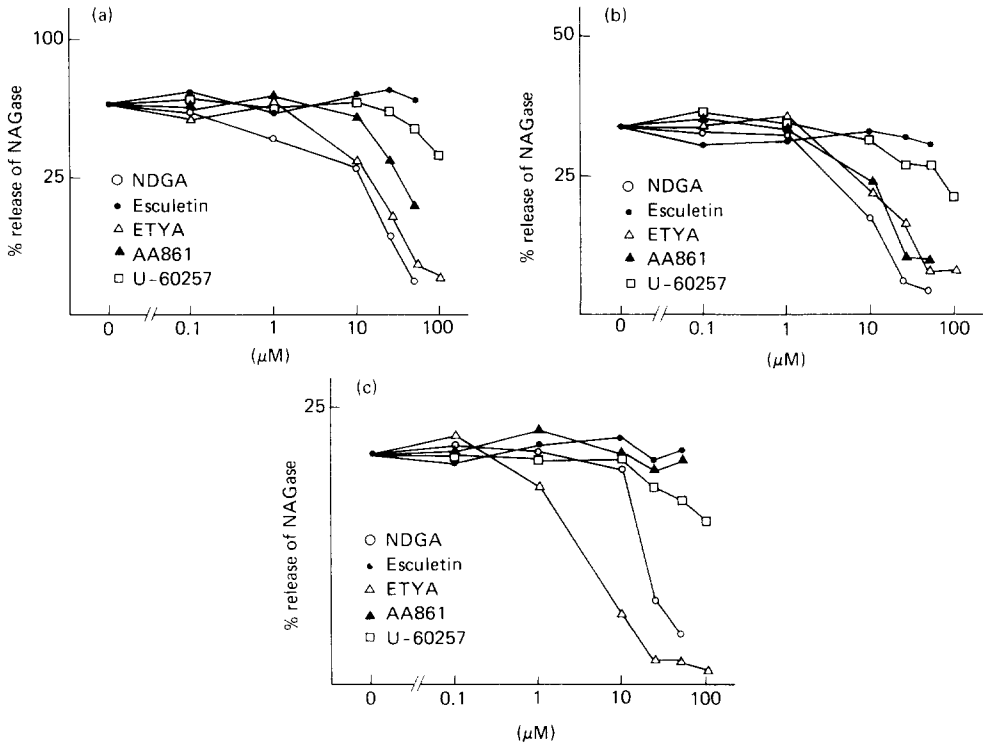

Fig. 2. Effects of lipoxygenase inhibitors on degranulation. Purified neutrophils (3 × 10^6) in 0.5 ml of KRP was preincubated for 5 min at 37° with CB (5 μg/ml) and the indicated concentrations of lipoxygenase inhibitors. After addition of the stimulator, the mixture was further incubated for 15 min. The resultant supernatant after centrifugation was assayed for the content of NAG. The results were compiled from three experiments and the data are expressed as percentages of total NAG available in detergent lysate of the cell suspension: (a) fMLP (1 μM) was used as the stimulator; (b) A23187 (1 μM); (c) PAF (1 μM).

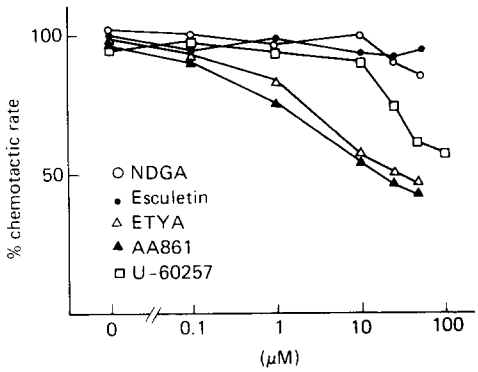

Fig. 3. Effects of lipoxygenase inhibitors on chemotaxis of neutrophils. Neutrophil chemotactic responsiveness was measured by the agarose plate method. Purified neutrophils were preincubated for 5 min with the indicated concentrations of a lipoxygenase inhibitor, and the agarose plate contained the same concentration of the inhibitor. Chemotaxis of neutrophils toward the higher gradients of fMLP was measured. The experiments were performed in duplicate and the data are expressed as percentages of the chemotactic responsiveness of the control cells.

of U-60,257 was rather weak, the highest rate of reduction being 23.3% at 100 μM. Escultein did not show any inhibitory effect at any concentration tested.

