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EFFECTS OF ANTI-ALLERGIC DRUGS ON HUMAN NEUTROPHIL SUPEROXIDE-GENERATING NADPH OXIDASE

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Abstract—The effects of anti-allergic drugs with or without H_1-receptor antagonism on the NADPH oxidase (EC 1.6.99.6) from human neutrophils in both whole-cell and fully soluble (cell-free) systems were investigated. Three anti-allergic drugs with H_1-receptor antagonism, azelastine, ketotifen and oxatomide, were found to inhibit the superoxide generation of human neutrophils exposed to phorbol myristate acetate in a whole-cell system and the activation of superoxide-generating NADPH oxidase by sodium dodecyl sulfate in a cell-free system. The concentrations of these drugs required for 50% inhibition of the oxidase (IC_{50}) were: azelastine—0.7 μM in the whole-cell system and 0.5 μM in the cell-free system; ketotifen—60 μM in the whole-cell system and 6.8 μM in the cell-free system; and oxatomide—25 μM in the whole-cell system and 9.7 μM in th cell-free system. In addition, in the cell-free system, these drugs did not change the K_m values for the NADPH of the oxidase. However, these drugs did not inhibit the superoxide generation of NADPH oxidase after its activation in whole-cell and cell-free systems, suggesting that these drugs do not have superoxide-scavenger actions. Concentrations of less than 200 μM of anti-allergic drugs without H_1-receptor antagonism, tranilast, repirinast and ibudilast, did not inhibit neutrophil NADPH oxidase in whole-cell and cell-free systems. The IC_{50} of hydrocortisone in the cell-free system was 60 μM. These results suggest that anti-allergic drugs with H_1-receptor antagonism inhibit activation of the solubilized membrane-bound enzyme by sodium dodecyl sulfate in cell-free systems and that they have much stronger anti-inflammatory action than hydrocortisone.

Neutrophils are a major component of the body's defense against microbial invasion [1]. Destruction of an invading microorganism occurs as a result of a complex sequence of events initiated by ingestion and sequestration of the microbe within the phagosome [2]. Concurrent with these processes, oxygen from the surrounding milieu is reduced to superoxide (O_2^-), which subsequently leads to the formation of other toxic metabolites [3]. Superoxide is produced primarily through the activation of plasma membrane-bound NADPH oxidase by stimulation with phagocytizable particles [4] or soluble agents [5]. The importance of this first step in the oxidative antimicrobial system of neutrophils is demonstrated by patients with chronic granulomatous disease, in which there is a defect in NADPH oxidase or its activating apparatus [6]. Several reports concerning the effects of therapeutic drugs on NADPH oxidase have been presented [7–10]. Anti-inflammatory drugs have been shown to modify or inhibit the generation of superoxide by neutrophils exposed to stimulating agents [7, 8]. I and my colleagues previously reported that steroids and non-steroidal anti-inflammatory drugs inhibit the NADPH oxidase from human neutrophils in whole-cell and cell-free systems [9, 10].

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Recent studies have suggested that inflammation may play a crucial role in the characteristic bronchial hyperresponsiveness and symptoms of chronic asthma [11, 12]. β_2-Agonists, with their potent mast cell stabilizing effect [13], are the drugs most widely prescribed for the treatment of asthma. β_2-Agonists, although they reduce symptoms, do not reduce the chronic inflammatory response or bronchial hyperresponsiveness of asthma and may mask underlying inflammation [11]. In addition to these drugs, anti-allergic drugs (anti-inflammatory drugs) without inhibitory effects on the cyclooxygenase system have also been widely used for prophylactic therapy in atopic asthmatic patients [14]. These anti-allergic drugs have been shown to reduce the release of chemical mediators such as leukotrienes and platelet-activating factors [15–24]. However, little information about the effects of these drugs on the activation of NADPH oxidase in cell-free systems is available.

In the present study, therefore, the effects of anti-allergic drugs on the oxygen free radical formation of human neutrophils, and especially those on the NADPH-dependent superoxide-generating oxidase, were investigated in both whole-cell and cell-free systems. Simultaneously, the effects of these drugs on the affinity of the oxidase for NADPH were also studied.

