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Effects of Non-Steroidal Anti-Inflammatory Drugs on the FMLP-Induced Migration of Neutrophil

Woo Mi Kim and Kooil Kang*

Department of Pharmacology, College of Medicine, Kosin University, Pusan, Korea

ABSTRACT

Enhancement or diminution of leukocyte migration to the specific site might be important factors for the development of inflammatory diseases. To investigate the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on chemotaxis of neutrophil, we obtained neutrophils by Hypaque-Ficoll step gradient centrifugation and tested the effects of seven drugs on the n-formyl-leucyl-phenylalanine (FMLP)-induced migration of neutrophil using a 48-well micro chemotaxis assembly. Oxyphenbutazone, phenylbutazone, sulindac, zomepirac, and ibuprofen suppressed the migration of neutrophil at the therapeutic concentrations, however, indomethacin showed stimulation effect. IC50s for inhibition of neutrophil migration by these drugs are less than 100uM. When drugs were preincubated with FMLP, no inhibition on migration of neutrophil was observed. These results indicated that inhibitory effects of these drugs on migration of neutrophil might be related to the receptor sites of neutrophil rather than molecular inactivation of chemoattractant (FMLP). In conclusion, we suggested that the property of inhibition effects on neutrophil migration of several NSAIDs might be another mode of pharmacological action for anti-inflammatory effect, which showed significant effects at concentrations below therapeutic levels, in addition to cyclooxygenase inhibition.

Key Words: Non-steroidal anti-inflammatory drugs, Migration of neutrophil, Inhibitory effect

INTRODUCTION

Chemotaxis of neutrophils to the inflammatory site is the essential step for the defense mechanism of host against various injuries (Maureen dale, 1984). At the inflammatory locus production of activated oxygen radicals and release of lysosomal enzymes leads to not only destruction of foreign materials but also results in degradation of host tissues (Cotran *et al.*, 1984; Janoff, 1972a; Starky *et al.*, 1977). Inhibitors of leukocyte migration may act as modulators of chronic inflammatory reactions. Therefore, we assume that enhancement or diminution of leukocyte migration to the specific site will be important factors for the improvement of inflammatory diseases.

Rescently, we investigated that some non-steroidal anti-inflammatory drugs, which were known as inhibitors of cyclooxygenase showed inhibitory effects on migration of neutrophil. Even though aspirin-like drugs have been prescribed for the treatment of chronic inflammatory diseases, mode of action has been pooly understood. Therefore, present report describes the effects of several agents on neutrophil migration in vitro system at concentrations of below, within, and above the

*To whom correspondence should be addressed.

range of therapeutic blood levels.

MATERIALS AND METHODS

Materials

Ficoll, sodium diatrizoate (Hypaque), n-formyl-methionyl-leucyl-phenylalanine (FMLP), acetyl salicylate, ibuprofen, zomepirac, phenylbutazone, oxyphenbutazone, indomethacin, sulindac were purchased from sigma chemical Co. (St. Louis, MO, USA). Wright stain and buffer solution were purchased from Yeoung Dong Pharm. Corp. (Seoul, Korea), and Hank's balanced salt solution (HBSS) was purchased from Gibco Corp. (Grand Island, N.Y., USA).

48 well micro chemotaxis chamber assmbly and polycarbonate filter membrane were purchased from Neuro Probe Inc. (Cabin Johon, Maryland, USA) and Poretics Co. (Livemore, CA, USA), respectively. All other chemicals were of the higest quality obtainable.

Separation of human neutrophils

Human neutrophils were obtained from heparinized blood of healthy donors (10 units of heparin per ml of blood) by modification of the method of Jeong's (Jeong *et al.*, 1987). 6 ml of Blood was centrifugated on Ficoll-Hypaque density gradients at 200 g for 25 min. The leukocyte rich layer was mixed with 3 vol of HBSS and centrifuged for 5 min at 500 g. The cell pellet was resuspended in 5 ml of ice-cold water and residual erythrocytes were hypotonically lysed. Leukocytes were resuspended and washed twice with 2 vol of HBSS. Final cell suspensions contained 95% of neutrophils which were greater than 98% of viability (assessed by tryphan blue dye exclusion).