(*b*) *A23187-induced release of NAG.* Neutrophils released 33.2% of the total cellular NAG upon stimulation by A23187. NDGA, ETYA, AA861, and U-60,257 in a dose dependent manner inhibited degranulation (Fig. 2b). The rank order of potency was NDGA > ETYA ≧ AA861 > U-60,257. Although NDGA, AA861, and ETYA almost completely inhibited degranulation at appropriate concentrations, the activity of U-60,257 was rather weak, the highest rate of reduction being 39.1%. Esculetin was without effect at any concentration tested.

(*c*) *PAF-induced degranulation.* Neutrophils released 21.2% of the total cellular NAG upon stimulation by PAF. NDGA, ETYA, and U-60,257 inhibited degranulation dose-dependently, potency being in the order of ETYA > NDGA > U-60,257 (Fig. 2c). AA861 and esculetin did not show any inhibitory effect at any concentration tested.

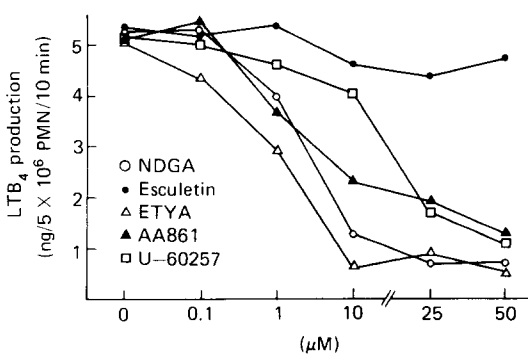

Fig. 4. Effects of lipoxygenase inhibitors on LTB_4 production by neutrophils. Purified neutrophils (5 × 10^6/l ml KRP) was preincubated for 5 min at 37° with the indicated concentrations of a lipoxygenase inhibitor. After addition of A23187 (1 μM), the mixture was further incubated for 10 min. The resultant supernatant after centrifugation was assayed for the content of LTB_4 by a RIA assay kit. The experiment was performed in duplicate and the data are expressed as percentages of the activity of the control cells.

Effects of the lipoxygenase inhibitors on chemotaxis

The average distance traveled by the moving front of neutrophils was 1.62 ± 0.24 mm/2 hr. ETYA, AA861, and U-60,257 inhibited the rate of chemotaxis (C.R.) dose-dependently, potency being in the order of AA861 = ETYA > U-60,257 (Fig. 3). NDGA had no effect below the concentrations of 25 μM, and even at the concentration of 50 μM, inhibited C.R. only by 15%. Esculetin had no inhibitory effect on C.R. at any concentration tested.

Effects of the lipoxygenase inhibitors on LTB_4 production

The average LTB_4 production by neutrophils induced by A23187 was 5.21 ng/5 × 10^6 PMN/10 min. ALl the lipoxygenase inhibitors except esculetin inhibited LTB_4 production dose-dependently (Fig. 4), potency being in the order of ETYA > NDGA > AA861 > U60,257. The inhibitory effect of U-60,257 was rather weak below the concentrations of 10 μM, but at 25 μM, it reduced LTB_4 formation by 67.3%. Compared with the other inhibitors, esculetin was extremely weak, the highest rate of reduction being 17.5% at 25 μM.

Effect of the lipoxygenase inhibitors on NADPH oxidase activity of particulate fraction of neutrophil membrane

Esculetin inhibited NADPH oxidase activity dose-dependently. ID_{50} of esculetin on NADPH oxidase activity was about 10 μM. ETYA, AA861 and U-60,257 had no inhibitory effect on NADPH oxidase activity at any concentration tested (Fig. 5). The