MATERIALS AND METHODS

The following chemicals were obtained from

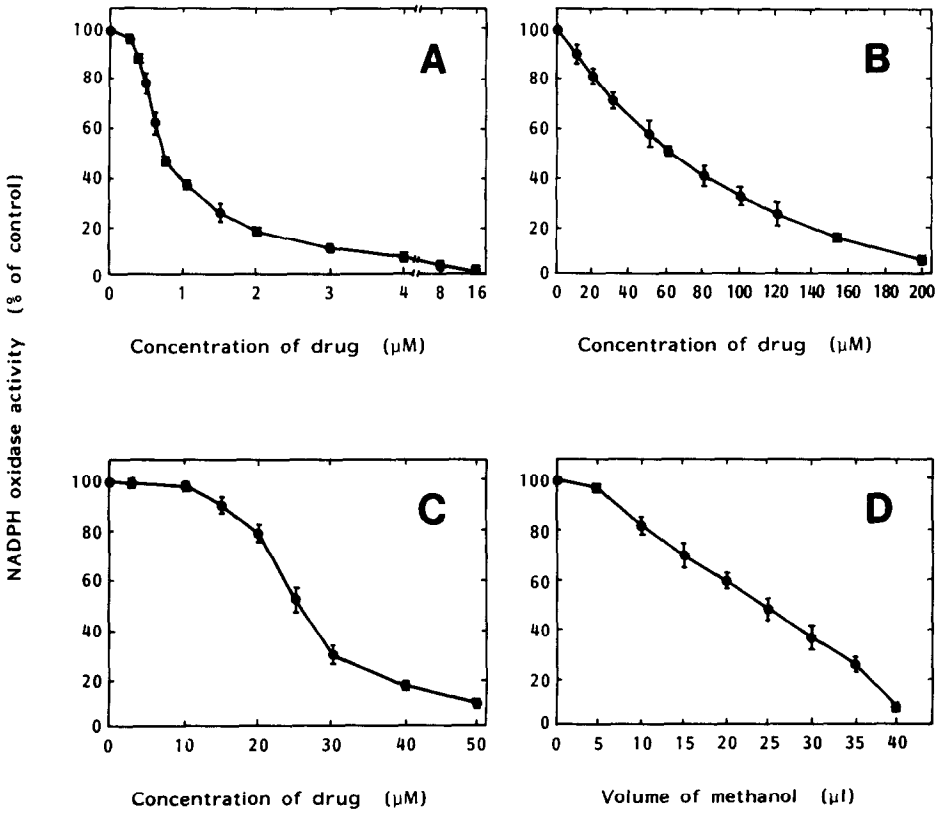

Fig. 1. Concentration-dependent changes due to anti-allergic drugs with H_1-receptor antagonism in superoxide generation of PMA-stimulated intact neutrophils. The assay method is described in Materials and Methods. Results are the means ± SD of three different experiments. Key: (A) azelastine; (B) ketotifen; (C) oxatomide; and (D) only methanol treatment. Control activity was 22.8 nmol $O_2^-/10^6$ cells/min (mean, N = 4).

commercial sources: bovine erythrocyte superoxide dismutase (SOD*), cytochome *c* (type III), β-NADPH (type I), sodium deoxycholate, phorbol 12-myristate 13-acetate (PMA), 1,4-piperazinediethanesulfonic acid (PIPES), [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), ATP, flavin adenine dinucleotide (FAD), sodium dodecyl sulfate (SDS), glycerol and sucrose (Sigma Chemical Co., St. Louis, MO); Ficoll–Paque (Pharmacia P-L Biochemicals, Piscataway, NJ); and Hanks' balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, NY). Sodium deoxycholate was recrystallized from ethanol before use. The following drugs were given by pharmaceutical companies: azelastine (Eisai Co., Ltd., Tokyo); ketotifen (Sandoz Pharmaceutical Corp., Tokyo); oxatomide (Kyowa Hakko Kogyo Co., Ltd., Tokyo); tranilast (Kissei Pharmaceutical Co., Ltd., Matsumoto); repirinast (Tokyo Tanabe Co., Ltd., Tokyo); ibudilast (Kyorin Medical Pharmacy Co., Ltd., Tokyo); and hydrocortisone sodium succinate (Nikken Chemicals Co., Ltd., Tokyo). Other chemicals were of the highest purity available from commercial sources.

Solubilized membranes and cytosolic fractions were prepared from resting human neutrophils as previously described [25, 26]. Protein concentration was determined according to the method of Lowry *et al.* [27] with bovine serum albumin as the standard. The protein concentrations were as follows: solubilized membranes, 30.2 ± 2.2 μg/10^7 cells (mean ± SD, N = 3); and cytosolic fractions, 160 ± 13 μg/10^7 cells (mean ± SD, N = 3).

Superoxide production by intact stimulated neutrophils (whole-cell system) was measured following the SOD-inhibitable reduction of cytochrome *c* at 550 nm [25, 26]. Neutrophils (2×10^5 cells/cuvette) were incubated in HBSS medium containing 0.12 mM cytochrome *c* and the desired concentrations of anti-allergic drugs, methanol or hydrocortisone for 2 min at 37° before the reactions were initiated by adding PMA (0.3 μg/cuvette). Assay mixtures were incubated for 4 min at 37°, in a total volume of 1.0 mL. The reference cuvette also received 20 μg of SOD.

* Abbreviations: SOD, superoxide dismutase; PMA, phorbol myristate acetate; PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FAD, flavin adenine dinucleotide; SDS, sodium dodecyl sulfate; and HBSS, Hanks' balanced salt solution.

