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Rebamipide suppresses mite-induced asthmatic responses in NC/Nga mice

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Murakami I, Zhang R, Kubo M, Nagaoka K, Eguchi E, Ogino K. Rebamipide suppresses mite-induced asthmatic responses in NC/Nga mice. *Am J Physiol Lung Cell Mol Physiol* 309: L872–L878, 2015. First published August 21, 2015; doi:10.1152/ajplung.00194.2015.—Allergic asthma caused by continuous allergen exposure evokes allergen-specific Th2 responses and is characterized by chronic airway inflammation and hyperresponsiveness. A previous report showed that rebamipide improved asthmatic symptoms in an ovalbumin/trypsin mice model. However, it is still unclear how rebamipide exerts its effects in asthma. In this study, rebamipide improved the asthmatic responses induced by mite exposure in NC/Nga mice, revealing the mechanism of this therapeutic effect. Rebamipide suppressed the infiltration of eosinophils into the airways and lung as well as attenuating the production of reactive oxygen species in tissues. In addition to these anti-inflammatory effects, rebamipide inhibited the production of IL-33, a member of the IL-1 family that drives the subsequent production of Th2-associated cytokines. These observations identify the point where rebamipide exerts its suppressive action on asthma and suggest that rebamipide has therapeutic potential in preventing mite-induced asthma.

rebamipide; asthma; eosinophil; IL-33; reactive oxygen species

ASTHMA IS A CHRONIC RESPIRATORY disease that affects about 300 million people worldwide (32). The major characteristics of asthma are airway hyperresponsiveness (AHR), moist cough, and variable airflow obstruction on the basis of airway inflammation (36). Such asthmatic symptoms are triggered by factors such as indoor allergens (house dust mites and pet dander), outdoor allergens (pollens, particulate matter, and molds), tobacco smoke, exercise, and viral respiratory infections (36). These factors evoke airway inflammation by means of the inflammatory mediators and cytokines derived from eosinophils, lymphocytes, and airway epithelial cells (3). In addition, when exposed to allergens or nonallergenic substances these cells can produce reactive oxygen species (ROS), which cause tissue damage via lipid peroxidation of cell membranes and nucleotide damage within DNA (8, 18).

Rebamipide (2-{4-chlorobenzoylamino-3-[2(1H)-quinolinon-4-yl]} propionic acid) is widely used for mucosal protection, healing of gastric ulcers, and treatment of gastritis. It has various biological effects that include upregulation of growth factors such as epidermal growth factor and hepatocyte growth

factor (31, 34); increased production of mucus and prostaglandins (34, 37); inhibition of oxygen radical production by neutrophils stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), opsonized zymosan, or *Helicobacter pylori* (20, 27, 39, 41); scavenging of hydroxyl radicals (40); and inhibition of the production of inflammatory cytokines and chemokines (4, 7). These effects of rebamipide suggest the possibility that rebamipide may be useful for the treatment and prevention for asthma. A previous report showed that oral administration of rebamipide improved asthmatic symptoms in an ovalbumin (OVA)/trypsin mice model; however, how rebamipide exerts these effects remains unclear (5).

The aim of this study was to examine whether rebamipide improved the asthmatic responses in a model in which NC/Nga mice were exposed to mites and to clarify the molecular mechanisms involved.

MATERIALS AND METHODS

Chemicals and reagents. Rebamipide was manufactured by Otsuka Pharmaceutical (Tokushima, Japan). Carboxymethyl cellulose sodium (CMC) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetylcholine chloride was purchased from Daiichi Sankyo Pharmaceutical (Tokyo, Japan). Gallamine triethiodide was purchased from Sigma-Aldrich (St. Louis, MO). Isoflurane was purchased from Intervet (Tokyo, Japan). Pentobarbital sodium was purchased from Kyoritsu Seiyaku (Tokyo, Japan).

Animals. Five-week-old male NC/Nga mice were obtained from Charles River Laboratories Japan (Kanagawa, Japan). The mice were maintained under specific pathogen-free conditions in 12:12-h light-dark cycle and had free access to standard laboratory food and water. They were acclimatized for at least 1 wk before the experiments. The care and handling of animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals at Shikata Campus of Okayama University. This study was approved by the Okayama University Institutional Animal Care and Use Committee.

Intranasal and intratracheal administrations. NC/Nga mice were sensitized to crude mite extract (*Dermatophagoides farinae*: Df, Cosmo Bio, Tokyo, Japan) based on a previously described protocol (29) as shown in Fig. 1. Briefly, mice were anesthetized with 3% isoflurane and treated with intratracheal 2% rebamipide (500 μ g in 25 μ l 0.1% CMC) for 14 consecutive days (days 0–13). One hour after each treatment, the anesthetized mice received intranasal instillation of Df crude extract (50 μ g in 25 μ l saline) for 6 days (days 0–4 and 11). Saline and 0.1% CMC were used as controls, for comparison with the groups given Df or rebamipide.