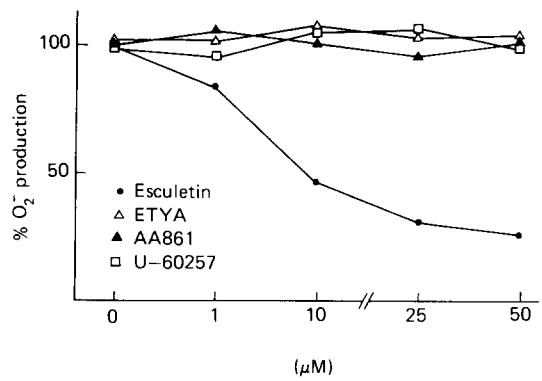

Fig. 5. Effects of lipoxygenase inhibitors on NADPH oxidase activity of particulate fraction of neutrophils. The NADPH oxidase activity of particulate fraction of neutrophils was assayed by NADPH-dependent O_2^- production. The assay mixture consisted of 0.05 M potassium phosphate buffer, pH 7.0, 0.2 mM NADPH, 0.08 mM cytochrome *c*, 0.2 mg/ml particulate fraction of neutrophils, and the indicated concentrations of a lipoxygenase inhibitor. The reaction was performed at room temperature, and the initial slopes for the first 4 min were used for calculations. The experiment was performed in duplicate, and the data are expressed as percentages of the activity of the control mixture.

Table 1a. Summary of the effects of five lipoxygenase inhibitors on neutrophil function

Lipoxygenase Inhibitor	O_2^- fMLP	PMA	A23187	PAF	·OH fMLP	PMA	A23187	PAF
NDGA	ND	ND	ND	ND	++++	++++	++++	++++
Esculetin	+++	++	+++	++	+++	+++	+++	+++
ETYA	+++	+	++	+++	++	−(+)	+++	+++
AA861	++	+	++	++	++	−(+)	++	−
U-60257	−(+)	−	−	+	++	−	+	+

Inhibitory effects of lipoxygenase on neutrophil function were evaluated at the concentration of 25 μM, and expressed as follows:

(++++) more than 60% reduction of the control activity;
(+++) 30–60% reduction;
(++) 15–30% reduction;
(+) 5–15% reduction;
(−) 0–5% reduction;
(−(+)) no inhibition was observed at 25 μM, but positive inhibitory effects were observed at higher concentrations.

Table 1b. Summary of the effect of five lipoxygenase inhibitors on neutrophil function

Lipoxygenase Inhibitors	Degranulation fMLP	A23187	PAF	C.R.	LTB_4	NADPH Oxidase	PLA_2
NDGA	++++	++++	++++	+	++++	++++	−
Esculetin	−	+	−	+	+	++++	−
ETYA	+++	++	+++	++	+++	−	−
AA861	++	+++	−	++	+++	−	−
U-60257	+	++	++	++	+++	−	−

Inhibitory effects of lipoxygenase on neutrophil function were evaluated at the concentration of 25 μM, and expressed as follows:

(++++) more than 60% reduction of the control activity;
(+++) 30–60% reduction;
(++) 15–30% reduction;
(+) 5–15% reduction;
(−) 0–5% reduction;
(−(+)) no inhibition was observed at 25 μM, but positive inhibitory effects were observed at higher concentrations.

effect of NDGA, which has a reducing activity on cytochrome *c*, was evaluated by NADPH-dependent ·OH production. The average ·OH production by 0.2 mg/ml particulate fraction of neutrophils was 9.45 ± 1.27 pmole/20 min. NDGA at 1 μM reduced ·OH production by 27.5 ± 3.6%, 76.8 ± 6.9% at 10 μM, and 91.9 ± 6.7% at 25 μM. The other lipoxygenase inhibitors had no significant effect on ·OH production.

Effect of the lipoxygenase inhibitors on phospholipase A_2 (PLA_2) activity of the microsomal fraction of neutrophil membrane

The average PLA_2 activity of the microsomal fraction of neutrophil membrane was 3.2 ± 0.3%/mg protein/min. None of the lipoxygenase inhibitors used in the present study had a significant inhibitory effect on PLA_2 activity at the concentrations of 1, 10 and 50 μM.

Tables 1a and 1b show the results of our work with the lipoxygenase inhibitors.

