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Original Article

Singlet oxygen -derived nerve growth factor exacerbates airway hyperresponsiveness in a mouse model of asthma with mixed inflammation

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Abbreviations:
4-ABAH, 4-aminobenzoic acid hydrazide; ANOVA, one-way analysis of variance; BDNF, brain derived neurotrophic factor; BAL, fluid broncho-alveolar lavage fluid; FGF, fibroblast growth factor; NHBE, normal human bronchial epithelial cells; HODE, hydroxyoctadecadienoic acids; LC-MS/MS, liquid chromatography/tandem mass spectrometry; Mch, methacholine; MPO, myeloperoxidase; NE, neutrophil elastase; NETs, neutrophil extracellular traps; NGF, nerve growth factor; Nrf2, nuclear factor-erythroid 2-related factor-2; PGP9.5, protein gene product 9.5; PPARγ, Peroxisome Proliferator-Activated Receptor γ; ROS, reactive oxygen species; R_{rs}, respiratory resistance; Trk, tropomyosin receptor kinase

ABSTRACT

Background: Refractory asthma, which is caused by several factors including neutrophil infiltration is a serious complication of bronchial asthma. We previously reported that nerve growth factor (NGF) is involved in AHR. NGF-derived induction of hyperalgesia is dependent on neutrophils; however, this relationship remains unclear in respiratory disease. In this study, we examined the roles of neutrophils and NGF in refractory asthma.

Methods: Using intranasal house dust mite sensitization, we established a mouse model of asthma with mixed inflammation (Mix-in). AHR, NGF production and hyperinnervation of the lungs were examined with or without different inhibitory treatments. The levels of the singlet oxygen markers, 10- and 12-(Z,E)-hydroxyoctadecadienoic acids (HODE) in the lungs, were measured by liquid chromatography-tandem mass spectrometry. An in vitro experiment was also performed to evaluate the direct effect of singlet oxygen on NGF production.

Results: NGF production and hyperinnervation were higher in Mix-in mice than in conventional eosinophilic-asthmatic mice and were positively correlated with AHR. Asthmatic parameters were inhibited by NGF neutralizing Abs and myeloperoxidase (MPO) inhibition. The 10- and 12-(Z,E)-HODEs levels were increased in the lungs and were positively correlated with MPO activity and NGF production. NGF was produced by bronchial epithelial cells in vitro upon stimulation with singlet oxygen.

Conclusions: Our findings suggest that neutrophil MPO-derived singlet oxygen induces increased NGF production, leading to AHR and 10- and 12-(Z,E)-HODEs production. These findings may help to develop new therapies targeting this mechanism and to establish a new biomarker for non-type 2 and refractory asthma.
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Introduction

Bronchial asthma is characterized by allergic airway inflammation, airway obstruction, and AHR. These features are related to various inflammatory cells including Th2 lymphocytes, eosinophils and neutrophils, and their cytokines, chemokines and growth factors. Severe asthma is a serious problem because it is refractory to inhaled steroid treatment and has a high mortality rate.

Various factors, such as exacerbation of airway remodeling, atypical glucocorticoid receptor expression,[1] activation of the IL-33-ILC2 axis,[2] and neutrophil infiltration[3,4] are considered to be involved in mechanism of refractory asthma. Neutrophils are elevated in the sputum and bronchial biopsy material of patients with severe asthma,[3,4] and neutrophilic airway inflammation induces persistent airflow limitation in asthma.[5] Several enzymes are released from neutrophils, for instance, by neutrophil extracellular traps (NETs).[6,7] Among these enzymes, neutrophil elastases (NE) and myeloperoxidase (MPO) are important factors for the allergic inflammation. NE mediates eosinophil activation and infiltration,[8] and induces smooth muscle cells proliferation.[9] In addition, we have previously reported that NE enhances airway epithelial permeability, leading to fibroblast growth factor (FGF)2-derived smooth muscle hyperplasia.[10] The IL-8 and MPO levels were higher in severe asthmatic patients,[11] and MPO-DNA complexes cause IL-8 production by macrophage via TLR9/NF-kB axis activation.[12] In addition, neutrophils play an important role in the production of reactive oxygen species (ROS), including superoxide anion radicals, hydroxyl radicals and singlet oxygen, which may cause damage to the airway membrane, leading to the exacerbation of allergic inflammation. We previously proposed the use of totally assessed hydroxyoctadecadienoic acids (HODEs) as lipid-derived oxidative stress markers, which can facilitate the estimation of the oxidative mechanism that occurs.[13] In particular, 10- and 12-(ZE) HODEs are yielded by singlet oxygen, mainly resulting from the MPO-H_2O_2-HOCI reaction in vivo[14] via nonradical and nonenzymatic mechanisms. Thus, neutrophils may play a critical role in refractory asthma via enzymatic reactions and by producing ROS.

Neurotrophins include brain-derived neutrophic factor (BDNF), nerve growth factor (NGF), and neurotrophins-3and -4. Tropomyosin receptor kinases (Trk) including TrkA, TrKB and TrkC, found on nerve fibers, are the major receptors that promote the development and survival of sensory neurons after binding to neurotrophins.[15] NGF levels are increased in the serum[16] and in the broncho-alveolar lavage (BAL) fluid of asthmatic patients.[17] In the airways, neurotrophins act as a survival and activation factors of eosinophils.[18] Furthermore, NGF has been shown to be involved in AHR in animal models.[19,20] Moreover, we have shown that NGF induces substance P-derived nerve fiber hyperinnervation, causing exacerbation of AHR.[21] This process is inhibited by administering anti-NGF Ab,[19] or in vivo siRNA of NGF[21] in a mouse model of asthma. Together, these findings demonstrated that NGF plays an important role in AHR and allergic inflammation.