Drug preparation

FMLP was dissolved in DMSO at a stock concentration of 1 mM and prepared final concentration with HBSS. Acetyl salicylate, ibuprofen, zomepirac, phenylbutazone, oxyphenbutazone, indomethacin, sulindac were dissolved in 0.1% DMSO and were subsequently diluted in HBSS.

Chemotactic assay

Neutrophil chemotaxis assay was carried out with a 48-well micro chemotaxis assembly (Falk *et al.*, 1980; Harvath *et al.*, 1980). 27 ul of 10 uM FMLP added to the bottom wells. A polycarbonate filter sheet (25 mm × 80 mm) with 5 um holes was placed on top of the wells in the bottom plate. The gasket and top plate were fixed in place and the top wells were filled with 47 ul of HBSS containing 1×10^5 neutrophils and various concentration of drugs. The assembly was incubated for 20 min at 37℃ in humidified air with 5% CO_2. After incubation, the top plate, gasket and filter were removed, cells on top of the filter were wiped off and the filter was fixed in methanol for 20 sec. The filter was air-dried on a glass slide and stained with Wright stain solution.

Migrated cells on filter sheets were evaluated by means of a Diastar Photomicroscope with Photostar automatic camera system (Reichert-Jung, Cambridge Ins. Co.). Duplicate chambers were used in each experiment, and 10 fields were examined in each chamber with a 24X objective.

Table 1. Effects of NSAIDs on FMLP-induced migration of neutrophil

Drugs	Migrated neutrophils (1×10^4)
Control	2.04±0.08
Phenylbutazone^{++}	1.28±0.08
Oxyphenbutazone^{++}	0.68±0.11
Acetylsalicylate	1.96±0.12
Ibuprofen^{++}	1.56±0.04
Zomepirac^{++}	1.30±0.02
Indomethacin^{++}	2.32±0.08
Sulindac^{++}	1.36±0.01

Neutrophils (1×10^5) were incubated in the upper or bottom wells of chemotactic chamber with several NSAIDs or 10uM FMLP in HBSS, respectively. After incubation for 20min at 37℃, the filter containing migrated neutrophils was removed, fixed, stained and counted. Each value is the mean±S.E.M. of three separate experiments.

$^{+}$Significant at $P<0.01$ vs control

$^{++}$Significant at $P<0.005$ vs control

Statistical analysis was done by t-test and significancy was accepted by p-value of less than 0.005.

RESULTS

The effects of several non-steroidal anti-inflammatory drugs on FMLP-induced migration of neutrophil were described in Table 1. At their therapeutic concentrations, oxyphenbutazone, phenylbutazone, sulindac, zomepirac, and ibuprofen showed significant suppression of neutrophil migration. However, acetyl salicylate showed no effect and indomethacin showed stimulation effect under the assay conditions.