Measurement of AHR. On day 14, the degree of bronchoconstriction was measured according to the overflow method (26). Briefly, mice were anesthetized with 50 mg/kg ip pentobarbital sodium and connected to an artificial ventilator following surgical incision of the

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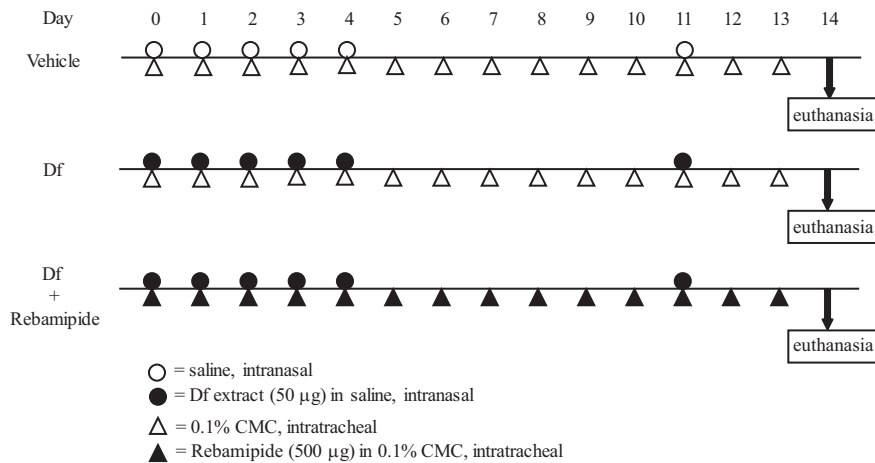


Fig. 1. Schematic representation of the experiment. The vehicle group received 25 μ l of saline intranasally on days 0–4 and day 11 and 25 μ l of 0.1% carboxymethyl cellulose sodium (CMC) intratracheally on days 0–13. The Df group received 50 μ g of *Dermatophagoides farinae* (Df) extract dissolved in 25 μ l of saline intranasally on days 0–4 and day 11 and 25 μ l of 0.1% CMC intratracheally on days 0–13. The Df plus rebamipide treatment group received 50 μ g of Df extract dissolved in 25 μ l of saline intranasally on days 0–4 and day 11 and 500 μ g of rebamipide dissolved in 25 μ l of 0.1% CMC intratracheally. Rebamipide or 0.1% CMC was administered 1 h before Df or saline administration.

trachea. A pulmotor system was constructed with a rodent ventilator (model 55-7058; Harvard Apparatus Canada, QC, Canada), a bronchospasm transducer (model 7020; Ugo Basile, Comerio-Barese, Italy), and a data recorder (model WR300; Graphtec, Kanagawa, Japan). Gallamine triethiodide (350 μ g/mouse) was immediately administered intravenously to eliminate spontaneous respiration and followed by repeated administration of acetylcholine with stepwise increases in the concentration from 62.5 to 2,000 μ g/kg. Dose-response curves were obtained for acetylcholine in anesthetized, mechanically ventilated mice. Bronchoconstriction was expressed as the respiratory overflow volume provoked by acetylcholine as a percentage of the maximal overflow volume (100%) obtained by totally occluding the tracheal cannula (16).

Bronchoalveolar lavage fluid. Immediately after the assessment of acetylcholine-induced AHR, the lungs were lavaged with 1 ml aliquots of cold Hanks' balanced salt solution (HBSS) without calcium and magnesium, containing 50 μ M EDTA. The collected bronchoalveolar lavage fluid (BALF) was then centrifuged at 200 g for 10 min at 4°C. The supernatant was collected and stored at –80°C for further analysis, and the cell pellet was resuspended in cold HBSS. The number of leukocytes in the BALF was determined by use of a hematology analyzer (model XT-200i; Sysmex, Hyogo, Japan). Another aliquot was used for cytospin preparations at 500 rpm for 5 min (Cytospin2; Thermo Fisher Scientific, Kanagawa, Japan) and was stained with Diff-Quik for cell count (Sysmex).

Lung specimens. The lung tissue was utilized for extraction of total RNA for RT-PCR and fixed in 10% buffered formalin for morphological examination. The remaining lung tissue was homogenized in a homogenizing buffer (T-PER tissue extraction reagent containing a protease inhibitor cocktail) with a Multi-Beads Shocker (model MB9010T; Yasui Kikai, Osaka, Japan) set to 1,900 rpm and 30 s, with repetition for two cycles.

Quantitative PCR analysis. Total RNA was isolated using the RNeasy mini kit (Qiagen, Redwood City, CA), and reverse transcription was performed with the High Capacity RNA-to-cDNA Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Quantitative RT-PCR was carried out by using the ABI 7500 Fast Real-Time PCR System and the TaqMan Fast Universal PCR Master Mix (Life Technologies). The reaction mixture was prepared with TaqMan Gene Expression Primer and Probe (Life Technologies) according to the manufacturer's protocol. Primers specific for interleukin-4 (IL-4, Assay ID: Mm00445259_m1), IL-5 (Mm00439646_m1), IL-13 (Mm00434204_m1), interferon- γ (IFN- γ , Mm99999071_m1), eotaxin-1 (Mm00441238_m1), eotaxin-2 (Mm00444701_m1), IL-33 (Mm00505403_m1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 4352932E), arginase-1 (Arg1, Mm00475988_m1), Fizz1 (Mm00445109_m1), Yml (Mm00657889_m1), and nitric oxide synthase 2 (NOS2, Mm00440502_m1) were used. The thermal cycling

conditions were at 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. The expression of mRNA was standardized to GAPDH mRNA, and the relative expression of each gene was quantified by the ddCt method, expressed as a ratio, related to the mean value for vehicle-treated lungs (17).