The induction of hyperalgesia by NGF is dependent on circulating neutrophils[22]; however, the relationship between NGF and neutrophils in respiratory disease remains unclear. Since neutrophils are involved in refractory asthma, we hypothesized that neutrophils may affect NGF production, thus contributing to AHR via NGF- derived hyperinnervation.

This study examines the role of neutrophils in AHR and their mechanism of action by using a mouse model of bronchial asthma with mixed inflammation.

Methods

Antigen preparation

The house dust mite antigen, mite- Dp extract, was purchased from Cosmo Bio (Tokyo, Japan). Endotoxin levels were reduced to <0.02 EU/mg using an Endotoxin Removal solution (Sigma–Aldrich, St. Louis, MO, USA).

Mouse experimental protocols

Six-week-old female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). The mice were maintained in the animal facility of Tokushima University under specific pathogen-free conditions, according to the guidelines and approval of the ethics committee of the university. In addition, the study protocol shown in Supplementary Figure 1 was approved by the ethics committee of Tokushima University. Each experiment was performed using two to five mice per group. We used two different sensitization protocols (Supplementary Fig. 1). The first protocol consisted of mouse intranasal sensitization (Mix-in model), on days 0, 1, 7, and 8 by intranasal administration of 10 μg of Dp dissolved in 80 μL saline. Each group of mice was administered 5, 10, and 25 μg of Dp dissolved in 80 μL of saline, respectively, intranasally three times a week, every other day from day 14 to day 39. Mix-in model caused mixed allergic inflammation, involving both eosinophils and neutrophils. The alternative protocol (Eo-ip model) involved sensitization on days 0 and 7 by intraperitoneal injections of 10 μg of Dp dissolved in 500 μL saline and mixed with 1 mg of alum (Sigma–Aldrich Japan). The Eo-ip model, which is a conventional model of chronic asthma, is characterized by eosinophilic allergic inflammation. The Eo-ip mice were then challenged intranasally with10 μg of Dp in 80 μL saline every other day, three days per week, from day 14–39. All mice were euthanized on day 42 (Supplementary Fig. 1).

Sample collection and analysis of airway resistance and AHR

Before euthanizing the mice, we assessed the respiratory resistance (R_{rs}) in all the groups, using flexiVent (SCIREQ Scientific Respiratory Equipment, Montreal, Canada) as previously reported.[23] R_{rs} values were expressed as percentages of the base line R_{rs}. As an index of AHR, the provocative concentration of methacholine (Mch) that caused a 200% increase in Rrs (PC200) was calculated by linear interpolation of the dose–response curves. After euthanizing the mice, BAL was performed for cell population analysis and to collect BAL fluid.[10] The lungs were harvested for histopathology, and the supernatants were obtained after lung homogenization[10] (details in the Supplementary Methods).

Measurements of cytokine concentrations and MPO activity assay

Levels of NGF in the BAL fluid and those of substance P and interleukins in the whole lung homogenate were determined using commercial ELISA kits. Myeloperoxidase (MPO) activity in the whole lung homogenate was measured using a Myeloperoxidase activity assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Pathological analysis

Lung tissue was fixed in 10% formalin and embedded in paraffin. Sections (3-μm thick) were prepared using a microtome and stained with hematoxylin and eosin. Eosinophils in one sequential section were identified using Luna modified staining (details in the Supplementary Methods). Nerve fiber hyperinnervation was evaluated using immunofluorescence. The numbers of nerve fibers stained by protein gene product 9.5 (PGP 9.5) around a bronchus were counted in 15–20 fields per lung lobe at a magnification of 200×.

Treatment with neutralizing Abs and inhibitors

Anti–IL-17, IgG isotype control (BioLegend, San Diego, CA, USA), and anti-NGF (ABCAM, Cambridge, UK) Abs were administered intraperitoneally (0.5 mg/kg) to Dp-sensitized mice.[24] The myeloperoxidase inhibitor, 4-aminobenzoic acid hydrazide (4-ABAH, 40 mg/kg; Cayman, Ann Arbor, MI, USA)[25] and the neutrophil elastase (NE) inhibitor, sivelestat (50 mg/kg, Ono Pharmaceutical, Osaka, Japan)[8] were also administered intraperitoneally to Dp-sensitized mice. The dose and the schedule of administration are described in Supplementary Figure 1.

Analysis of oxidative stress markers by liquid chromatography tandem mass spectrometry

HODEs levels in the supernatant of lung homogenates were measured using a slightly modified version of a previously reported method.[26,27] HODEs were measured by using liquid chromatography/tandem mass spectrometry (LC-MS/MS) on a TSQ Quantum Access Max system (Thermo Fisher Scientific, Waltham, MA, USA) after reduction hydrolysis of the reaction mixture (details in the Supplementary Methods).

Cell analysis and in vitro experiments

The human bronchial epithelial cell line, BEAS-2B, transformed with Simian virus 40, was provided by ATCC (Manassas, VA, USA). Primary cultured normal human bronchial epithelial cells (NHBE) cells were purchased from Lonza (Allendale, NJ, USA). Cells were plated in 24-well tissue culture plates with serum free RPMI-1640 or BEGM (Lonza)/RPMI-1640 medium. Endoperoxide (EP: 3-(1, 4-epidioxy-4-methyl-1, 4-dihydro-1-naphthyl) propionic acid; Wakenbtech, Shiga, Japan) which stably generates singlet oxygen above 35 °C,[28] was applied to the culture medium and cells were incubated at 37 °C under 5% CO_2 in air for 48 h. NE (Millipore, Billerica, MA, USA) and recombinant IL-17 (Thermo Fisher Scientific, Waltham, MA, USA) were also applied at similar conditions. Supernatants were collected and the NGF levels were measured.

