Inhibitions of Metabolic Responses of Polymorphonuclear Leukocytes by Anti-allergic Drugs

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Sites of the inhibitory actions of antiallergic and antihistaminic drugs on polymorphonuclear leukocytes were examined by means of measurements of arachidonic acid release, superoxide anion generation and changes in membrane potential. The results obtained in this study were as follows. (i) Azelastine, an antiallergic drug, inhibited arachidonic acid release as well as superoxide generation. However, high concentrations of azelastine diminished the charge of the cells. (ii) Clemastine, used as an antihistamine, was as effective as azelastine on the metabolic bursts of leukocytes, including changes of membrane potential in leukocytes. (iii) The selective inhibition of arachidonic acid release was achieved by ketotifen without appreciable effect on superoxide generation. The relationship among the changes of membrane potential, superoxide generation and arachidonic acid release is discussed.

Keywords — leukocytes; metabolic response; arachidonic acid release; superoxide anion; membrane potential; antiallergic drug

Introduction

Polymorphonuclear leukocytes (PMNs) respond to certain membrane stimuli by producing superoxide radicals and arachidonic acid (AA) metabolites. The metabolic burst of PMNs is known to play roles in host defense mechanisms including bactericidal function and the inflammatory process. Recently, there has been increased interest in the roles of leukocytes, and it has been shown that several types of leukocytes release a variety of biological response modifiers including prostaglandins, leukotrienes, histamine and a number of peptides. Although the precise mechanisms are a matter of debate, recent studies have demonstrated that stimulations of PMNs are responsible not only for inflammation but also for the allergic process, and the reactivity and sensitivity of the cells to allergens were suggested to be related to the releases of histamine and leukotrienes. It has been shown that chemical mediator release from a variety of leukocytes is involved in allergen-induced asthma, and some newly developed antiallergic drugs including azelastine and ketotifen are known to be inhibitors of chemical mediator release, with an H receptor-blocking property.

We have previously demonstrated that some antiinflammatory drugs as well as antihistamines inhibit superoxide generation from activated PMNs. Furthermore, there are some indications that the inhibitory effects of some drugs on AA release are closely related to the inhibition of superoxide generation.

In this study, the effects of some antihistaminic agents as well as antiallergic drugs were examined in terms of AA release, superoxide generation and change in membrane potential of PMNs.

Materials and Methods

Preparation of PMNs — A rabbit was injected intraperitoneally with 250 ml of a 0.2% (w/v) solution of glycogen in sterile saline. After 14—16 h, peritoneal exudates containing PMNs were collected and filtered through three layers of cheesecloth. The cell suspension was centrifuged (400 × g, 5 min), and the pellet was resuspended in Hanks’ solution (Nissui Pharmaceutical Co., Tokyo, Japan). Two volumes of ice-cold distilled water was added, and the suspension was left to stand for 1 min to lyse contaminating erythrocytes. After restoration of the osmolarity with 1.8% (w/v) NaCl solution, the suspension was centrifuged (400 × g, 2 min) and washed with Hanks’ solution (3
After collection, the cells were suspended in Gey's balanced salt solution containing 0.1% (w/v) bovine serum albumin and 0.01 M Hepes buffer at pH 7.4 (modified Gey's solution), at a concentration of $1 \times 10^7$ cells/ml. This procedure yielded leukocytes ($5 \times 10^8 - 10^9$) containing over 90% of PMNs.

**Chemicals** — [3H]Arachidonic acid (80 Ci/mmol) was obtained from New England Nuclear. Azelastine, clemastine and ketotifen were obtained from Eisai Co. (Tokyo, Japan). N-Formyl-methionyl-leucyl-phenylalanine (FMLP) and ferri cytochrome c (type III) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

**Preparations of [3H]AA-labeled Cells** — [3H]AA (final concentration, 0.25 μCi/ml) was transferred into a plastic test tube, the ethanol was evaporated off under a stream of nitrogen, and immediately a known volume of cell suspension ($10^7$ cells in 1 ml) was added. The cell suspension was incubated at 37°C for 10 min. At the end of the incubation period, five volumes of ice-cold modified Gey's solution was added, and the suspension was centrifugated ($400 \times g$, 2 min). The pellet was washed with modified Gey's solution (2 $\times$ 10 ml) and resuspended in an appropriate volume of the solution to give a final concentration of $1 \times 10^7$ cells/ml [110000 $\pm$ 14000 dpm/10^8 cells/ml ($n = 16$)].