EIA analysis. The concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was determined by using a DNA/RNA oxidative stress EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. The detection limit of this assay was 10.3 pg/ml.

ELISA analysis. Protein concentrations were determined by the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, Waltham, MA) using bovine serum albumin standards. The concentrations of eotaxin-1, eotaxin-2, and IL-33 were determined by ELISA kit or DuoSet (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions and these were adjusted by total protein. The detection limits of the assays for eotaxin-1, eotaxin-2, and IL-33 were 15.6 pg/ml each.

Histopathological observations. The fixed lung tissues were dehydrated, embedded in paraffin, and sectioned. Hematoxylin and eosin (H&E) staining was used to assess the degree of inflammation (11). Periodic acid-Schiff (PAS) staining was also performed to observe interstitial goblet cell hyperplasia (30). Images of lung tissue sections stained with H&E and PAS were acquired with a BZ-9000 microscope (Keyence, Osaka, Japan) equipped with a $\times 40$ objective lens. The levels of inflammation in peribronchial areas of the lung were determined according to an ordinal scale of grades 0 to 3, as described elsewhere (33), in a blinded fashion with examination of at least five airway cross sections per slide. A value of 0 indicated that no inflammation was detectable; a value of 1, occasional cuffing with inflammatory cells; a value of 2, most bronchi were surrounded by a thin layer (1–5 cells thick) of inflammatory cells; and a value of 3, most bronchi were surrounded by a thick layer (more than 5 cells thick) of inflammatory cells. Goblet cell hyperplasia was semiquantitatively scored in a blinded fashion in at least five airway cross sections per slide on a scale of grades 0 to 4 (absence, <25% of epithelial lining cells, 25–50% of epithelial lining cells, 50–75% of epithelial lining cells, >75% of epithelial lining cells, respectively) as previously described (21).

Statistical analysis. All data, unless otherwise noted, are expressed as means $\pm$ SE. One-way ANOVA followed by Dunnett's test was used to evaluate the significance of differences between more than two groups. A mixed model for repeated measures method was used to determine significant differences in the dose-response studies. The threshold for statistical significance was set at $P < 0.05$. Statistical analyses were performed with SAS software release 9.3 (SAS Institute, Tokyo, Japan).

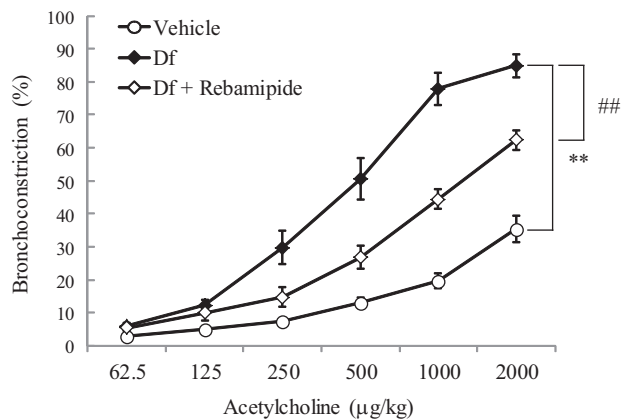


Fig. 2. Airway hyperresponsiveness (AHR) to acetylcholine. Effect of rebamipide on Df-induced increase of AHR in NC/Nga mice. Data were obtained from 5–12 mice/group. The bronchoconstriction (%) is expressed as means $\pm$ SE. $**P < 0.01$ Df group vs. Vehicle group. $##P < 0.01$ Df plus rebamipide group vs. Df group. A mixed model for repeated measures method was used.

RESULTS

Effect of rebamipide on AHR. Mite antigen is one of the major allergic substrates that induce asthma. Here we used *Dermatophagoides farinae* (Df) for the induction of asthma in NC/Nga mice. To confirm asthmatic features, AHR, which is a basic and reproducible indicator of asthma, was measured. First we examined whether Df treatment changes AHR in response to acetylcholine. In the vehicle group, AHR increased in a dose-dependent manner and such increments in AHR were further enhanced by Df administration ($P < 0.01$, Fig. 2). We

then evaluated the effects of rebamipide on Df-treated mice and found that rebamipide reduced AHR significantly ($P < 0.01$, Fig. 2). Thus intratracheal administration of rebamipide improved AHR in response to acetylcholine in Df-treated NC/Nga mice.