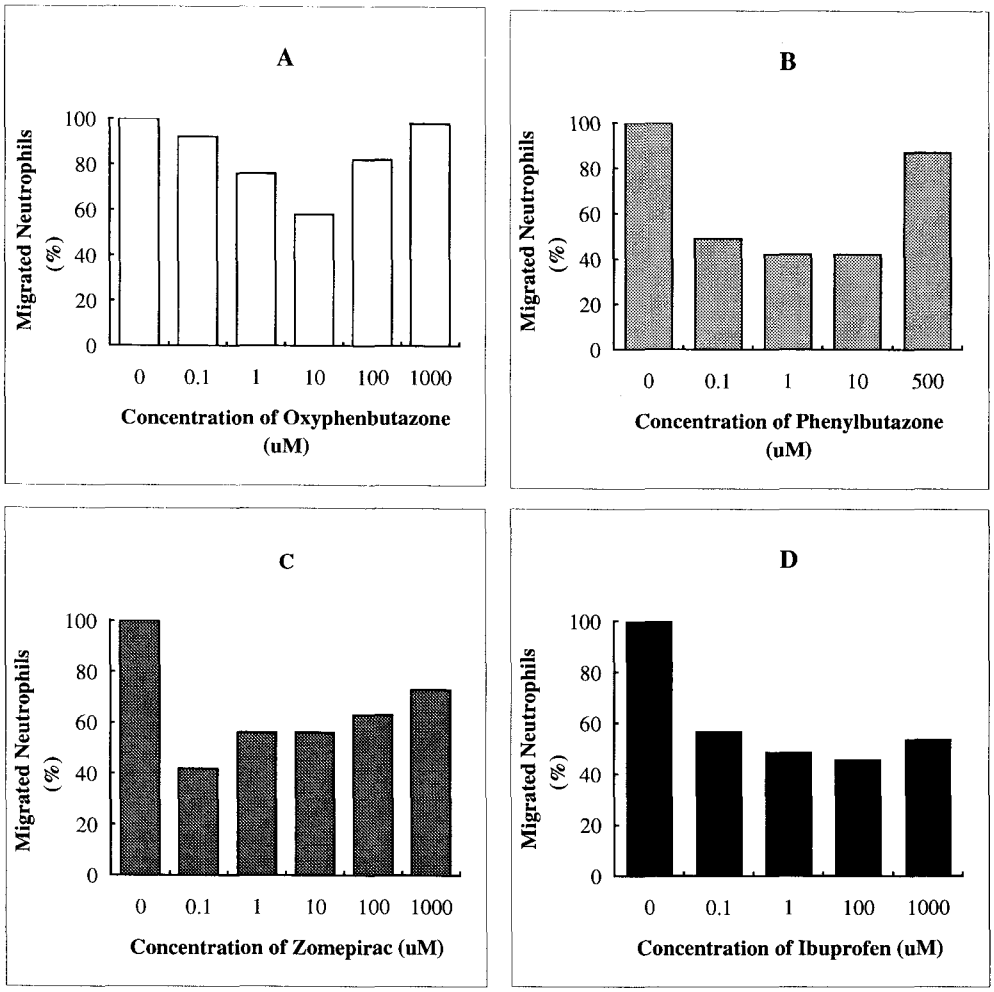

Fig. 1. Effects of oxyphenbutazone, phenylbutazone, zomepirac, ibuprofen on the FMLP-induced migration of neutrophil.

Neutrophils (1×10^5) were incubated in the upper or bottom wells of chemotactic chamber with various concentrations of oxyphenbu-tazone(A), phenylbutazone(B), zomepirac(C), ibuprofen(D) or 10 uM FMLP in HBSS respectively. After incubation for 20 min at 37℃, the filter containing migrated neutrophils was removed, fixed stained and counted. Each bar represents the mean of 10 determinants.

Subsequent studies about the inhibitory capacity of these drugs on the migration of neutrophil at various concentrations were carried out (Fig. 1, A ~D). At concentrations below therapeutic blood levels, oxyphenbutazone, phenylbutazone, zomepirac, ibuprofen inhibited neutrophil migration as much as 40~60% of control values. Especially, all of them also had maximal inhibition effect at the concentration of less than 100 uM.

When oxyphenbutazone was tested at concentrations of 0.1uM~10uM, neutrophil migration was inhibited in a dose dependent manner (Fig. 1, A). At a concentrations of 100 uM, 1 mM, however, there was only a little or no effect on neutrophil chemotaxis. The effects of phenylbutazone on neutrophil chemotaxis were similar to those of oxyphenbutazone (Fig. 1, B). Concentrations of 0. 1 uM, 10 M of phenylbutazone caused than 50% of control values, but the concentrations at 500 uM showed no inhibition effect on neutrophil chemotaxis. Zomepirac also showed inhibition effect of 100 uM as much as 44%, 44%, 37% of control values, respectively (Fig. 1, C).

At concentration of 0.1 uM, zomepirac showed maximal inhibition effect on migration of neutrophil. In contrast, ibuprofen caused significant suppression of neutrophil migration at the concentrations between 0.1 uM~1 mM (Fig. 1, D).

To determine whether these effects come from direct interaction between FMLP and drugs in solution or not, ibuprofen was incubated with FMLP in the lower chamber prior to start chemotactic assay, the results were depicted in Fig. 2. When drugs were preincubated with chemoattractant in the lower chamber, no inhibition of leukocyte migration was observed.