DISCUSSION

The inhibitors used in this study, NDGA, esculetin, ETYA, AA861, and U-60,257 have all been shown to inhibit the synthesis of lipoxygenase products in several tissue preparations and intact cells. Esculetin, AA861, and U-60,257 have been reported to be specific for lipoxygenase, without much effect on cyclooxygenase, while NDGA and ETYA inhibits both lipoxygenase and cyclooxygenase [29–35]. Generally, these agents are less potent in inhibiting lipoxygenase in whole cells than isolated cell-free preparations, and there is a wide range of inhibitory potency among the different cell types and the species of animals [36]. In the present study, ETYA, NDGA, AA861, and U-60,257 at the concentration of 25 μM inhibited LTB_4 production by human neutrophils by more than 60%, potency decreasing in that order. Esculetin had virtually no effect on LTB_4 production by human neutrophils. Esculetin has been found to possess an inhibitory effect on 5-lipoxygenase of mouse mast tumor cells with ID_{50} of 4×10^{-6}, without a significant effect on cyclooxygenase [30]. Neutrophil lipoxygenase of man may be more resistant to esculetin than that of mouse mast tumor cells, or there may be some other factors such as permeability of human neutrophil membrane to esculetin, that renders esculetin ineffective [30, 37].

The inhibitory effects of the five lipoxygenase inhibitors tested differed in the rate of reduction and the range of parameters of neutrophil function. Moreover, the inhibitory effects of these lipoxygenase inhibitors on neutrophil function did not correlate closely with the degree of inhibition on LTB_4 production; U-60,257, at concentrations where LTB_4 production was significantly inhibited, showed virtually no inhibitory effect on oxygen radical production and degranulation by neutrophils. ETYA, NDGA, and AA861, which are equally potent inhibitors of LTB_4 production, also differed in some parameters. Kitchen and Dawson reported similar findings with the effects of NDGA, BW755C and benoxaprofen on chemotaxis [15]. Two important points arise from these findings. First, the question remains as to the role of LTB_4 as a secondary mediator of neutrophil oxygen radical production and degranulation. It is reported that a high concentration of LTB_4 is required for the induction of O_2^- production [37], and LTB_4 production is not correlated with degranulation [12]. In agreement with these findings, we found that the reduced production of LTB_4 by U-60,257 did not modulate neutrophil function induced by the stimulators used in this study. Taken together, we assume that LTB_4 contributes little, if at all, to neutrophil oxygen radical production and degranulation. On the other

hand, LTB_4 is one of the most potent chemoattractants, and it is proposed to be a mediator of neutrophil accumulation in inflammatory sites [2, 4, 5]. The results of our study do not detract from the hypothesis that LTB_4 may be a mediator of neutrophil chemotaxis; U-60,257 moderately inhibited neutrophil chemotaxis. Then, the question arises as to whether the putative lipoxygenase inhibitors modify neutrophil function by the mechanism originally expected. Our findings in conjunction with the others suggest that the effects of lipoxygenase inhibitors on neutrophil function cannot be totally attributed to their inhibitory effects on lipoxygenase and their effects on other systems should be taken into consideration. A number of previous works used lipoxygenase inhibitors to examine whether arichidonic acid metabolites through the lipoxygenase pathway were involved in cell function. The role of the lipoxygenase pathway may have been overestimated, and some of them may need reevaluation.

Although there must be a number of enzyme systems that could modulate neutrophil function and each one of these should be evaluated, we evaluated the effects of these inhibitors on two well-known systems, NADPH oxidase and PLA_2 activity of particulate fraction of neutrophils.

NADPH oxidase complex is a key enzyme in oxygen radical production by neutrophils, and inhibition of this enzyme by an agent would result in a decreased rate of oxygen radical production by the whole cells. Of the five inhibitors used in the present study, NDGA and esculetin inhibited NADPH oxidase activity. Therefore, the inhibitory effects of the other three inhibitors on oxygen radical production by neutrophils could be attributed to the enzyme(s) other than NADPH oxidase complex. PLA_2, which releases arachidonic acid from phospholipids, is a key enzyme in metabolism of arachidonic acid. In the whole cells, inhibition of this enzyme as well as that of lipoxygenase would result in reduction of lipoxygenase products, including LTB_4. To exclude the possibility of these inhibitors reducing LTB_4 production by inhibiting PLA_2, we evaluated the effect of these inhibitors on PLA_2 activity of particulate fraction of the neutrophil membrane. None of the inhibitors inhibited PLA_2 activity at the concentrations required for almost complete inhibition of LTB_4 production by neutrophils. Although a smaller concentration of the inhibitor is required in a cell-free system than a whole-cell system, none of the agents tested in the present study inhibited PLA_2 activity of the cell-free system even at the concentrations at which LTB_4 production by neutrophils was almost completely inhibited. Therefore, we assume that the inhibitory effect of these lipoxygenase inhibitors on LTB_4 production by neutrophils is virtually independent of inhibition on PLA_2. By what mechanism do the putative lipoxygenase inhibitors modify neutrophil function? The state of our knowledge at present is limited, and it need further evaluations.