Effect of rebamipide on histopathological findings. Histopathological changes in lungs of mice treated with Df were examined to estimate the degree of inflammation, using H&E staining. Df treatment increased the inflammation score to about twice that of the vehicle group (0.9 ± 0.0 vs. 2.0 ± 0.2 , $P < 0.01$, Fig. 3, A, B, and G) and such Df-induced inflammation was suppressed by rebamipide (2.0 ± 0.2 vs. 1.4 ± 0.1 , $P < 0.01$, Fig. 3, C and G). Since it is known that airway inflammation causes hyperplasia of goblet cells, we attempted to estimate it using PAS staining. Similar to the results of inflammatory scoring, the goblet cell hyperplasia score in the Df-treated group was higher than that in the vehicle group (0.0 ± 0.0 vs. 2.5 ± 0.4 , $P < 0.01$, Fig. 3, D, E, and H), whereas the score in the rebamipide group was lower than in the Df-treated group (2.5 ± 0.4 vs. 1.6 ± 0.2 , $P < 0.05$, Fig. 3, F and H). These results confirmed that rebamipide exhibits anti-inflammatory effects on Df-elicited inflammation in lung.

Effect of rebamipide on the accumulation of immune cells in BALF. To explore the mechanisms of inflammation in the airway and lung, we collected BALF and analyzed the number of accumulated cells and types in BALF using a hematology analyzer. Df treatment increased total cell number about nine times that in the vehicle group ($2.64 \pm 0.43 \times 10^4$ cells vs. $23.58 \pm 4.07 \times 10^4$ cells) (Fig. 4). We further analyzed BALF to identify the cell types present and found that the numbers of inflammatory cells including eosinophils, macrophages, neu-

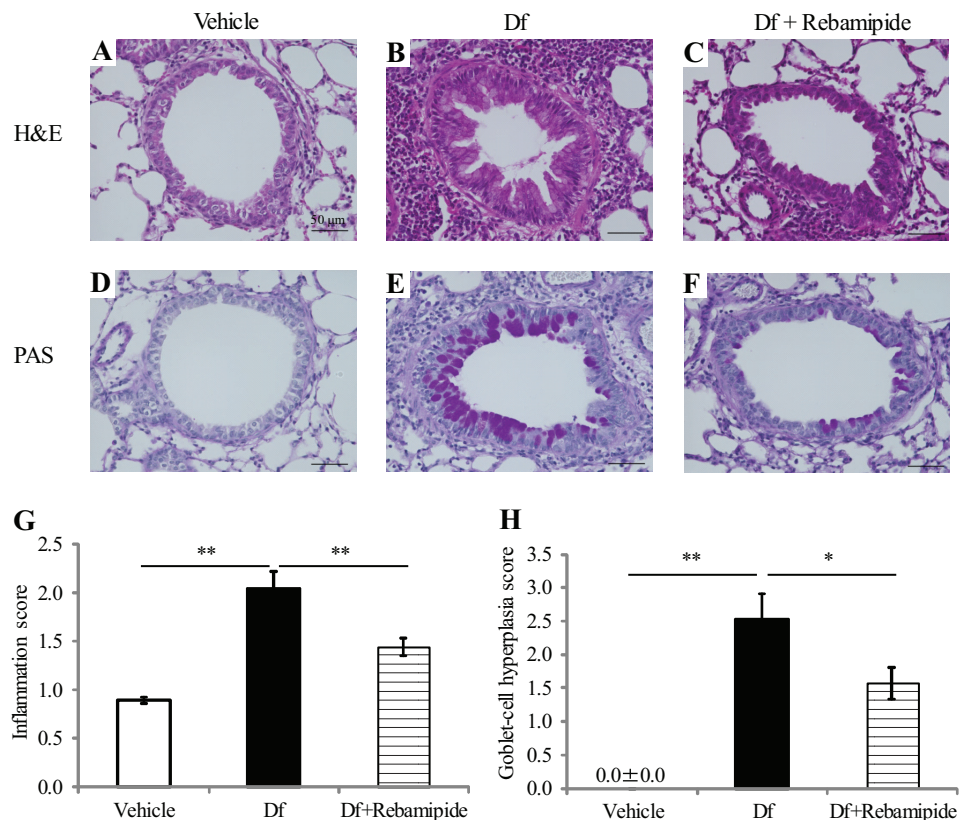


Fig. 3. Histopathology of hematoxylin and eosin (H&E)-stained lungs or periodic acid-Schiff (PAS)-stained lungs from Vehicle group (A and D), Df group (B and E), and Df plus rebamipide group (C and F). For A, B, C, D, E, and F, scale bars are 50 μ m and pictures were taken at $\times 40$ magnification. Inflammation score (G) and goblet cell hyperplasia score (H) are expressed as means $\pm$ SE. Data were obtained from 5–12 mice/group. $*P < 0.05$, $**P < 0.01$ vs. Df group.