**Assay of Arachidonic Acid Release** — The labeled cell suspension was preincubated with various drugs at 37°C for 5 min. The reaction was started by addition of 100 nM FMLP and was carried out for a further 5 min before termination by addition of 3.5 ml of methanol/chloroform (2:1; v/v). Extraction and separation of radioactivity were carried out according to the method described by Bligh and Dyer. The chloroform extract was evaporated under a stream of nitrogen and the residue was spotted on a thin layer chromatography (TLC) plate (silica gel, 20 $\times$ 20 cm), which was developed in petroleum ether/diethyl ether/acetic acid (80:20:1, v/v). Spots detected by exposure to iodine vapor were scraped into counting vials and the radioactivity was measured in a liquid scintillation counter after the addition of 10 ml of Scintisol (Wako Pure Chemical Industries, Osaka, Japan) to each vial. The values of FMLP-stimulated release of AA were $17900 \pm 4800$ dpm ($n = 4$). In the absence of stimuli, the control value was $6200 \pm 1500$ dpm ($n = 4$).

**Assay of Superoxide Generation** — Superoxide anion production was measured as described previously using 100 nm FMLP, 20 μM ferricytochrome c and $10^6$ cells in the presence of various drugs.

**Measurement of Membrane Potential Change** — The fluorescence intensity of 3,3-dipropylthiobibocyanine iodide [di-O-C₆(3)] (Nihonkankoshikiso Co., Shimoishi, Okayama, Japan) was followed at 510 nm, with excitation at 460 nm, using a spectrofluorometer (Shimadzu, type RF510). The reaction mixture contained 2.45 ml of Hank's solution, 20 μl of cell suspension ($10^6$ cells) and 5 μl of the cyanine dye (125 μM) at 37°C. When the fluorescence intensity reached equilibrium, a 5μl stimulus was applied.

**Results**

As shown in Fig. 1, the dose dependence of the effects of azelastine, clemastine, ketotifen and diphenhydramine on the FMLP-induced AA release from PMNs was examined using the PMNs prelabeled with [3H]AA. The effects of drugs on the superoxide release from the FMLP-stimulated PMNs are included in the same figure. The result are expressed relative to no addition of drugs as 100%. The order of potency of the inhibitory effects of the drugs in terms of the AA release was as follows: azelastine, clemastine, ketotifen and diphenhydramine.

The absolute amount of superoxide production by FMLP was $1.4 \pm 0.2$ nmol/min/10^6 cells. Among the drugs examined in this study, azelastine and clemastine were equally effective in the concentration ranges of 10 μM to 50 μM, but ketotifen and diphenhydramine were not effective even at the concentration of 100 μM.

The cell viabilities were examined by means of the trypan blue exclusion test. Since the numbers of positive cells were not significantly increased after the incubation period even at the highest drug concentrations, the decreased cel-
lular responses were not due to cellular damage.

Although ketotifen at the concentration of 20 μM inhibited almost 50% of the AA release from PMNs, the effect on the superoxide generation was minimal even at 5 times the concentration which completely depressed the AA release. In other words, ketotifen (50 μM) inhibited the AA release without affecting the generation of superoxide from PMNs.

We next examined the effects of these drugs on the cellular membrane potential to investigate the modes of action of the drugs on PMNs. As can be seen in Fig. 2, addition of cells to the reaction medium containing di-O-C₄ (3) enhanced the fluorescence intensity, and the changes reached equilibrium in a few minutes. The metabolic stimulator, FMLP, caused changes of the fluorescence intensity indicating the discharge and restoration of membrane potential of PMNs. When 50 μM azelastine was applied, the membrane potential gradually decreased over a few minutes, resulting in the disappearance of the changes in membrane potential during the stimulation with FMLP (shown by dotted lines in Fig. 2). Ketotifen and diphenhydramine, however, were ineffective on the resting PMNs. In fact, the addition of 100 μM ketotifen inhibited neither the membrane potential of resting PMNs by itself nor the cellular response to FMLP. That is, azelastine and clemastine were equally effective on the changes of membrane potential by FMLP, but high concentrations of azelastine diminished the charge of the resting PMNs.

Together with these results, it appeared that the superoxide generation correlated with the stimuli-induced changes of membrane potential. Ketotifen and diphenhydramine did not inhibit the changes of membrane potential up to 100 μM.

Although the data are not presented in this

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**Fig. 1. Effects of Antiallergic Drugs on Arachidonic Acid Release and Superoxide Generation from FMLP-Stimulated PMNs**

Arachidonic acid release and superoxide anion generation were measured after addition of FMLP as described in the text. Results were expressed relative to no addition of drug as 100%. Absolute value of arachidonic acid release and superoxide anion generation by FMLP were 12000±3000 dpm/10⁶ cells and 1.4±0.2 nmol/min/10⁶ cells.

○, arachidonic acid release; ▲, superoxide anion generation.

