Inhibitory Effect of Epinastine on Superoxide Generation by Rat Neutrophils

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ABSTRACT—We studied the effects of antiallergic drugs, epinastine, ketotifen, oxatomide, mequitazine and cromolyn sodium on superoxide anion (O_2^-) generation from rat neutrophils. Epinastine, ketotifen, oxatomide and mequitazine dose-dependently prevented the *N*-formyl-Met-Leu-Phe- and phorbol 12-myristate 13-acetate-induced O_2^- generation, but cromolyn sodium did not prevent it. When membrane and cytosol fractions were incubated with each drug, epinastine, ketotifen and mequitazine prevented O_2^- generation. On the other hand, when only the membrane fraction was incubated with each drug, ketotifen and mequitazine prevented O_2^- generation, but epinastine did not. Epinastine may inhibit the NADPH oxidase system through the obstruction of NADPH oxidase-associated cytosol components.

Keywords: O_2^- generation, Epinastine, Neutrophil (rat)

Epinastine (WAL 801 CL) has been reported to show a potent inhibitory effect on the immediate type hypersensitivity reaction and antigen-induced histamine or SRS-A release from the lung pieces of actively sensitized guinea pigs (1, 2). Epinastine may be more potent than terfenadine or oxatomide in inhibiting the histamine-induced contraction of isolated guinea pig ileum. Furthermore, epinastine is more effective than ketotifen and terfenadine in inhibiting histamine-induced skin wheal in rats, when the drugs were administered intravenously. However, epinastine has been reported to be less effective than ketotifen and mepyramine in inhibiting anaphylactic histamine release from rat peritoneal mast cells (3).

The activated neutrophils generate superoxide anion (O_2^-). O_2^- is generated primarily through the activation of the plasma membrane-bound NADPH oxidase system containing FAD, cytochrome b_{558} (4) and other cytosolic soluble proteins (5). There is some evidence that O_2^- may be responsible for allergic reactions such as asthma (6). There are only a few reports that have examined the relationship between O_2^- and antiallergic drugs. In this study, we examined the effects of epinastine, ketotifen, oxatomide, mequitazine and cromolyn sodium on the O_2^- generation from the rat neutrophils, using cypridina luciferin analog (MCLA)-dependent chemilumines-

cence as a probe for O_2^- generation.

Male Wistar rats (SLC, Hamamatsu), each weighing 300 to 400 g, were used. Under ether anesthesia, whole blood was collected from the carotid artery and diluted twice with Hanks' balanced salt solution (HBSS) (pH 7.4). Neutrophils were purified to a level higher than 85% by Percoll (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. O_2^- generation by rat neutrophils was measured by the MCLA (Tokyo Kasei Kogyo Co., Ltd., Tokyo)-dependent chemiluminescence method (7). Neutrophil suspensions (10^6 cells/ml) were incubated for 3 min in HBSS containing 0.4 mM of MCLA and various concentrations of antiallergic drugs at 37°C in the dark. Cell suspensions were transferred into a glass vial, and then photon counting was started. Five seconds later, *N*-formyl-Met-Leu-Phe (fMLP; Sigma Chemical Co., Ltd., St. Louis, MO, USA) ($2.5 \mu\text{M}$) or phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., Ltd.) ($20 \mu\text{g/ml}$) was added into the vial. MCLA-dependent chemiluminescence was monitored with a Lumicounter ATP-237 (Advantec Co., Ltd., Tokyo).

Cell disruption was carried out according to the previously described method (8). Disrupted cells were centrifuged at $1,000 \times g$ for 10 min at 4°C to remove unbroken cells, nuclei and large debris. The supernatant was centrifuged at $135,000 \times g$ for 1 hr at 4°C. The pellet containing the membrane fraction was incubated with each

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drug for 3 min at 37°C and then centrifuged at $135,000 \times g$ for 1 hr at 4°C. The pellet was mixed with the $135,000 \times g$ supernatant. FAD (Sigma Chemical Co., Ltd.), sodium dodecyl sulfate (SDS; Sigma Chemical Co., Ltd.) and MCLA were added, and photon counting was started. Five seconds later, 10 mM NADPH (Sigma Chemical Co., Ltd.) was added to initiate O_2^- -generation. The assay was done in the Lumicounter ATP-237.