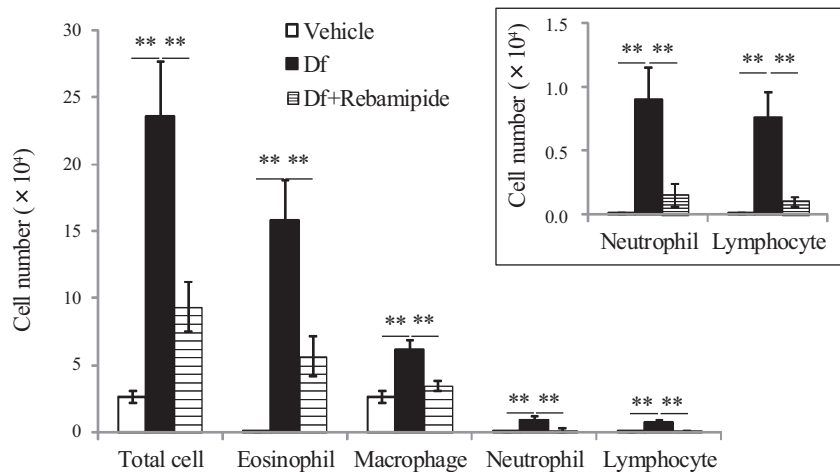


Fig. 4. Effect of rebamipide on bronchoalveolar lavage fluid (BALF) cell numbers. Enlarged view displays results of neutrophil and lymphocyte (inset). Data were obtained from 5–12 mice/group. The total cell, eosinophil, macrophage, neutrophil, and lymphocyte counts are expressed as means $\pm$ SE. ** P < 0.01 vs. Df group.

trophils, and lymphocytes were notably increased by Df treatment (Fig. 4). In particular, the number of eosinophils in the BALF increased $\sim 4,000$ -fold compared with vehicle control ($0.04 \pm 0.04 \times 10^3$ cells vs. $157.89 \pm 29.82 \times 10^3$ cells), suggesting that Df-induced an inflammatory response mainly mediated by excess numbers of infiltrating eosinophils in the airways and lungs. This increased number of inflammatory cells was reduced for every cell type when rebamipide was administered intratracheally for 14 days, where the attenuation rate in eosinophil number was $\sim 64\%$.

Effect of rebamipide on expression of eotaxins. Next we measured the expression of eotaxin-1 and eotaxin-2 in lung tissue to confirm the involvement of eosinophils, as suggested in the results of BALF (Fig. 4). We found that Df challenge increased eotaxin-1 mRNA expression ~ 20 -fold and eotaxin-2 mRNA 80-fold compared with those in the vehicle group (Fig. 5A). Elevated protein expression was also detected for both eotaxin-1 and eotaxin-2 (Fig. 5B; eotaxin-1: 36.1 ± 9.1 vs. 219.0 ± 32.8 pg/mg protein, P < 0.01; eotaxin-2: 59.2 ± 6.0 vs. 679.5 ± 185.6 pg/mg protein, P < 0.01). When rebamipide was administered, augmented mRNA expression induced by Df was reduced about one-half and one-quarter for eotaxin-1 and eotaxin-2, respectively. In addition, increased protein expression of both eotaxin-1 and eotaxin-2 were also significantly decreased by rebamipide (eotaxin-1: 90.8 ± 12.7 pg/mg protein, P < 0.01; eotaxin-2: 174.7 ± 31.5 pg/mg protein, P < 0.05).

These results demonstrate that main cell infiltrating the lung was the eosinophil, which was also the most abundant cell type in BALF after Df treatment; rebamipide suppressed this infiltration.

Effect of rebamipide on 8-OHdG in BALF. ROS are detected extensively in patients with asthma, where they are mainly produced by granulocytes and epithelial cells. Because rebamipide is a direct scavenger of ROS (40) and also inhibits ROS production by activated neutrophils through the competitive inhibition of the formyl peptide receptor (20), we measured 8-OHdG in BALF as an indicator of ROS production to examine the suppressive effect of rebamipide in our model. The level of 8-OHdG in BALF increased after Df treatment (78.0 ± 12.6 vs. 159.6 ± 24.6 pg/ml BALF, P < 0.05) and this increase in 8-OHdG was significantly reduced to the same level seen with the vehicle control when rebamipide was administered (74.3 ± 9.4 , P < 0.01) (Fig. 6).

Effect of rebamipide on expression of IL-33 and cytokines. It has been revealed that the epithelium-derived cytokine IL-33 plays important roles in asthma in Th2-dependent and independent ways (23), where IL-33 activates effector cells such as eosinophils, mast cells, and basophils directly and also induces activation of these cells via IL-4, IL-5, and IL-13 produced by Th2 cells (13, 19). We thus examined changes in the expression of IL-33 and Th2 cytokines in lung, before and after Df treatment.