Statistical analysis

Experimental results are presented as means ± SE. The experimental groups were compared using a one-way analysis of variance (ANOVA). If statistical significance was identified by ANOVA, a Tukey's post-hoc test was used to correct for multiple comparisons.

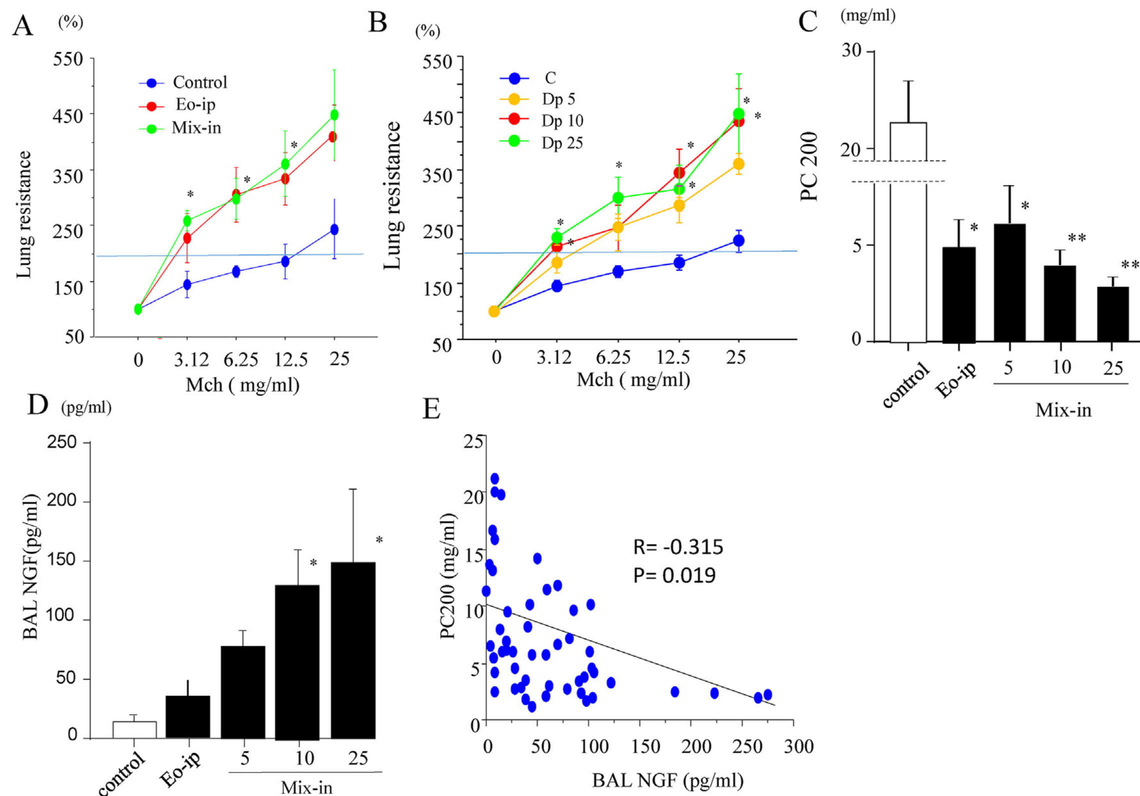

Fig. 1. Airway hyperresponsiveness and NGF production in MIX-in and Eo-ip mice. **A)** Respiratory resistance in Eo-ip and 10 μg of Dp challenged Mix-in mice. **B)** Respiratory resistance in 5–25 μg of Dp challenged Mix-in mice **C)** PC200 values after Dp challenge. Respiratory resistance was measured using flexiVent. **D)** NGF levels in BAL fluid as measured using ELISA. Results are expressed as the means ± SEs from three different experiments. Two to five mice per group were used in each experiment (n = 8–11). *P < 0.05, compared to control. **P < 0.01, compared to control. **E)** Correlation between NGF levels and PC200. n = 45. BAL fluid, bronchoalveolar lavage fluid; NGF, nerve growth factor; PC200, provocate concentration that caused a 200% increase in R_{rs}.

For airway resistance measurements, a two-way repeated measures ANOVA was used, followed by a Tukey–Kramer test. Statistical analyses were performed using the GraphPad PRISM software (5.01; GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $P < 0.05$.

Results

NGF levels and AHR are positively correlated in the Mix-in model

First, we evaluated and compared the respiratory resistance among Mix-in, Eo-ip, and control mice. As shown in Figure 1A, the respiratory resistance values were higher in Mix-in and Eo-ip mice challenged with 10 μg of Dp than in control mice ($F [2, 15] = 5.84$; $P = 0.013$), but there was no difference between Mix-in and Eo-ip mice ($F [8, 60] = 1.69$; $P = 0.12$, Fig. 1A). As shown in Figure 1B, there were significant differences among the curves for all groups of Mix-in mice ($F (3, 36) = 6.50$; $P = 0.001$). ANOVA also showed an interaction between the groups of Mix-in mice and the Mch dose ($F [15, 180] = 3.66$; $P < 0.001$). Percentage of R_{rs} values for groups challenged with 10 μg and 25 μg of Dp significantly increased at a dose of 3.12 mg/ml Mch or higher (Fig. 1B). Similarly, PC200 values decreased in a dose dependent manner in case of Dp challenge in Mix-in mice (Fig. 1C). PC200 values for Mix-in mice challenged with 10 μg of Dp were lower than that for Eo-ip mice challenged similarly, although there was no statistical significance (Fig. 1C).