Fig. 1. Effects of epinastine (○), oxatomide (●), cromolyn sodium (△), mequitazine (□) and ketotifen (■) on the generation of superoxide anion (O_2^-) induced by *N*-formyl-Met-Leu-Phe (fMLP) and phorbol 12-myristate 13-acetate (PMA) by rat neutrophils. Neutrophil suspensions (10^6 cells/ml) were incubated for 3 min in Hanks' balanced salt solution (HBSS) containing MCLA (0.4 μM) and various concentrations of anti-allergic drugs at 37°C, and fMLP (2.5 μM) (A) or PMA (20 μg/ml) (B) was added. A: Epinastine, oxatomide, mequitazine and ketotifen dose-dependently inhibited fMLP-induced O_2^- generation. The IC_{50} was 0.03, 0.27, 14.59 and 0.03 mM, respectively. B: Epinastine, oxatomide, mequitazine and ketotifen also dose-dependently inhibited PMA-induced O_2^- generation. The IC_{50} was 5.23, 49.41, 6.09 and 0.76 mM, respectively. Each point indicates the mean $\pm$ S.E. ($n=8$). * $P<0.05$, ** $P<0.01$ vs control.

The results are reported as the means $\pm$ standard error of the means (S.E.). Statistical significance was evaluated by the unpaired Student's *t*-test, with $P<0.05$ being regarded as significant. IC_{50} values were calculated according to the method of Litchfield-Wilcoxon.

Addition of fMLP at concentrations between 0.4 and 40 μM increased the peak photon counts of MCLA-dependent chemiluminescence in a dose-dependent fashion (9045–42780 cpm). Pretreatment with superoxide dismutase (SOD, 1–100 U/ml) dose-dependently inhibited these increases (3860–2415 cpm vs 30885 cpm at SOD(–)). Figure 1A shows the inhibitory effects by epinastine, oxatomide, mequitazine and ketotifen on

Fig. 2. Effects of epinastine, ketotifen, mequitazine and oxatomide on sodium dodecyl sulfate (SDS)-induced O_2^- generation in the reconstructed NADPH oxidase system by rat neutrophils. Drugs were used at the maximal inhibitory concentration in the intact cells. A: Membrane and cytosol fractions were preincubated with each drug for 3 min at 37°C, and FAD, MCLA, SDS and NADPH were added. B: The membrane fraction was incubated with each drug for 3 min at 37°C and then centrifuged at $135,000 \times g$ for 1 hr at 4°C. The pellet was mixed with the cytosol fraction, FAD, MCLA, SDS and NADPH. Each point indicates the mean $\pm$ S.E. ($n=8$). * $P<0.05$, ** $P<0.01$ vs control.

fMLP-induced O_2^- generation from rat neutrophils. The IC_{50} was 0.03, 0.27, 14.59 and 0.03 μM , respectively. However, cromolyn sodium could not prevent O_2^- generation. On the other hand, epinastine, oxatomide, mequitazine and ketotifen also prevented PMA-induced O_2^- generation in a dose-dependent fashion (Fig. 1B), with an IC_{50} of 0.52, 49.41, 6.09 and 0.76 μM , respectively. Cromolyn sodium was also unable to prevent PMA-induced O_2^- generation.

The $1,000 \times g$ supernatant of the disrupted neutrophils could generate O_2^- when it was stimulated by SDS (100 μM : 16137 ± 1289 cpm vs 645 ± 58 cpm), but the $135,000 \times g$ supernatant could not generate O_2^- . When the membrane and cytosol fractions were pretreated with antiallergic drugs, O_2^- generation was significantly prevented by epinastine, ketotifen and mequitazine. However, oxatomide did not inhibit it (Fig. 2A). On the other hand, when only the membrane fraction was pretreated, O_2^- generation was not prevented by epinastine, but prevented by ketotifen and mequitazine (Fig. 2B).