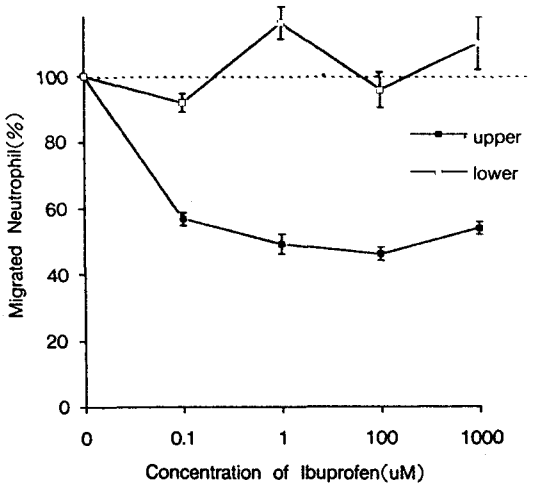

Fig. 2. Effect of ibuprofen in the lower chamber or upper chamber on the FMLP-induced migration of neutrophil.

Neutrophils (1×10^5) were placed in the upper wells of chemotactic chamber and bottom wells were filled with 10uM FMLP in HBSS. Various concentrations of ibuprofen were added to the upper (■−■) or bottom wells (□−□). After incubation for 20min at 37℃, the filter containing migrated neutrophils was removed, fixed, stained and counted. Each point represents the mean±S.E.M. of 10 determinants.

DISCUSSION

Aspirin-like drugs are known as inhibitors of prostaglandin biosynthesis for anti-inflammatory activity (Insel *et al.*, 1990), since anti-edema and anti-erythema actions of these drugs correlate closely with their ability to inhibit the production of vasodilator prostaglandins in inflammation (Dawson *et al.*, 1984). Recently, several laboratories reported that ibuprofen and naproxen showed inhibitory effects on leukocyte function (Borel, 1973; Geioud *et al.*, 1982; Huy *et al.*, 1985). We observed interesting results with 7 species of non-steroidal anti-inflammatory drugs for their effects on neutrophil migration at therapeutic concentration. Phenylbutazone, oxyphenbutazone, sulindac, ibuprofen, and zomepirac inhibited the FMLP-induced migration of neutrophil (Table 1). However, acetyl salicylate and indomethacin showed no effect or stimulation effect on neutrophil migration. It was interesting point that indomethacin, aspirin, phenylbutazone, oxyphenbutazone, sulindac, ibuprofen, and zomepirac, which showed inhibition effects onPGs synthesis, showed differential effect on the accumulation of neutrophil in inflammation, and the inhibition effect of these drugs on neutrophil migration was not correlated with inhibition effect on cyclooxy

genase activity. Therefore, we suggest that inhibition of prostaglandin biosynthesis by NSAIDs is one unique mechanism of action as well as those drug can also modulate neutrophil migration. This suggestion has also been defined by subsequent experiments (Fig. 1, A-D). Concentrations required for maximum inhibition effect on neutrophil migration were lower than those of inhibition on PGs synthesis. At concentrations less than therapeutic blood levels, oxyphenbutazone, phenylbutazone, zomepirac, ibuprofen showed marked suppression of neutrophil chemotaxis. IC50s for inhibition of neutrophil migration by these drugs are less than 100uM. (Fig. 1, A-D).

The differential activity of these drugs on leukocyte migration and prostaglandin synthesis could lead to different clinical effects according to the concentration. Thus selective inhibition of cyclooxygenase would be expected to result in relief of symptoms caused by the vasodilator and hyperalgesic properties of prostaglandins (Dawson *et al.*, 1984). Increased numbers of leukocytes at an inflammatory site would result in higher concentrations of lysosmal enzymes and enhanced tissue destruction (Janoff, 1972a; Starky, 1977). At therapeutic concentrations, it is often stated that indomethacin and acetyl salicylates do nothing to reverse the underlying causes of chronic inflammatory diseases (Insel *et al.*, 1990). This may be due to use of the drug at doses which give good symptomatic relief but actually enhance the accumulation of inflammatory cells. In this respect, ibuprofen, which do not increase migration, would be expected to have an improved therapeutic effect, particularly during leukocyte accumulation, before the onset of tissue destruction. Consequently, these results may have some advantages to determine the drug of choice and/or the theapeutic dose of drug for the treatment of inflammatory diseases.