Next, we examined the correlation between BAL fluid NGF levels and AHR in Mix-in mice. NGF levels increased in response to Dp challenge in a dose-dependent manner in Mix-in mice (Fig. 1D), and the degree of NGF production was higher in Mix-in mice than in Eo-ip mice when both were similarly challenged by 10 μg of Dp (Fig. 1D). Moreover, NGF levels were significantly negatively correlated with PC200 values (Fig. 1E). These results suggested that NGF contributes to AHR exacerbation in Mix-in mice, more strongly than in Eo-ip mice.

AHR is exacerbated in the Mix-in model through nerve fiver hyperinnervation

To examine the effect of NGF, nerve fiber hyperinnervation was examined in mix-in mice by immunohistochemical analysis of the nerve fiber marker PGP 9.5. In addition, since substance P production by nerve fibers is involved in bronchocontractile activity,[29] substance P levels in the lung were also examined. As shown in Figure 2, nerve fiber hyperinnervation was observed around the bronchus in Eo-ip mice and Mix-in mice (Fig. 2A). Substance P expression was also observed in hyperinnervated nerve fibers in Eo-ip and Mix-in mice challenged with 10 μg and 25 μg of Dp. Statistical analysis showed that the number of nerve fibers around the bronchus was significantly increased in Mix-in mice challenged with 10 μg and 25 μg of Dp compared to the control (Fig. 2B). In addition, substance P levels in Mix-in mice challenged with 10 μg and 25 μg of Dp were significantly higher than those in

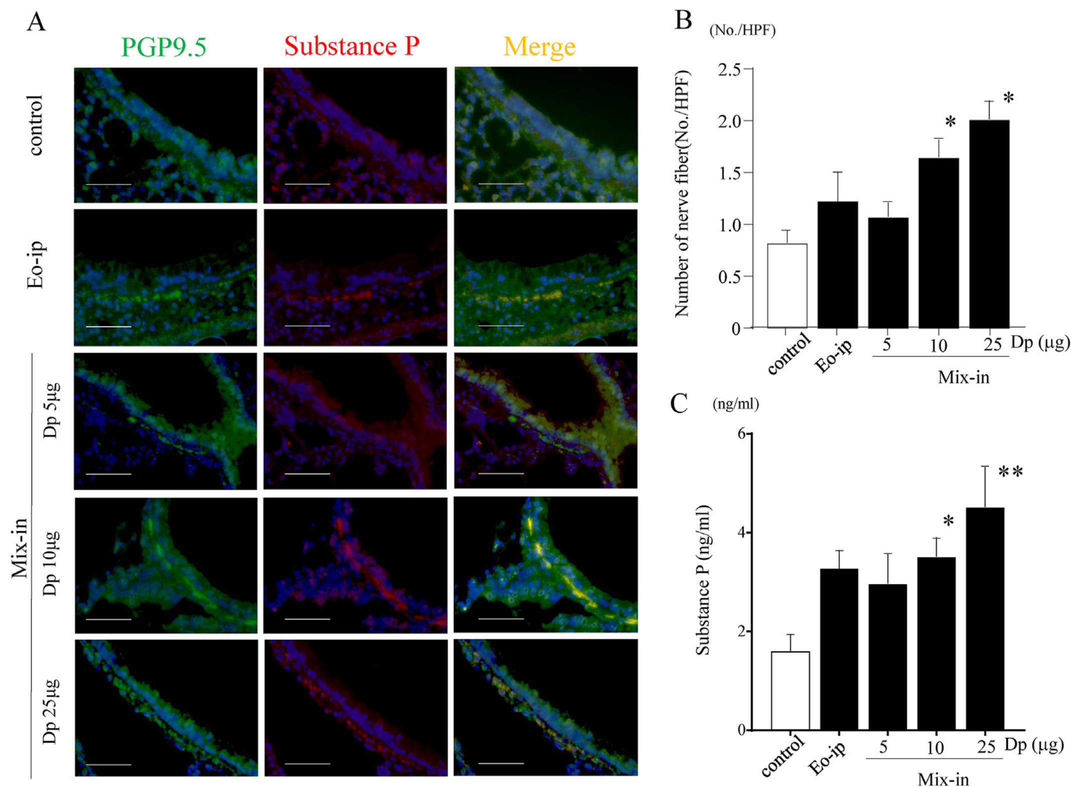

Fig. 2. Nerve hyperinnervation in Mix-in and Eo-ip mice. **A)** Immunofluorescence of nerve fibers. Photomicrographs are representative of three different experiments. Green, PGP9.5; red, SP; yellow, merge of PGP9.5 and SP. Original magnification: 400×. Scale bar = 100 μm. **B)** Number of PGP9.5-positive nerve fibers per field (magnification: 200×). **C)** SP levels in whole-lung homogenates. Results are presented as the means ± SE from three different experiments. Two to five mice per group were used in each experiment (n = 8–12). *$P < 0.05$, compared to control. **$P < 0.01$, compared to control. HPF, high power field; PGP9.5, protein gene product 9.5; SP, substance P.

the control (Fig. 2C). The number of nerve fibers and the levels of substance P were higher in Mix-in mice than in Eo-ip mice, which was not different from that of the control (Fig. 2B, C). These findings suggested that, in the Mix-in model, nerve fiber hyperinnervation and substance P production may be involved in the exacerbation of AHR, as described previously,[21] and that the contribution of these factors to AHR is stronger than that in the Eo-ip model.

NGF mediates AHR and nerve fiber hyperinnervation in Mix-in model

To confirm these findings, we neutralized NGF in Mix-in mice using an anti-NGF Ab. The anti-NGF Ab (0.5 mg/kg) did not increase the NGF production, which was elevated in the BAL fluid of Mix-in mice after challenge with 10 μg of Dp (Fig. 3A). Challenge with 10 μg of Dp significantly increased the R_{rs} and AHR (PC200), but the neutralizing Ab restored these (Fig. 3B, C). In addition, the anti-NGF Ab significantly reduced the number of nerve fibers around the bronchus (Fig. 3D) and the levels of substance P in Mix-in mice (Fig. 3E). On the other hand, anti-NGF Ab treatment did not affect the airway inflammation including eosinophils and neutrophils infiltration around the bronchus (Fig. 3F). In BAL cells, results seemed to be similar as that of the pathological analysis (Supplementary Fig. 1B, C). Thus, these results confirmed that the exacerbation of AHR in Mix-in mice is due to NGF production-derived nerve hyperinnervation in the lungs.