We first examined IL-33 expression by measuring mRNA and protein levels. Df treatment increased IL-33 expression significantly compared with the vehicle group (690.1 ± 73.7 vs. $3,306.5 \pm 493.8$ pg/mg protein, P < 0.01) (Fig. 7, A and B). We then tested the effect of rebamipide on the expression of IL-33. Rebamipide reduced IL-33 expression by about half, with respect to both mRNA and protein ($1,742.1 \pm 178.0$ pg/mg protein, P < 0.01). We next measured the expression of IL-4, IL-5, and IL-13 as a representative Th2 cytokines and

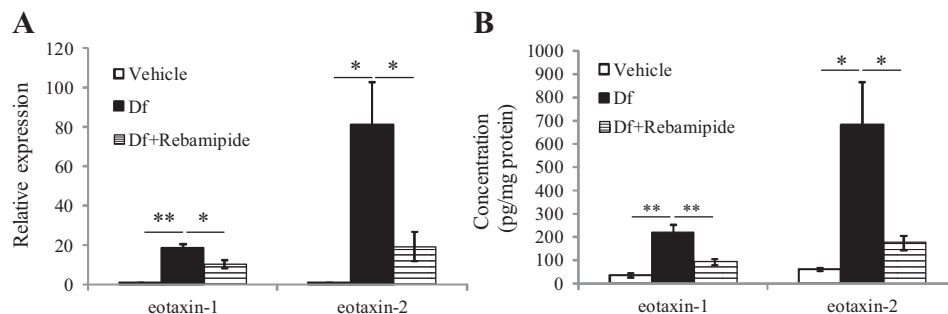


Fig. 5. Effect of rebamipide on Df-induced mRNA (A) and protein expression (B) of eotaxin-1 and eotaxin-2 in lung tissues. The expressions of mRNA for eotaxin-1 and eotaxin-2 were standardized to GAPDH mRNA and the relative expression of each gene was quantified by the ddCt method. The mean expression levels in lung tissues exposed to vehicle were normalized to 1. Data were obtained from 5–12 mice/group. Concentrations of eotaxin-1 and eotaxin-2 are expressed as means $\pm$ SE. * P < 0.05, ** P < 0.01 vs. Df group.

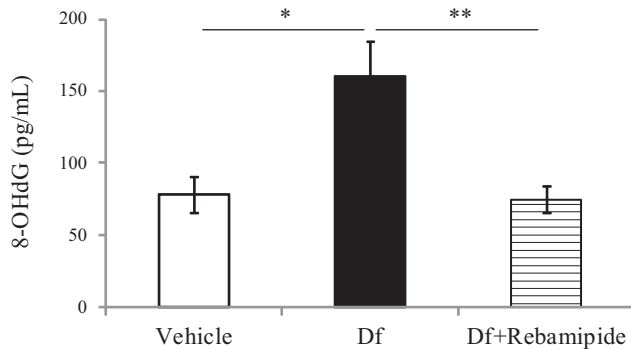


Fig. 6. Effect of rebamipide on Df-induced 8-hydroxy-2'-deoxyguanosine (8-OHdG) production in BALF. Data were obtained from 5–12 mice/group. Concentrations of 8-OHdG in BALF are expressed as means $\pm$ SE. * P < 0.05, ** P < 0.01 vs. Df group.

IFN- γ as a Th1 cytokine, respectively. For all cytokines examined except IFN- γ , mRNA expression was elevated, suggesting that Df treatment selectively evokes a Th2 response. These augmented Th2 responses were dramatically suppressed when rebamipide was administered (Fig. 8).

Effect of rebamipide on macrophage polarization. On the basis of the result of enhanced Th2 cytokine productions shown in Fig. 8, we further analyzed macrophage polarization to confirm Th2 response by testing the expression of series of polarization marker. Here we utilized arginase-1 (Arg1), found in inflammatory zone-1 (Fizz1), and chitinase-like 3 (Ym1) as a marker for M2 macrophage and nitric oxide synthase 2 (NOS2) was selected for M1 macrophage marker (25, 38). All mRNA levels in M2 macrophage markers increased after Df treatment. On the other hand, M1 macrophage maker did not show any changes (Fig. 9). These enhanced expression of M2 macrophage makers were remarkably decreased after rebamipide treatment, suggesting the possibility that rebamipide suppresses M2 macrophage polarization through the inhibition of production of Th2 cytokines.

DISCUSSION

Previous studies reported that rebamipide attenuated the allergic response and respiratory symptoms by improving AHR, reducing leukocyte numbers in BALF, and suppressing goblet cell hyperplasia regardless of the different animals, allergens, and routes of administration of rebamipide. For example, Lee et al. (12) reported that oral administration of rebamipide reduced the number of infiltrating leukocytes and the amount of TNF- α in BALF and downregulated MUC5AC mucin synthesis in the airway epithelium in a rat model of cigarette smoke-induced mucin production. Another group