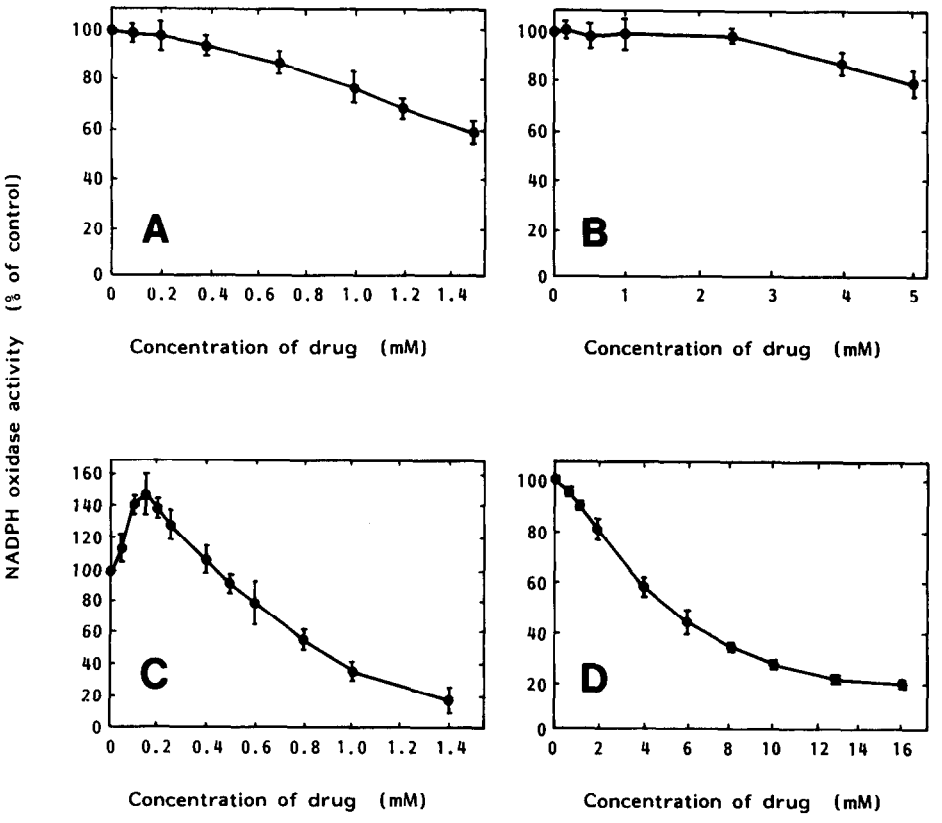

Fig. 2. Concentration-dependent changes due to anti-allergic drugs without H_1-receptor antagonism in superoxide generation of PMA-stimulated intact neutrophils. The assay method is described in Materials and Methods. Results are the means ± SD of three different experiments. Key: (A) tranilast; (B) repirinast; (C) ibudilast; and (D) hydrocortisone treatment. Control activity was 21.9 nmol $O_2^-/10^6$ cells/min (mean, N = 4).

Superoxide production in cell-free systems was assayed as previously described [25, 26]. Assay mixtures contained 0.1 mM cytochrome *c*, 3.6 mM $MgCl_2$, 89 mM KCl, 2.7 mM NaCl, 0.5 mM PIPES (pH 7.3), 0.9 mM ATP, 1.2 mM EGTA, 0.5 μM FAD, 6×10^6 cells of cytosolic fractions, 1.5×10^6 cells of membranes solubilized in deoxycholate (0.94 mM), the desired concentrations of drugs, 0.04 mM SDS and 0.16 mM NADPH, with alterations as noted in the figure and table legends, in a total volume of 0.75 mL. The reference cuvette contained 40 μg of SOD. Basically, all of the constituents except NADPH were mixed in the cuvette and then were placed in the reference and sample cuvettes. Absorbance at 550 nm was followed for 3 min at room temperature (23–24°). Then the reactions were started by adding 25 μL of NADPH solution to each cuvette, and the change in absorbance at 550 nm was followed for 3–5 min on a Cary model 118 double-beam spectrophotometer. Superoxide production was calculated using an extinction coefficient $E^{mM}_{550\,nm} = 19.6\,mM^{-1}\cdot cm^{-1}$ [28]. The concentrations (IC_{50}) of drugs for 50% inhibition of oxidase were estimated from the results of each concentration-dependent inhibition curve obtained.