Infiltration of neutrophils and IL-17 and keratinocyte derived chemokine (KC) production were observed in Mix-in mice

We previously reported that the Mix-in mice were characterized by allergic inflammation with neutrophils infiltration and IL-17 production.[10] We examined the observations from pathological analysis and cytokine profiles in whole lungs of Mix-in mice. As shown in Supplementary Figure 2, we confirmed that apart from eosinophils, infiltration of neutrophils also increased in a dose-dependent manner in the BAL fluid of Mix-in mice, and that these were higher than in the Eo-ip mice. IL-17 concentration in whole lungs was similarly observed (Supplementary Fig. 3C). KC, IL-8 homolog which is a strong chemoattractant for neutrophils in mice, was also found to have increased in a dose dependent manner in response to Dp challenge in Mix-in mice (Supplementary Fig. 3D). The trend in KC production was similar to that of neutrophil infiltration in the BAL fluid (Supplementary Fig. 2C, 3D).

IL-17 neutralization is limited in NGF-derived AHR

Previously, it has been reported that IL-17 is important for neutrophil infiltration through either IL-8 or KC production in the lungs.[30,31] We hypothesized that IL-17 may be an important cytokine for Mix-in mice and may stimulate the bronchial epithelial cells to produce NGF, based on the result presented in Supplementary Figure 2 and 3. Thus, we examined the effect of

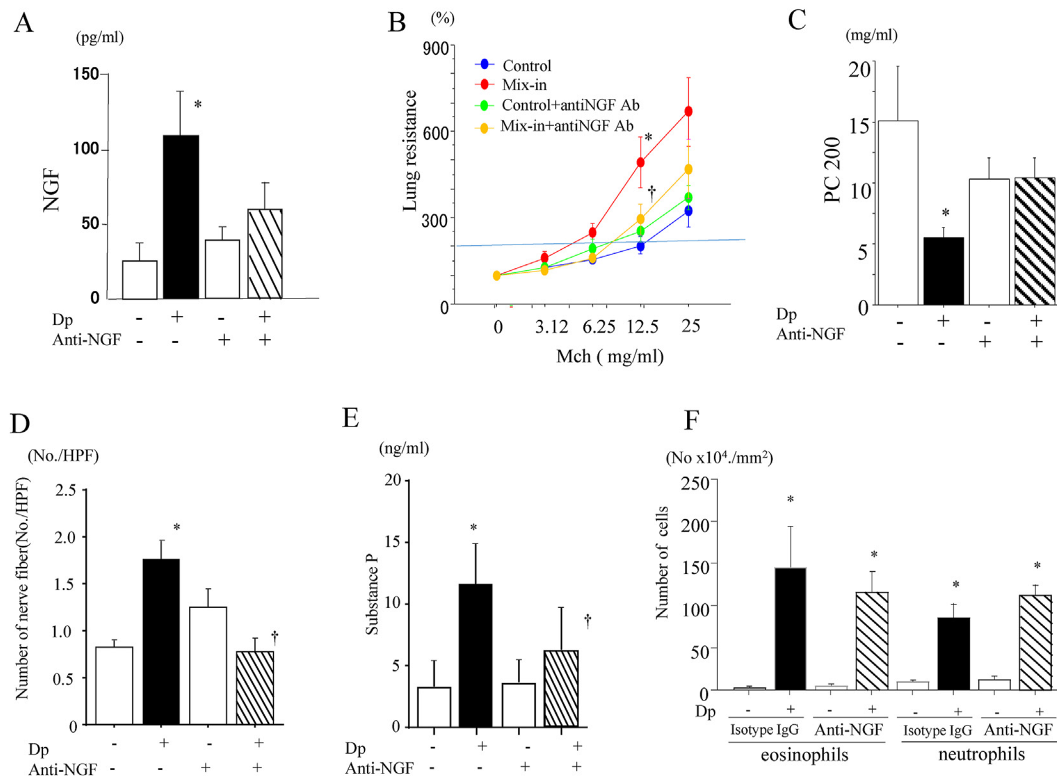

Fig. 3. Effect of NGF neutralization in Mix-in mice. NGF neutralizing Ab was administered intraperitoneally (0.5 mg/kg). **A)** NGF production in the BAL fluid. **B)** Respiratory resistance. **C)** PC200 values after Dp challenge. **D)** Number of PGP9.5- positive nerve fibers per field (magnification: 200×). **E)** SP levels in whole-lung homogenates. **F)** Eosinophil and neutrophil infiltration in the lung histological observation. Results are presented as the means ± SEs from three different experiments. Two to five mice per group were used in each experiment (n = 6–10). White bars, control; black bars, untreated Mix-in mice; shaded bars, Mix-n + NGF Ab. *P < 0.05, compared to control. †P < 0.05, compared to untreated Mix-in mice. BAL fluid, bronchoalveolar lavage fluid; HPF, high power field; NGF, nerve growth factor; PC200, provocate concentration that caused a 200% increase in R_{rs}; PGP9.5, protein gene product 9.5; SP, substance P.