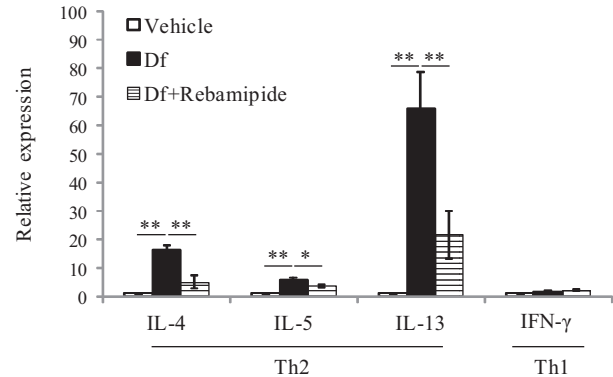
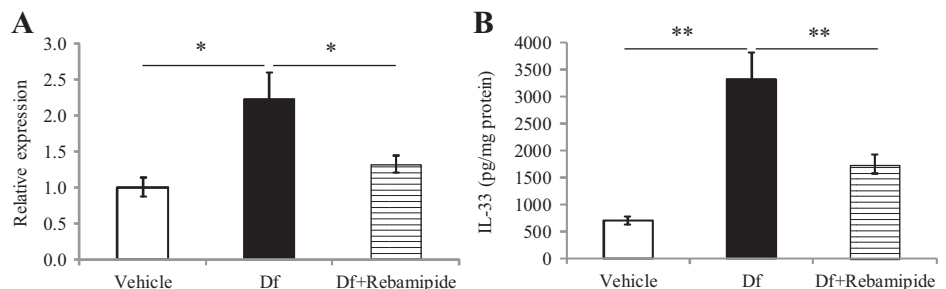


Fig. 8. Effect of rebamipide on Df-induced mRNA expression of cytokines in lung tissues. The expression of mRNA for IL-4, IL-5, IL-13 (Th2), and IFN- γ (Th1) were standardized to GAPDH mRNA and the relative expression of IL-33 was quantified by the ddCt method. The mean expression levels of lung tissue exposed to vehicle were normalized to 1. Data were obtained from 5–12 mice/group. Data are expressed as means $\pm$ SE. ** P < 0.01 vs. Df group.

used a different approach, examining the effects of rebamipide on an OVA/trypsin-induced asthmatic mouse model, and found that oral administration of rebamipide decreased the eosinophil number in BALF and improved the respiratory rate, air-flow rate, and tidal volume (5). In this study, we used a mite-induced asthmatic model in NC/Nga mice, which resembles human asthma, to examine the therapeutic effects of rebamipide and to clarify the molecular mechanisms involved (26, 29, 30).

Our results suggest that rebamipide improves features of asthma in NC/Nga mice through three pathways (Fig. 10). First, rebamipide suppressed eosinophil invasion of the airways and lungs (Figs. 4 and 5). It is known that an excess number of infiltrating eosinophils in respiratory tissues is one of the characteristic features of asthma and it is strongly associated with the development of AHR. Such chemotaxis of eosinophils toward inflamed tissue may be inhibited by rebamipide directly or indirectly. Since previous reports have shown that rebamipide exerts an antagonistic effect on the formyl peptide receptor (FPR) expressed in neutrophils and eosinophils, an essential receptor for the expression of chemotaxis (20, 28), there is a possibility that rebamipide suppresses chemotactic behavior of eosinophils through the inhibition of the FPR. In addition to the direct effects of rebamipide on eosinophils, we found that rebamipide reduced the expression of eotaxin-1 and eotaxin-2 in lung (Fig. 5). The eotaxin family has been identified as chemoattractant for eosinophils, implying that rebamipide may suppress chemotaxis of eosinophils by eliminating the chemoattractant source. Second, rebamipide

Fig. 7. Effect of rebamipide on Df-induced mRNA (A) and protein expression (B) of IL-33 in lung tissues. The expression of mRNA for IL-33 was standardized to GAPDH mRNA and the relative expression of IL-33 was quantified by the ddCt method. The mean expression levels of vehicle lung tissues were normalized to 1. Data were obtained from 5–12 mice/group. Data are expressed as means $\pm$ SE. * P < 0.05, ** P < 0.01 vs. Df group.



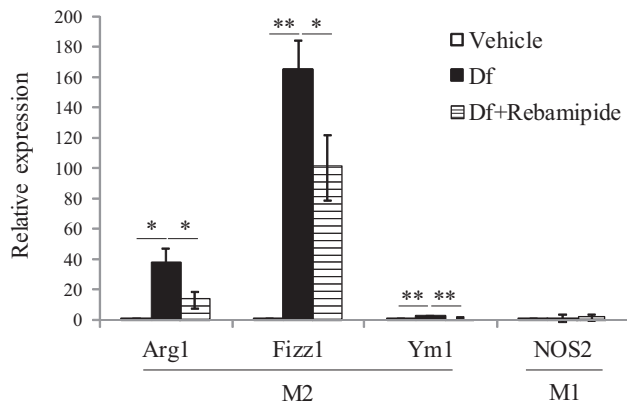


Fig. 9. Effect of rebamipide on Df-induced mRNA expression of M2 and M1 macrophage markers in lung tissue. The expression of mRNA for Arg1, Fizz1, Ym1, and NOS2 was standardized to GAPDH mRNA and the relative expression of each gene was quantified by the ddCt method. The mean expression levels of lung tissue exposed to vehicle were normalized to 1. Data were obtained from 5–12 mice/group. Data are expressed as means $\pm$ SE. * $P < 0.05$, ** $P < 0.01$ vs. Df group.