In both whole-cell and cell-free systems, a stock solution of 0.1 to 0.8 mM azelastine–methanol, 1–5 mM ketotifen–HBSS buffer, 1–10 mM oxatomide–methanol, 50–200 mM tranilast–methanol, 50–200 mM repirinast–HBSS buffer, 50–300 mM ibudilast–methanol, or 100–400 mM hydrocortisone–HBSS buffer was used. In the experiments using azelastine–methanol, oxatomide–methanol or ibudilast–methanol solution, each final volume of methanol was less than 6 μL (= 150 mM methanol). In the experiments using tranilast–methanol solution, when each final volume of methanol was more than 5 μL (= 125 mM methanol), inhibition rates of oxidase due to tranilast were modified by the subtractions of methanol-induced inhibition rates of oxidase.

RESULTS

Figure 1 shows concentration-dependent changes due to anti-allergic drugs with H_1-receptor antagonism in the superoxide generation of intact neutrophils stimulated by PMA (whole-cell system). Micromoles of azelastine (A), ketotifen (B) and oxatomide (C) concentration-dependently inhibited

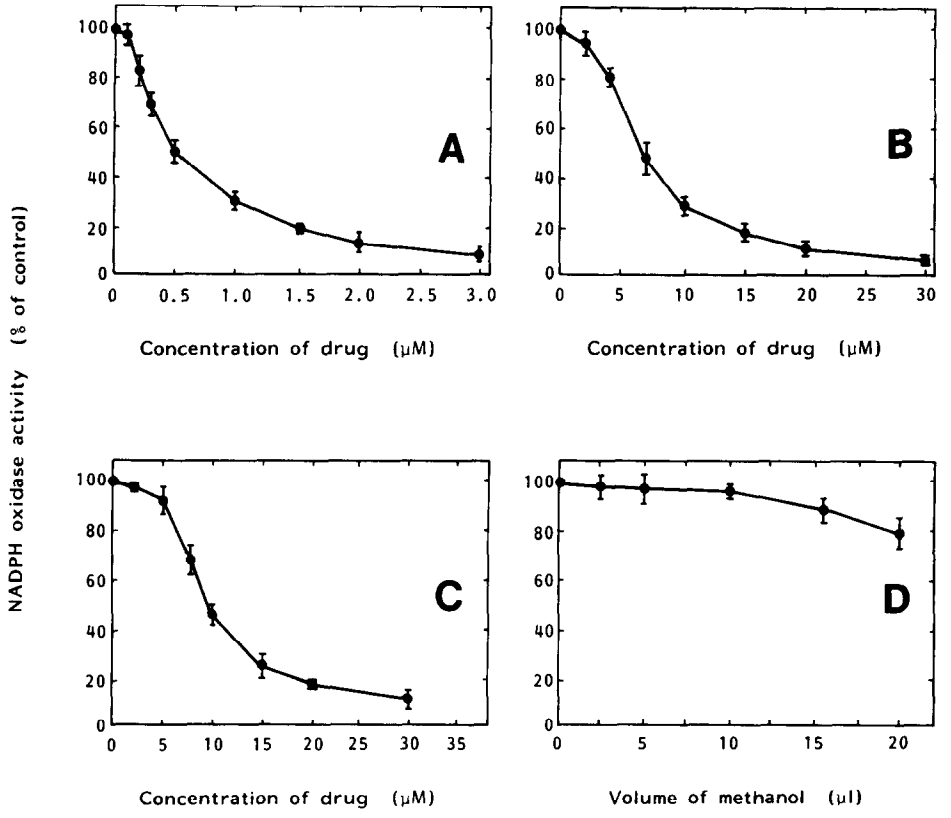

Fig. 3. Concentration-dependent changes due to anti-allergic drugs with H_1-receptor antagonism in the activation of NADPH oxidase in the cell-free system. The assay method is described in Materials and Methods. Results are the means ± SD of three different experiments. Key: (A) azelastine; (B) ketotifen; (C) oxatomide; and (D) only methanol treatment. Control activity was 20.4 nmol $O_2^-/10^7$ cells/min (mean, N = 5).

NADPH oxidase, with the concentrations of these drugs required for 50% inhibition of oxidase (IC_{50}) being 0.7, 60 and 25 μM, respectively. However, the IC_{50} value for methanol alone (D) required a volume of 23 μL (= 575 mM).

Figure 2 shows concentration-dependent changes due to anti-allergic drugs without H_1-receptor antagonism in neutrophil NADPH oxidase activities in the whole-cell system. Tranilast (A), repirinast (B) and hydrocortisone (D) concentration-dependently inhibited the oxidase, while each IC_{50} value was in millimole order: tranilast—more than 1.5 mM, repirinast—more than 5 mM, and hydrocortisone—5.1 mM. Concentrations of up to 0.15 mM ibudilast (C) increased neutrophil NADPH oxidase activity, but higher concentrations inhibited the oxidase activity. The IC_{50} value was 0.84 mM.

Figure 3 shows the concentration-dependent changes due to drugs in the SDS-induced activation of neutrophil NADPH oxidase in the cell-free system. Anti-allergic drugs with H_1-receptor antagonism concentration-dependently inhibited activation of NADPH oxidase. Concentrations of these drugs required to obtain IC_{50} values in the cell-free system were as follows: azelastine—0.5 μM (A), ketotifen—6.8 μM (B), and oxatomide—9.7 μM (C). The IC_{50} value for methanol alone (D) required a volume of 20 μL (= 500 mM).