IL-17 on NGF production using IL-17 antibody treatment in Mix-in mice. As shown in Supplementary Figure 4, the effect of IL-17 neutralization by anti-IL-17 Ab was limited. Parameters except number of nerve fibers in the Mix-in with anti-IL-17Ab treatment were lower than that in untreated Mix-in mice, but there was no statistical significance (Supplementary Fig. 4). To confirm the effect of IL-17 stimulation, we performed an in vitro experiment using bronchial epithelial cells. The NGF levels were slightly increased by IL-17 treatment both in BEAS2B cells and NHBE cells; however, the increases in NGF production by IL-17 treatment was very low and there was no dose dependency observed (Supplementary Fig. 5A, B).

MPO inhibition decreases NGF production and may be involved in NGF-derived AHR

Based on the result described above, we examined the effect of neutrophil elastase (NE) on in vivo and in vitro NGF production. Although NE was shown to be one of the key enzymes that exacerbates bronchial asthma in Mix-in model of bronchial asthma,[10] its inhibition by sivelestat did not affect the NGF production in the whole lungs of Mix-in mice. Similarly, in an in vitro experiment performed using BEAS-2B cells (bronchial epithelial cell line), NGF production was not increased by NE stimulation (Supplementary Fig. 5C, D). Therefore, we focused upon and investigated the effect of MPO inhibition on NGF production in Mix-in mice. The MPO inhibitor, 4-ABAH, significantly decreased R_{rs} and AHR (PC 200), as compared against untreated Mix-in mice (Fig. 4A, B), although AHR by 4-ABAH was not suppressed in the Eo-ip model (Supplementary Fig. 6A, B). Furthermore, 4-ABAH significantly reduced nerve fiber hyperinnervation, NGF levels, and substance P production in the Mix-in mice compared to the untreated Mix-in mice (Fig. 4C–E). On the other hand, in the airway inflammation, eosinophils and neutrophils infiltration was not inhibited by 4-ABAH treatment (Fig. 4F and Supplementary Fig. 6C, D). These findings suggested that MPO may be directly involved in NGF-derived AHR exacerbation.

The levels of MPO-derived singlet oxygen-induced peroxidation products and NGF are positively correlated

We then focused on MPO-derived ROS released by neutrophils, because MPO-derived oxidative stress induced inflammation in chronic respiratory disease. As shown in Figure 5A, MPO activity in Mix-in mice was increased in a Dp dose- dependent manner, and was significantly decreased by 4-ABAH treatment, an MPO inhibitor (Fig. 5A). We then examined oxidative stress markers derived from linoleates, focusing on MPO-specific reactions. As shown in Table 1 and Figure 5B, MPO activity was correlated with the levels of many HODE isomers. Among these, 10- and 12-(Z,E)- HODEs, which are the specific markers of singlet oxygen, were significantly correlated with BAL fluid NGF levels (Table 1 and Fig. 5C). These findings suggested that singlet oxygen is a key MPO-derived ROS involved in the production of NGF. Furthermore, 10- and 12-(Z,E)- HODEs were increased in Mix-in mice in Dp dose dependent manner, and significantly decreased by 4-ABAH, MPO inhibitor treatment (Fig. 5D).