**Fig. 2. Effects of Antiallergic Drugs on Change of Membrane Potential of FMLP Stimulated PMNs**

Membrane potential change was measured as described in text.
TABLE 1. Effects of Antiallergic Drugs on Arachidonic Acid Release and Superoxide Generation from FMLP-Stimulated PMNs

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC\textsubscript{50} (\textmu M)</th>
<th>Arachidonic acid release</th>
<th>Superoxide anion generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azelastine</td>
<td>19 ± 1 (3)</td>
<td>23 ± 4 (4)</td>
<td></td>
</tr>
<tr>
<td>Clemastine</td>
<td>18 ± 4 (4)</td>
<td>20 ± 3 (3)</td>
<td></td>
</tr>
<tr>
<td>Ketotifen</td>
<td>28 ± 2 (3)</td>
<td>50 &gt;&gt;</td>
<td></td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>50 &gt;&gt;</td>
<td>50 &gt;&gt;</td>
<td></td>
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Absolute value of arachidonic acid release and superoxide anion generation by FMLP were 12000 ± 3000 dpm/10\textsuperscript{5} cells and 1.4 ± 0.2 nmoI/min/10\textsuperscript{6} cells, respectively. Each value represents the mean ± S.D. of 3—4 independent experiments.

report, we have observed that activations of PMNs by other types of stimuli, such as phorbolmyristate acetate (PMA) and calcium ionophore (A23187), gave similar results concerning the inhibitory effects of the antiallergic agents. The IC\textsubscript{50} values of the antiallergic drugs and antihistamines are summarized in Table I, indicating that azelastine and clemastine showed similar effects on the response of PMNs, but ketotifen inhibited only the AA release without influencing on other cellular responses.

Discussion

Although it has been well established that mast cells play a central role in type I reaction of allergy, recent studies have centered on the role of stimulation of PMNs in the reactivity and sensitivity of the cells to allergens.\textsuperscript{5,6} Since arachidonic metabolites as well as proteolytic enzymes are known to be important mediators of the allergic process, it is worth examining the metabolic responses of PMNs.\textsuperscript{11} Some newly developed antiallergic drugs including azelastine and ketotifen are inhibitors of chemical mediator release.\textsuperscript{7} However, the action sites of these drugs have not been clarified in terms of the inhibition of the release of chemical mediators. The activation of the metabolic burst of PMNs starts at the level of the interaction of stimulus with surface receptors, which activate the breakdown of phosphatidyl inositides by endogenous phospholipase C, followed by enhanced Ca\textsuperscript{2+} entry and mobilization.\textsuperscript{12} The products of phospholipase C may serve as a branch point controlling a variety of Ca\textsuperscript{2+}-regulated, protein kinase C-dependent, or arachidonate-dependent metabolic pathways.\textsuperscript{13} However, two separate enzymes (glyceride lipase and phospholipase A\textsubscript{2}) have been proposed for the release of AA from these two products of the phosphoinositide response.\textsuperscript{14}

The results in this study showed that azelastine inhibited AA release as well as superoxide generation, but ketotifen diminished only the former metabolic response, although both drugs are classified as antiallergic agents. On the other hand, clemastine which is classified as an antihistamine,\textsuperscript{15} suppressed the two metabolic responses. As mentioned in the results section, these inhibitory effects were neither due to cellular injury, nor the blocking of the receptors for FMLP, since the by-passed activations of PMNs with phorbolmyristate acetate for superoxide generation and with calcium ionophore for AA release showed similar inhibitory effects of the drugs as with FMLP.\textsuperscript{16} The effects of these drugs on the changes in membrane potential are
Arachidonic Acid Release from Leukocytes

associated with the superoxide generation, indicating that the suppression of the changes of membrane potential is a consequence of inhibition of the respiratory burst, or the discharge of PMNs causes inactivation of the oxidase. Although these is no conclusive evidence so far, it seems likely that interactions of drugs with membrane components, such as phospholipids, enhance the suppression of the oxidase resulting in the observed changes of membrane potential. However, further quantitative discussion is difficult due to experimental limitations. Furthermore, it is interesting to note that the metabolic inhibition by these drugs was restored by addition of an anionic amphiphile, indicating that the drug actions were reversible.177

As shown in Fig. 3, since the chemical structures of azelastine, clemastine and ketotifen commonly possess a tertiary amino group and aromatic rings, these drugs may act as cationic amphiphiles. Therefore, we have the impression that the amino group with a lipophilic group may serve as the inhibitor of AA release and the moiety between these two groups might exert the antihistaminic action of clemastine, azelastine and ketotifen.

Although the structure-action relationship remains to be examined in detail, these four chemicals including diphenhydramine showed unique actions on PMNs as follows. (i) A selective inhibition of AA release is achieved by ketotifen. (ii) Superoxide generation and AA release, as well as changes of membrane potential, are suppressed by azelastine and clemastine. (iii) Diphenhydramine does not affect the metabolic burst of PMNs up to concentration of 50 μM. From these observations in this study together with reported data, it seems likely that many clinically employed drugs inhibit the physiological responses of leukocytes in different manners, which might be reflected in the drug actions, or side effects. To clarify the precise mechanisms involved, further studies on the effects of the drugs on other cellular types are necessary.

References