Figure 4 shows the concentraion-dependent changes due to anti-allergic drugs without H_1-receptor antagonism in the activation of neutrophil NADPH oxidase in the cell-free system. These drugs concentration-dependently inhibited activation of NADPH oxidase. Concentrations of these drugs required to obtain IC_{50} values were as follows: tranilast—0.9 mM (A), repirinast—more than 3.5 mM (B), ibudilast—0.25 mM (C), and hydrocortisone—60 μM (D). The IC_{50} values for azelastine, ketotifen and oxatomide in the whole-cell (Figs. 1 and 2) and cell-free (Figs. 3 and 4) systems were much lower than those for hydrocortisone.

Figure 5 shows time-dependent changes in the activation of NADPH oxidase in the cell-free system after preincubation with anti-allergic drugs of concentrations required for IC_{50} and with methanol (5 μL = 125 mM). Three drugs, azelastine (A), ketotifen (B) and oxatomide (C), time-dependently inhibited the NADPH oxidase. Methanol alone (D), however, caused no significant change in NADPH oxidase.

Table 1 shows the effects of drugs on the K_m and V_{max} values for the NADPH of the NADPH oxidase

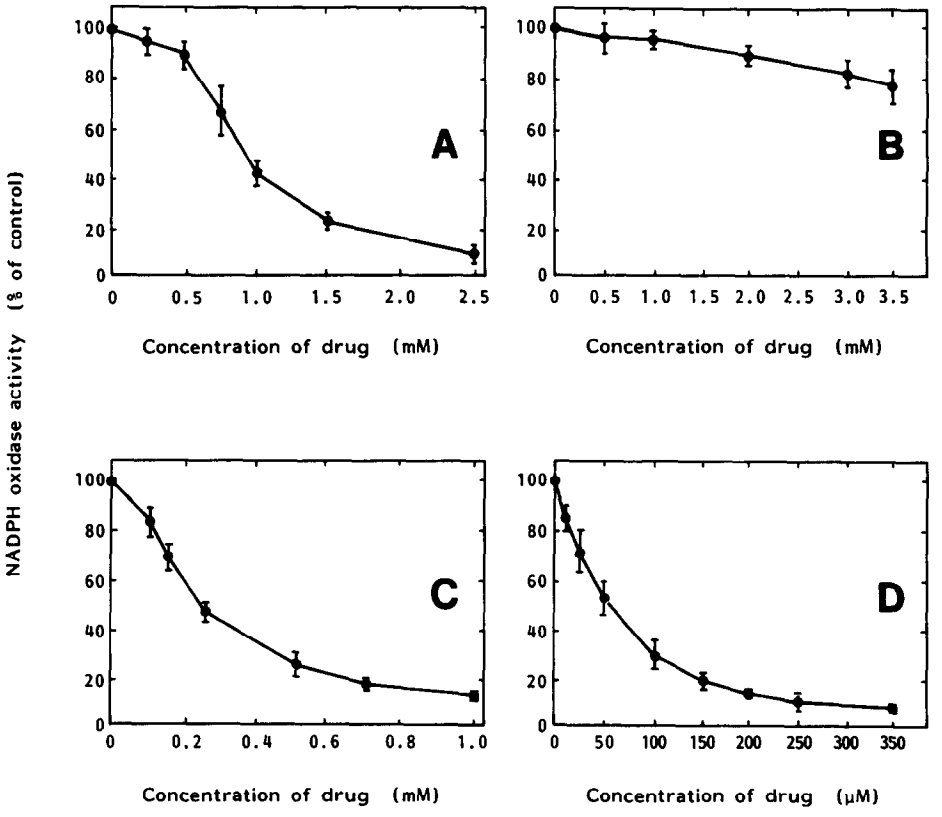

Fig. 4. Concentration-dependent changes due to anti-allergic drugs without H_1-receptor antagonism in the activation of NADPH oxidase in the cell-free system. The assay method is described in Materials and Methods. Results are the means ± SD of three different experiments. Key: (A) tranilast; (B) repirinast; (C) ibudilast; and (D) hydrocortisone treatment. Control activity was 21.0 nmol $O_2^-/10^7$ cells/min (mean, N = 5).