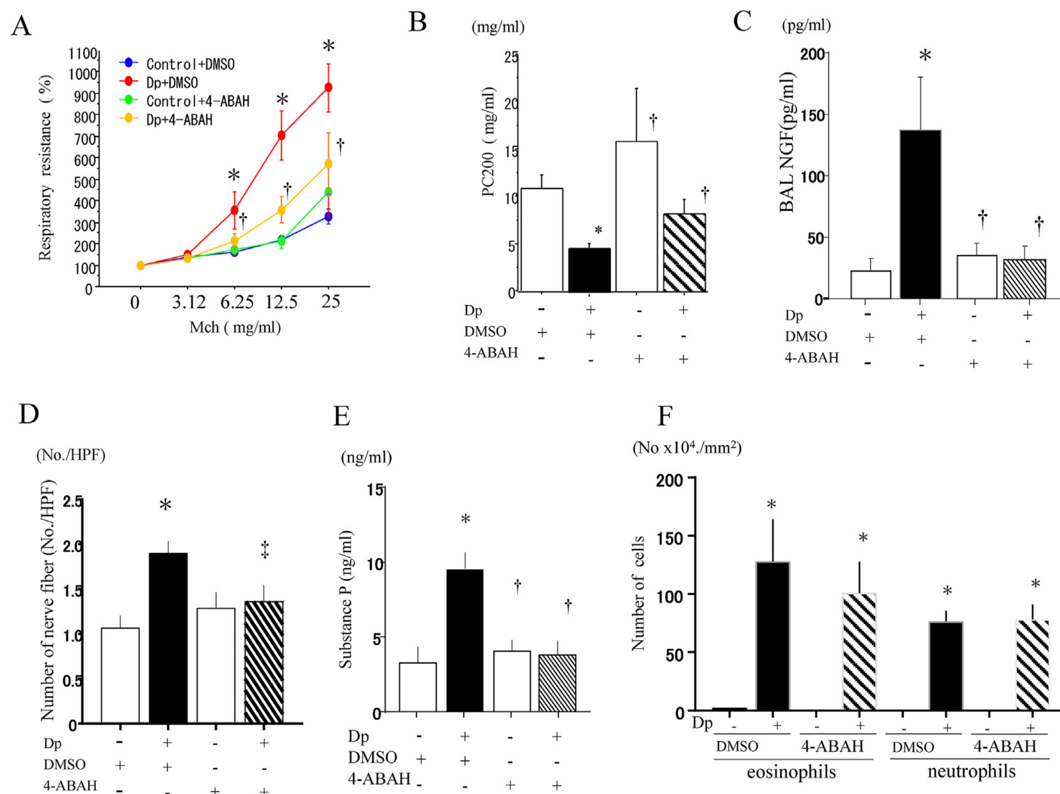

Fig. 4. Effect of IL-17 neutralization and MPO inhibition in Mix-in mice. The MPO inhibitor, 4-ABAH (40 mg/kg) were administered intraperitoneally. **A)** Respiratory resistance. **B)** PC200 values after Dp challenge. **C)** NGF levels in BAL fluid. **D)** Number of PGP9.5-positive nerve fibers per field (magnification: 200×). **E)** SP levels in whole-lung homogenates. **F)** Eosinophil and neutrophil infiltration in the histology of lungs. Data represents the means ± SE from three different experiments. Three to five mice per group were used in each experiment (n = 9–15). White bars, control; black bar, untreated Mix-in; shaded bars, Mix-in + 4-ABAH. *$P < 0.05$ compared to control. †$P < 0.05$ compared to untreated Mix-in mice. 4-ABAH, 4-aminobenzonic acid hydrazide; BAL fluid, bronchoalveolar lavage fluid; HPF, high power field; MPO, myeloperoxidase; NGF, nerve growth factor; PC200, provocate concentration that caused a 200% increase in R_{rs}; PGP 9.5, protein gene product 9.5; SP, substance P.

Singlet oxygen generation increases NGF production by bronchial epithelial cells

To confirm the results mentioned above, we examined the singlet oxygen-derived NGF production by bronchial epithelial cells using endoperoxide. Since endoperoxide produces singlet oxygen at 37 °C, we added it to the cultured medium of bronchial cells. Stimulation with 0.5 and 1 mM of endoperoxide significantly increased NGF production by BEAS-2B cells (Fig. 6A). Stimulation with endoperoxide of a concentration above 1 mM resulted in decreased NGF production due to cell death (data not shown). Application of 1 mM of endoperoxide caused an increase in NGF production in a time-dependent manner (Fig. 6B); 0.5 mM of endoperoxide also increased NGF production in a time-dependent manner, but the magnitude was low (Fig. 6B). Similarly, stimulation with 0.2 and 0.5 mM of endoperoxide significantly increased NGF production by NHBE cells (Fig. 6D) and increased them in a time-dependent manner (Fig. 6E). These findings suggested that singlet oxygen directly induced NGF production by bronchial epithelial cells.

Singlet oxygen generation increases IL-8 production by bronchial epithelial cells

We also examined the IL-8 production by bronchial epithelial cells using endoperoxide which generated singlet oxygen. In both BEAS2B cells and primary culture ofNHBE cells, IL-8 production was increased in proportion to the dose of endoperoxide (Fig. 6C, F). It suggests that singlet oxygen stimulation directly led to production of IL-8 by bronchial epithelial cells.

Discussion

AHR is thought to be caused by allergic inflammation associated with eosinophilia and Th2 responses. Previously, we reported that increased production of NGF causes AHR by inducing substance P-derived nerve fiber hyperinnervation in an animal model.[21] In present findings, we demonstrated that singlet oxygen, which is produced by MPO, is released from neutrophils and regulates the production of NGF by bronchial epithelial cells, leading to the exacerbation of AHR in bronchial asthma in mixed inflammation mouse models. Given that neutrophils are steroid resistant, this is the first report that a part of asthma refractoriness is associated with neutrophils. In addition, our study revealed that IL-17 derived neutrophils and singlet oxygen may be potential therapeutic targets in severe asthma. Present findings also suggested that HODEs may be prominent biomarkers for non-type 2 asthma and refractory asthma.

Determining the source of the increased NGF production was of crucial importance. We found that although NE is a key molecule in smooth muscle actin hyperplasia,[10] it did not contribute to NGF production in this model (Supplementary Fig. 5A, B). Further, although IL-17 appeared to be involved, its effect was rather limited (Supplementary Fig. 5C, D). Therefore, we focused on MPO