Nakano showed that MCLA reacted with O_2^- or $^1O_2$ to emit light, probably via the dioxetanone analog (7). SOD or NaN_3 can be used to differentiate between O_2^- - and $^1O_2$ -dependent luminescence (7). In this experiment, we examined the effect of antiallergic drugs on the O_2^- generation by rat neutrophils using MCLA-dependent chemiluminescence as a probe of O_2^- generation. fMLP increased MCLA-dependent chemiluminescence in a dose-dependent fashion, which was extinguished by SOD, and the luminescence was not affected by the presence of NaN_3 (1 mM), a quencher of $^1O_2$ (data not shown). These findings suggested that this assay system using MCLA could measure only O_2^- , and MCLA-dependent chemiluminescence were not contaminated with $^1O_2$.

Antiallergic drugs with H_1 -receptor antagonism, epinastine, oxatomide, mequitazine and ketotifen, abolished the MCLA-dependent luminescence from rat neutrophils induced by fMLP and PMA in a dose-dependent fashion, but cromolyn sodium, without H_1 -receptor antagonism, did not abolish it. These findings suggest 1) that these drugs trap O_2^- from neutrophils in the medium or 2) that the drugs inhibit the O_2^- generating NADPH-oxidase system. To determine whether these drugs act as a scavenger or not, we examined the effect of KO_2 that generates O_2^- chemically. Consequently, it was found that epinastine, oxatomide, mequitazine and ketotifen have no effect on O_2^- itself (data not shown), and these drugs may not be able to trap O_2^- . Therefore, we studied whether these drugs might affect NADPH oxidase directly or not. The $1,000 \times g$ supernatant of the disrupted neutrophils containing the membrane and cytosol

fractions could generate O_2^- when it was stimulated by SDS, but the $135,000 \times g$ supernatant containing only the cytosol fraction could not. However, when the $135,000 \times g$ pellet was mixed with the supernatant, the O_2^- generation was elicited (9). The NADPH-oxidase system contains membrane associated proteins such as cytochrome b_{558} and cytosolic proteins such as p47-phox and p67-phox (5, 10–12), and the activation of NADPH oxidase in the cell-free system requires the association of several cytosolic components together with membrane associated cytochrome b_{558} (5). Therefore, using a $1,000 \times g$ supernatant, FAD, NADPH and SDS as both membrane solubilizer and stimulant of this system, we examined the direct inhibitory effect of these drugs on the NADPH-oxidase system. When both membrane and cytosolic components were pretreated with epinastine, ketotifen, mequitazine and oxatomide, epinastine, ketotifen and mequitazine inhibited O_2^- generation by the SDS-stimulated NADPH-oxidase system, but oxatomide did not. Epinastine, ketotifen and mequitazine might have a direct inhibitory effect on the O_2^- generating-NADPH oxidase system. Ketotifen and mequitazine inhibited superoxide generation from the NADPH-oxidase system that was reconstructed from the membrane fraction exposed to the drug, the cytosol fraction, FAD, NADPH and SDS, but epinastine and oxatomide did not affect this NADPH oxidase system. Ketotifen and mequitazine have been shown to decrease membrane fluidity (13). The NADPH-oxidase system may be partially inhibited due to the membrane stabilizing action of these drugs. However, epinastine had no influence on the membrane fluidity (2). Epinastine augmented the adenylate cyclase activity, and inhibited Ca^{2+} release from the intracellular Ca store of rat peritoneal mast cells and calmodulin activity (2). The increase in cyclic AMP content prevents O_2^- generation from neutrophils (unpublished data, N. Fukuishi and M. Akagi), and the initiation of O_2^- generation may be attributable to the Ca^{2+} -calmodulin dependent process(es) (14). Therefore, epinastine may partially inhibit O_2^- generation through the increase in cyclic AMP content and the inhibition of a Ca^{2+} -calmodulin dependent process(es).

In conclusion, our studies suggest that epinastine may inhibit NADPH oxidase through the inhibition of cytosolic proteins, p67-phox and p47-phox, and ketotifen and mequitazine, through the obstruction of membrane-associated factors, cytochrome b_{558} . On the other hand, oxatomide may inhibit superoxide generation through an indirect influence on the NADPH oxidase system.

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