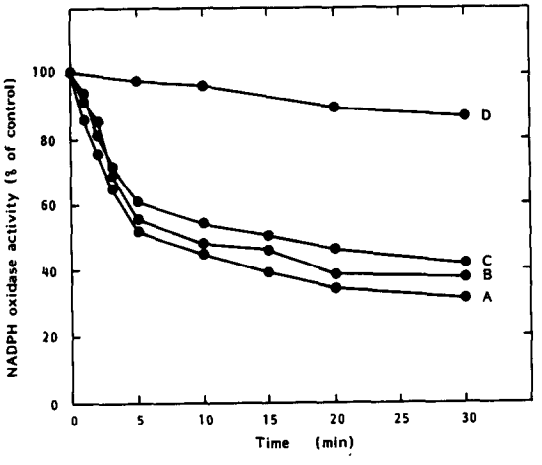

Fig. 5. Time-dependent changes in the activation of NADPH oxidase in the cell-free system after preincubation with anti-allergic drugs but before the addition of SDS and NADPH. The assay method is described in Materials and Methods. Results are the means of three different experiments. Key: (A) azelastine; (B) ketotifen; (C) oxatomide; and (D) only methanol treatment. Control activity was 19.7 nmol $O_2^-/10^7$ cells/min (mean, N = 4).

in the cell-free system. Although the mean V_{max} values for the NADPH of the oxidase after treatment with the four drugs at concentrations required to obtain IC_{50} values were about half those in the control assay, these drugs did not change the K_m value for the NADPH of the oxidase.

Figure 6 shows the effects of anti-allergic drugs with H_1-receptor antagonism on the superoxide generation of NADPH oxidase after its activation by PMA in the whole-cell system. In *curve 1* (control curve), the cells were exposed to cytochrome *c* in HBSS medium. After the addition of PMA to this mixture, oxidase activity was immediately apparent. In a whole-cell system containing cytochrome *c*, cells and PMA, three drugs, azelastine (A, *curve 2*), ketotifen (B, *curve 3*) and oxatomide (C, *curve 4*), did not abolish the superoxide generation of NADPH oxidase.

DISCUSSION

The results obtained here suggest that azelastine, ketotifen, oxatomide, tranilast, repirinast and ibudilast, which are potent anti-allergic drugs, inhibit the superoxide generation by PMA-stimulated neutrophils in whole-cell systems and SDS-induced activation of neutrophil NADPH oxidase in cell-free

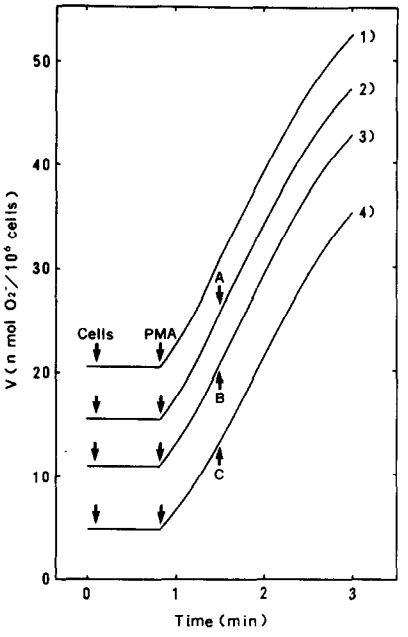

Fig. 6. Effects of anti-allergic drugs with H_1-receptor antagonism on the superoxide generation of NADPH oxidase of PMA-stimulated neutrophils when the drugs were added to the cuvettes after adding PMA. In *curve 1* (control curve), the cells (2×10^5 cells) were exposed to cytochrome *c*–HBSS buffer. Thereafter, PMA (0.3 μg/cuvette) was added to the cuvette. In *curves 2, 3* and *4*, the same procedure was followed, with each further addition of drugs. One experiment. Key: (A) in *curve 2*, azelastine; (B) in *curve 3*, ketotifen; and (C) in *curve 4*, oxatomide. Control activity was 18.1 nmol $O_2^-/10^6$ cells/min (*curve 1*).

systems. The rank of potencies was as follows: azelastine ≫ ketotifen = oxatomide ≫ ibudilast > tranilast > repirinast. In the inhibition of superoxide generation of NADPH oxidase, azelastine and oxatomide were active at concentrations lower than 0.5 and 10 μM, respectively, which correspond to the plasma levels currently reached when these drugs are administered for therapeutic purposes. Anti-allergic drugs, including azelastine, ketotifen and oxatomide, inhibited the superoxide generation of neutrophil NADPH oxidase more strongly than hydrocortisone. However, tranilast, repirinast and ibudilast, acidic anti-allergic drugs without H_1-receptor antagonism, did not inhibit superoxide generation of the NADPH oxidase at therapeutic doses.