improved asthmatic symptoms by reducing ROS production by eosinophils and epithelial cells. A marker of oxidative stress formed by ROS is 8-OHdG (10). Several studies demonstrated that 8-OHdG expression was induced and enhanced in the lungs as a result of several types of oxidative stress (1, 24, 35). Similar to previous studies, the 8-OHdG level in BALF was elevated by Df treatment and was reduced significantly by rebamipide administration, suggesting that rebamipide may work as a scavenger of ROS (Fig. 6). Alternatively, rebamipide may reduce ROS produced by activated eosinophils through inhibition of FPR agonist-induced eosinophil activation as described above. Finally, rebamipide exhibited a suppressive

action on the Th2 response by inhibition of IL-33 production. Previous reports have demonstrated the involvement of IL-33 in asthmatic symptoms (6, 9, 22). The IL-33 level is elevated in asthmatic patients compared with healthy individuals, and a similar tendency was also observed in mice (6, 9, 22), where IL-33 is released from bronchial epithelial cells in response to allergen exposure and this secreted IL-33 then induces Th2 responses from macrophages, eosinophils, and mast cells (19). Direct administration of IL-33 induces eosinophilic inflammation and AHR in mice, and administration of neutralizing antibodies against IL-33 and IL-33 receptor ST2 attenuates eosinophilic inflammation and AHR in an OVA-induced airway inflammation model in mice (2, 9, 14, 15). In the present study, rebamipide decreased the expression of IL-33 in lung tissue (Fig. 7) and also attenuated the expression of Df-elicited Th2 cytokines, including IL-4, IL-5, and IL-13 (Fig. 8). Taken together, the inhibition of IL-33 production by rebamipide appears to suppress subsequent Th2 responses. Another approach used to check Th2 response involves examining the polarization of macrophages: macrophages have been classified as M1 (classically activated) or M2 (alternatively activated) macrophages. M1 macrophages express inducible nitric oxide synthase (NOS2) and proinflammatory cytokines, and these are essential for protection against infection. Conversely, M2 macrophages express arginase-1 (Arg1), chitinase-like 3 (Ym1), and found in inflammatory zone-1 (Fizz1) and chemokines such as chemokine (C-C motif) ligand 17 (CCL17) and CCL24 (eotaxin-2), and these play important roles in responses to parasite infection, tissue remodeling, angiogenesis, and tumor progression (25, 38). Gene expression levels of M2 macrophage markers such as Arg1, Fizz1, and Ym1 were significantly upregulated by Df administration and downregulated by rebamipide treatment, whereas M1 macrophage marker NOS2 showed no change (Fig. 9). Because both IL-33 and IL-13 can polarize macrophages from M1 to M2 types, it is likely that rebamipide impairs the polarization toward M2 macrophages owing to the decreased expression of IL-33 and IL-13.

In conclusion, our present study indicates the therapeutic potential of rebamipide in the prevention of mite-induced asthma. Our data demonstrate that rebamipide improves mite-induced asthmatic symptoms through the inhibition of eosinophil infiltration, as well as attenuating oxidative stress and IL-33 production.

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DISCLOSURES

I. Murakami is employed by Otsuka Pharmaceutical.

AUTHOR CONTRIBUTIONS

I.M. and K.O. conception and design of research; I.M., R.Z., M.K., and K.N. performed experiments; I.M., E.E., and K.O. analyzed data; I.M., R.Z., M.K., K.N., E.E., and K.O. interpreted results of experiments; I.M. prepared figures; I.M. drafted manuscript; I.M., R.Z., M.K., K.N., E.E., and K.O. approved final version of manuscript; M.K., K.N., E.E., and K.O. edited and revised manuscript.

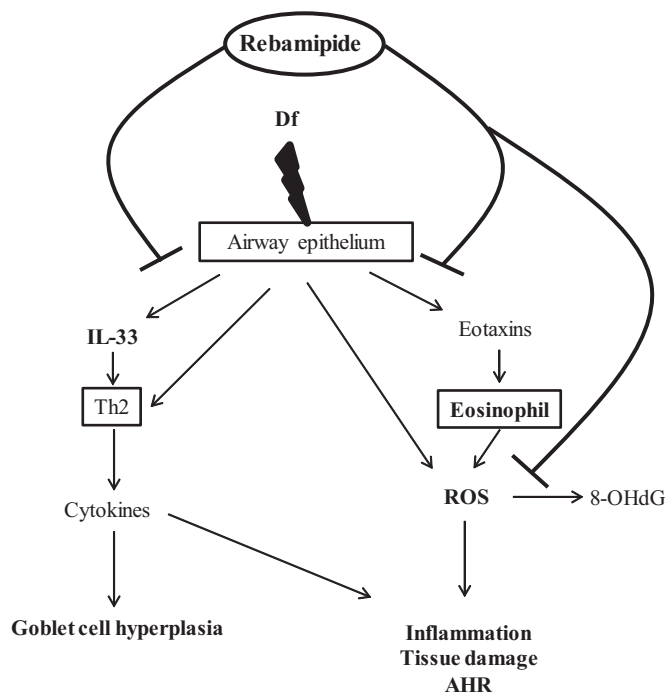


Fig. 10. Schematic representation of mechanisms by which rebamipide affects Df-induced asthmatic symptoms. ROS, reactive oxygen species.

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