NDGA had a potent inhibitory effect on oxygen radical production, degranulation, and LTB_4 production. Although its inhibitory effect on NADPH oxidase activity of neutrophil membrane suggests that a part of its inhibitory effect on oxygen radical production by the whole cells is independent of its inhibitory effect on intracellular processes, its effect on the latter two indicates that NDGA does modulate certain intracellular processes that follow activation by the stimulators. In contrast to its effect on degranulation and LTB_4 production, it had only weak inhibitory effect on chemotaxis induced by fMLP. The different effects of NDGA on neutrophil function suggest that it may inhibit intracellular processes that finally lead to oxygen radical production and degranulation but not those of chemotaxis. These findings support the concept that several aspects of neutrophil function are separately regulated [38, 39]. In a previous study dealing with rabbit neutrophils, NDGA was found to have a strong inhibitory effect on chemotaxis, with ID_{50} of 3–5 μM [17]. This discrepancy may be due to the species differences; NDGA fails to modulate migration of rat neutrophils and guinea pig neutrophils.

The effect of ETYA and AA861 on several aspects of neutrophil function we evaluated is quite similar for PAF-induced neutrophil activation; ETYA and AA861 at similar concentrations inhibited oxygen radical production, degranulation, chemotaxis, and LTB_4 production, induced by fMLP or A23187. ETYA strongly inhibits oxygen radical production and degranulation induced by PAF, while AA861 had virtually no inhibitory effect on PAF-induced degranulation and only weak inhibitory effect on PAF-induced oxygen radical production. There is increasing evidence for the presence of several different pathways of activation involved by various stimulators [8, 9, 10, 40]. Although PAF is known to bind to specific receptors, to mobilize membrane-bound Ca^{2+} and to stimulate arachidonic acid metabolism [41–43], the pathway by which PAF activates neutrophils remains largely unknown. Our findings indicate that PAF activates neutrophils by a certain process that could be inhibited by ETYA and NDGA, but not by AA861. We did not evaluate PAF binding to neutrophil membrane, and therefore cannot exclude the possibilities that those lipoxygenase inhibitors except AA861 counteract PAF-induced activation of neutrophils by inhibiting PAF binding to neutrophil membrane, as is shown in the case of platelets; in platelets, non-steroidal anti-inflammatory agents have been shown to inhibit PAF binding to the membrane [44], although relatively high concentrations of the agents [50–100 μM) were required for this effect. Nonetheless, the ineffectiveness of AA861 on PAF-induced neutrophil activation appears to be of great importance, and it will serve as a good tool in investigating neutrophil activation induced by PAF.

ETYA and AA861 had a relatively weak inhibitory effect on PMA-induced oxygen radical production, while they moderately or strongly inhibited oxygen radical production and degranulation induced by A23187 or fMLP. Our findings appear to be in good agreement with the previous report that non-steroidal antiinflammatory agents did not inhibit PMA-induced neutrophil activation [45]. ETYA and AA861 may affect the signal transduction system employed by fMLP and A23187, but not that of PMA, and therefore, inhibit neutrophil activation induced by fMLP or A23187, but not by PMA.

Esculetin effectively reduced neutrophil oxygen radical production by all the stimuli. However, its potent inhibitory effect on NADPH oxidase activity suggest that the reduction is not caused by its effect on certain intracellular processes of neutrophils. Its failure to show inhibitory effects on degranulation, chemotaxis, and LTB_4 appears to support this hypothesis.

The results of our work on lipoxygenase inhibitors show that the potency of inhibition on LTB_4 production is not closely related to inhibition of neutrophil function. They raise the question as to the mechanism by which these agents actually exert their inhibitory effects on neutrophil function. It is also proposed that LTB_4 contributes little, if at all, to neutrophil oxygen radical production and degranulation induced by the stimuli used in the present study. Although the mechanism of their inhibitory effects on neutrophil function is not clearly understood, our findings showed that some of the lipoxygenase inhibitors have unique features that would help us to understand the mechanism by which neutrophils are activated by stimuli.

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