Asthma is a disease characterized by episodic bronchoconstriction, hypersecretion of mucus, and inflammation of the airways. Evidence suggests that substances derived from the action of the enzyme 5-lipoxygenase on arachidonic acid may play a role in mediating the physiologic events in asthma. The metabolites derived from the 5-lipoxygenase pathway include the sulfidopeptide leukotrienes, 5-hydroxy-eicosatetraenoic acid, and leukotriene B_4, which have been shown individually and collectively to be potent bronchoconstrictors [29], mucous secretagogues [30], and chemotactic agents [31]. Products of the 5-lipoxygenase pathway have been detected during spontaneous attacks of asthma [32]. Recent studies have suggested that these chemical mediators may play an important role in the characteristic bronchial hyperresponsiveness and symptoms of chronic asthma [11, 12]. Recently, in addition to the β_2-agonists with potent mast cell stabilizing effects, anti-allergic drugs (anti-inflammatory drugs) without inhibitory effects on the cyclooxygenase system have also been widely used for prophylactic therapy in atopic asthmatic patients [14].

Although the chemical structures of the anti-allergic drugs are quite different as seen in Fig. 7, the agents may be classified into two groups: (i) the basic anti-allergic drugs (azelastine, ketotifen and oxatomide) containing amines in their structures, and (ii) the acid anti-allergic drugs (tranilast, repirinast and ibudilast) which have carbonic acid in their molecules. Both basic and acidic anti-allergic drugs, such as azelastine [15], ketotifen [17, 18], oxatomide [19, 20], tranilast [22], repirinast [23] and

Table 1. Effects of anti-allergic drugs on the K_m and V_{max} for NADPH in a cell-free system

Additions	N*	K_m (μM)	V_{max} (nmol $O_2^-/10^7$ cells/min)
None	3	40.1 ± 2.3	21.4 ± 3.3
Azelastine (0.6 μM)	3	38.4 ± 2.6	11.5 ± 1.9†
Ketotifen (7.0 μM)	3	41.1 ± 1.8	10.6 ± 2.0†
Oxatomide (10 μM)	3	37.8 ± 1.2	12.3 ± 1.0†
Hydrocortisone (60 μM)	3	39.2 ± 3.0	11.2 ± 2.4†

The assay method is described in Materials and Methods, except that NADPH, at various concentrations, was used in these experiments. Kinetic constants were calculated by linear regression analysis of Lineweaver–Burk plots. Values are means ± SD.

* The number of experiments performed.

† $P < 0.001$, compared with the control (no additions).

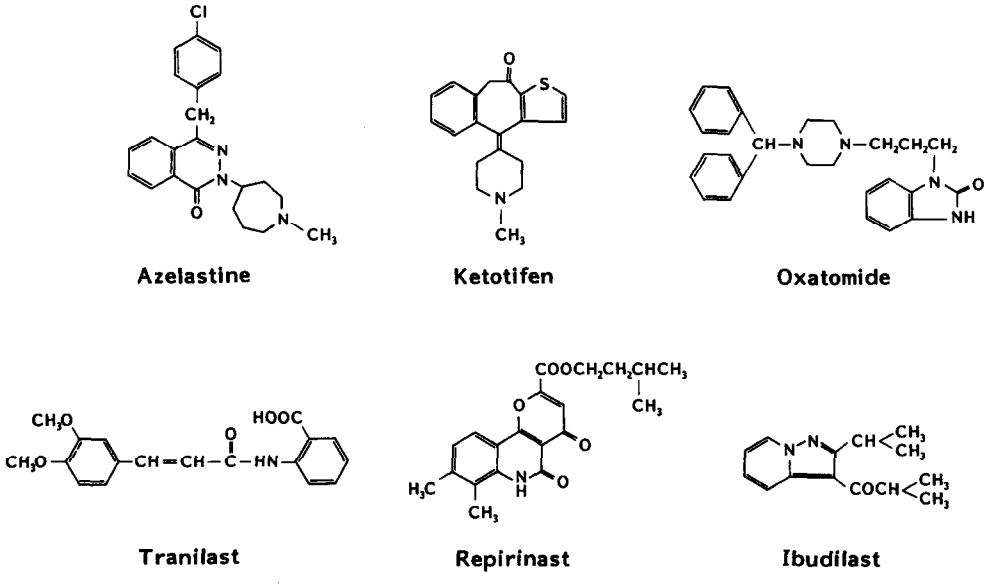

Fig. 7. Chemical structures of azelastine, ketotifen, oxatomide, tranilast, repirinast and ibudilast.