Possible mechanism of action of inhibition on the FMLP-induced migration of neutrophil by NSAIDs could be: 1) drug interacts with FMLP in solution interferring its binding to the receptor site on neutrophil surface, 2) drug directly modulates the receptor sites of neutrophils (Bender *et al.*,1987., Daniel *et al.*, 1986; Sullivan *et al.*, 1984; Williams *et al.*, 1977). To determine whether FMLP interacts with drug in solution or not, drugs were incubated with neutrophil or FMLP. When neutrophils were pretreated with drug, their migration toward chemotactic factor was effectively inhibited. When drugs were preincubated with chemoattractant, however, no inhibition of leukocyte migration was observed as shown in Fig. 2. These results indicated that inhibitory effects of these drugs on the migration of neutrophil might be related to receptor sites of neutrophil rather than molecular inactivation of chemoattractant. Still we have to do fine experimental works for the details on the mechanism of action of these drugs on the receptor sites of neutrophil.

Overall, aspirin-like drugs, which were known as antipyretic, analgesic, and anti-inflammatory agents, showed individual different pharmacological activities (Insel *et al.*, 1980). We speculated that these various pharmacological effects of NSAIDs might come from different mechanisms of actions of these drugs, like interferring cyclooxygenase, migration of neutrophil, and release of proteases (Roch-Arveiller *et al.*, 1979; Smithj *et al.*, 1980). In conclusion, we suggested that the property of inhibition effects on neutrophil migration of several NSAIDs might be another mode of pharmacological action for anti-inflammatory effect, which showed significant effects at concentrations below therapeutic levels, in addition to cyclooxygenase inhibition.

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=국문초록=

비스테로이드성 항염증제가 FMLP에 의한 사람 중성구의 이동에 미치는 영향

고신대학교 의과대학 약리학 교실

김 우 미·강 구 일

본 연구는 7종의 비스테로이드성 항염증제가 FMLP에 의한 사람 중성구의 이동에 미치는 영향을 약물의 농도별로 관찰하고자, Hypaque-Ficoll step gradient centrifugation 방법에 의하여 중성구를 분리하고, 48-well micro chemotaxis assembly를 이용하여 chemotactic assay를 시행하여 다음과 같은 결과를 얻었다. Oxyphenbutazone, phenylbutazone, zomepirac, ibuprofen은 약물의 치료 농도하에서 중성구의 이동에 대한 강력한 억제작용을 나타내었으며, indomethacin은 중성구의 이동을 오히려 증가시키는 작용을 나타내었다. 이 약제들은 모두 100uM 미만의 약물 농도에서 각각 IC50를 나타내었으며, oxyphenbutazone, phenylbutazone은 10uM에서 최대 억제 효과를 나타내었고, zomepirac, ibuprofen은 각각 0.1uM과 100uM에서 가장 강한 억제 작용을 나타내었다. 또한 상기 약제를 FMLP와 함께 하단 구획에 첨가하였을 때에는 세포와 함께 상단 구획에 첨가하였을 때와는 상이하게 세포의 이동에 전혀 영향을 미치지 못하였다. 이러한 결과는 이 약제가 FMLP와의 분자적 상호 작용을 통하여 FMLP의 작용을 저하시키는 것보다는 세포에 직접적인 영향을 미침으로서 세포의 이동을 억제하였음을 나타내어 준다. 이상의 연구 결과에서, oxyphenbutazone등의 약제가 100uM미만의 저농도에서 FMLP에 의한 중성구의 이동을 강력하게 억제하는 작용이 있음을 보고, 이 작용은 지금까지 비스테로이드성 함염증제의 작용 기전으로 알려진 cyclooxygenage 억제 작용과는 별개의 기전으로 사료되므로, 이를 상기 약제가 세포 수준에서 나타내는 제 2의 약리 기전으로 제시한다.