ibudilast [24], have action which reduces the release of products of the 5-lipoxygenase pathway, but they do not have inhibitory effects on the cyclooxygenase pathway. Acidic anti-allergic drugs, including tranilast, repirinast and ibudilast, have no H_1-receptor antagonism, but basic anti-allergic drugs, including azelastine, ketotifen and oxatomide, have both H_1-receptor antagonism and anti-allergic action [15–20, 33, 34]. Our interest in the non H_1-effects of antihistamines on allergic mechanisms goes back more than a decade. It was discovered that exogenous histamine regulated the release of basophil histamine and the slow-reacting substance of anaphylaxis downward in response to IgE cross-linking in an *in vitro* system. This effect was shown to be caused by the blocking effect of H_2-receptor antagonists on H_2-receptors. On the other hand, neither H_1-receptor agonists nor antagonists had any effect [35]. However, Togias *et al.* [36] reported that the H_1-antihistamines at concentrations of 10^{-5} to 10^{-4} M inhibit the release of histamine and leukotriene C_4 from antigen-stimulated lung mast cells. Histamine is an important mediator of asthma symptoms. Most of the histamine in the lung is located in the secretory granules of mast cells in the airway, and specific challenge by allergens or nonspecific challenge by exercise or cold air stimulates the release of this preformed mediator of inflammation and contributes to airway obstruction [33]. After bronchoprovocation by an inhaled allergen, early and late constrictor responses are associated with increased plasma concentrations of histamine. Increased circulating histamine concentrations have also been reported in association with spontaneous episodes. Histamine produces asthma symptoms by constriction of smooth muscle, which occurs directly via stimulation by H_1-receptors and indirectly via stimulation of vagal reflexes.

In addition to their H_1-blocking activity, some of the new H_1-receptor antagonists, such as azelastine, ketotifen and oxatomide, have anti-allergic properties; that is, they decrease the release of mediators of inflammation, such as histamine and leukotrienes, from inflammatory cells [15–20]. This effect occurs *in vitro* at physiological concentrations of the H_1-receptor antagonists, and also *in vivo*, after usual therapeutic doses. Cetirizine, a second-generation H_1-receptor antagonist, has an anti-inflammatory effect and inhibits the recruitment of inflammatory cells, including eosinophils, neutrophils, and basophils, to the site of a type-1 hypersensitivity reaction [37, 38]. It seems to ameliorate both the early and late phase response to allergens [37]. However, little information about the effects of H_1-receptor antagonists on neutrophil NADPH oxidase closely associated with defense mechanisms is available.

In the present study, azelastine (more than 0.1 μM), ketotifen (more than 2 μM) and oxatomide (more than 5 μM) inhibited the SDS-induced activation of neutrophil NADPH oxidase in the cell-free system, suggesting that these drugs may partially produce some conformational changes in the solubilized oxidase enzyme. The discrepancy between the inhibitory effects of ketotifen and hydrocortisone on NADPH oxidase activities in the whole-cell (IC_{50}, 60 μM in ketotifen and 5.1 mM in hydrocortisone) and cell-free (IC_{50}, 6.8 μM in ketotifen and 60 μM in hydrocortisone) systems may be due to the hydrophilicity of the drugs. The H_1-receptor antagonists, azelastine, ketotifen and oxatomide, were able to inhibit the activation of oxidase by PMA in the whole-cell system when they were added to the cuvettes before adding PMA (Fig. 1), but each of the drugs at the same concentrations failed to inhibit the superoxide generation of neutrophil NADPH oxidase when they were added after adding PMA (Fig. 6). In addition, these drugs at concentrations required to obtain IC_{50} values failed to inhibit the activation of oxidase when they were added to the cuvettes after adding SDS (data not

shown). These results suggest that these drugs could not inhibit the NADPH oxidase previously activated by PMA or SDS, and that they may bring about modifications in the oxidase activation system rather than having a direct effect on the oxidase enzyme or a superoxide-scavenger effect. However, a possible direct effect of the drugs on NADPH oxidase may not be discounted.

In the results obtained in the cell-free system, three H_1-receptor antagonists decreased the V_{max} for the NADPH of the oxidase, but they did not change the K_m value for NADPH, suggesting that these drugs may not change the affinity of NADPH oxidase for NADPH. Further investigation, however, will be required to obtain a more detailed picture of the mechanisms of the inhibition of activation of the NADPH oxidase in cell-free systems caused by the H_1-receptor antagonists.

Rat and human mast cells have been shown to exhibit parallel release of superoxide and histamine induced by anti-IgE [39]. Furthermore, rat peritoneal mast cells release histamine on exposure to xanthine oxidase plus hypoxanthine and also after exposure to H_2O_2 [40, 41]. Ogasawara *et al.* [42] reported that human basophils release histamine after exposure to H_2O_2. To varying degrees, the H_1-receptor antagonists protect against the early and late responses to allergens [43, 44]. Protection against the late response is probably not an H_1-receptor blocking effect, but seems to be related more to the anti-allergic and anti-inflammatory effects of the new H_1-receptor antagonists. The new H_1-receptor antagonists have bronchodilator activity [45] and provide relief from the seasonal or chronic asthma symptoms of patients with mild asthma [46]. H_1-Receptor antagonists (anti-allergic drugs) are not the drugs of first choice for asthma, but previous concerns about their potential adverse effects in asthma have been exaggerated greatly. Patients with chronic asthma or seasonal asthma who require H_1-receptor antagonists for treatment of concurrent rhinoconjunctivitis or urticaria will not be harmed by H_1-receptor antagonist treatment and may even gain some modest anti-asthma benefit from the H_1-receptor antagonists.

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