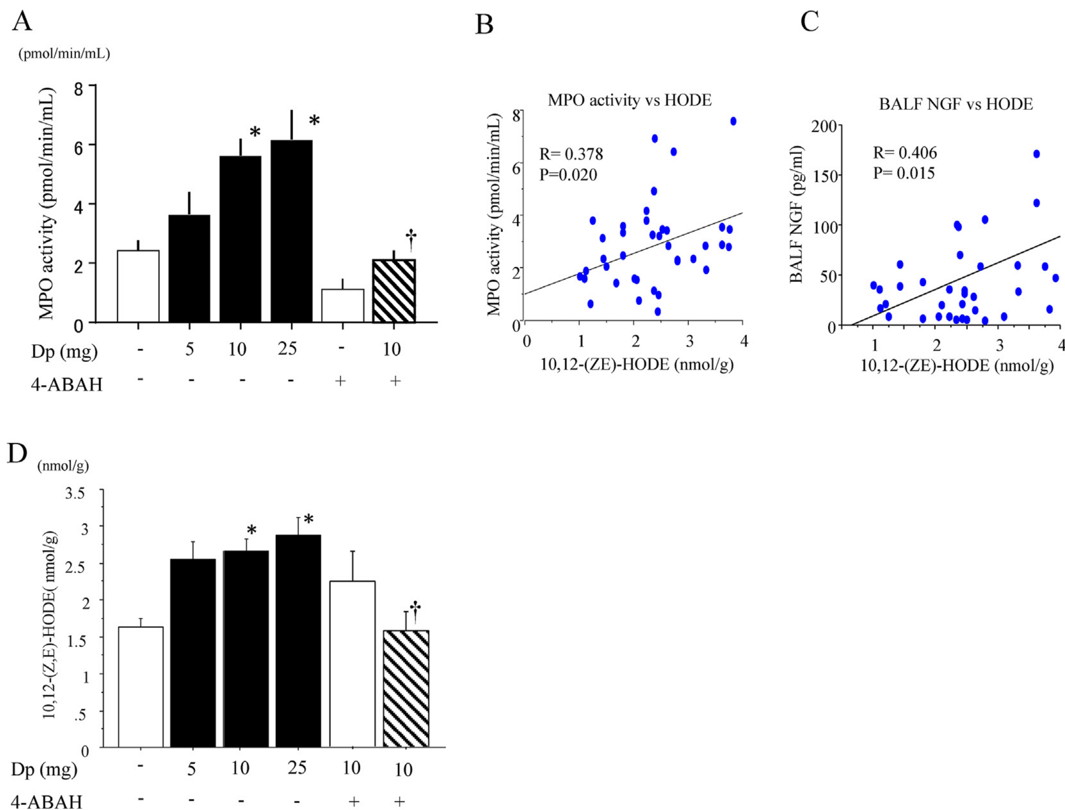

Fig. 5. MPO activity and 10- and 12- (Z,E) HODEs production in Mix-in mice. MPO activity in whole-lungs homogenates was measured using MPO activity assay kit. Linoleate-derived 10- and 12-(Z,E) HODEs produced by singlet oxygen were measured by LC-MS/MS. **A)** MPO activity. **B)** Correlation between MPO activity and 10- and 12- (Z,E) HODE levels (n = 38). **C)** Correlation between NGF levels in BAL fluid and 10- and 12- (Z,E) HODE levels (n = 38). **D)** 10- and 12- (Z,E) HODEs production in whole lungs. Results are presented as the means ± SE from 3 different experiments. Two to five mice per group were used in each experiment (n = 5–11). White bars, control; black bar: untreated Mix-in (5–25 µg of Dp challenged); shaded bars, Mix-in + 4-ABAH. *P < 0.05 compared to control. †P < 0.05 compared to untreated and 10 µg of Dp challenged Mix-in. 4-ABAH, 4-aminobenzonic acid hydrazide; 10-and 12-(Z,E)-HODEs, 10- and 12-(Z,E) hydroxyoctadecadienoic acid; BAL fluid, bronchoalveolar lavage fluid; LC-MS/MS, liquid chromatography/tandem mass spectrometry; MPO, myeloperoxidase; NGF, nerve growth factor.

Table 1
Correlation between HODEs in the lung and neutrophils, MPO activity and BALF NGF.

Stress marker	neutrophil		MPO		NGF	
	r	p	r	p	r	p
9ZE-HODE	0.104	0.532	0.173	0.308	0.156	0.375
9EE-HODE	−0.335	0.036*	−0.222	0.187	0.030	0.863
13ZE-HODE	0.128	0.442	0.465	0.003*	0.180	0.304
13EE-HODE	−0.254	0.120	−0.050	0.763	0.111	0.530
10ZE-HODE	0.443	0.049*	0.604	<0.001*	0.352	0.044*
12ZE-HODE	0.512	<0.001*	0.558	<0.001*	0.325	0.065
10- and 12-(ZE)-HODE	0.099	0.552	0.378	0.020*	0.406	0.015*

We examined the seven kinds of HODEs produced by the oxidation of linoleic acid in the lungs. HODEs in supernatant of whole lung homogenates were analyzed by using liquid chromatography-tandem mass spectrometry. The data are representative of three independent experiments. *P < 0.05.
HODEs, hydroxyoctadecadienoic acids; r, Correlation coefficient.

and singlet oxygen. Singlet oxygen is mainly produced by the MPO-H_2O_2-HOCl reaction in neutrophils,[14] and it influences intracellular signaling systems by attacking phospholipids in biological membranes, including linoleic acid, arachidonic acid, and cholesterol. Umeno *et al.* reported that 10- and 12-(Z,E)-HODEs, which are produced from linoleates by singlet oxygen, are detected in human plasma after reduction and saponification of the samples.[27,32] Since the mouse model of bronchial asthma with mixed inflammation (Mix-in model) presented a neutrophilic response, we hypothesized that MPO could be involved in the pathogenesis of asthma, and that singlet oxygen might contribute to NGF production in this model. Strikingly, the levels of 10- and 12- (Z,E)-HODEs were positively correlated with MPO activity and BAL fluid NGF levels. The singlet oxygen producer, endoperoxide, increased NGF production by bronchial epithelial cells in vitro. Furthermore, the MPO inhibitor, 4-ABAH, inhibited 10- and 12- (Z,E)-HODE production, NGF-induced AHR, and related parameters such as nerve hyperinnervation and substance P production in the Mix-in model, although it did not affect the airway inflammation including eosinophil and neutrophils infiltration. On the other hand, the HODE isomers 9- and 13-(Z,E) and (E,E)-HODEs, which are formed by radical and enzymatic oxidation, did not show a significant correlation with MPO activity and BAL fluid NGF levels. These results suggested that singlet oxygen is involved in NGF-related AHR in the Mix-in model.

Our findings clarify the mechanism whereby mixed allergic inflammation leads to NGF-related AHR. In mixed allergic inflammation, IL-17/IL-8-derived neutrophil infiltration leads to MPO release around the bronchus, which is supported by findings that NE-and MPO-containing NETs may be related to asthma severity.[6,7] MPO-derived singlet oxygen may injure the membrane of bronchial epithelial cells, leading to the production of NGF. Then, bronchial epithelium- derived NGF induces nerve hyperinnervation, causing the exacerbation of AHR via substance P secretion.[21] To our knowledge, this is the first study reporting NGF-derived AHR in bronchial asthma with mixed inflammation.

Interestingly, Umeno *et al.* showed that levels of 10- and 12-(Z,E)-HODEs correlated with HbA1c levels, glucose level during oral glucose tolerance test, insulin secretion, and resistance index in pre diabetic patients.[27] Further, Murotomi *et al.* found that their levels

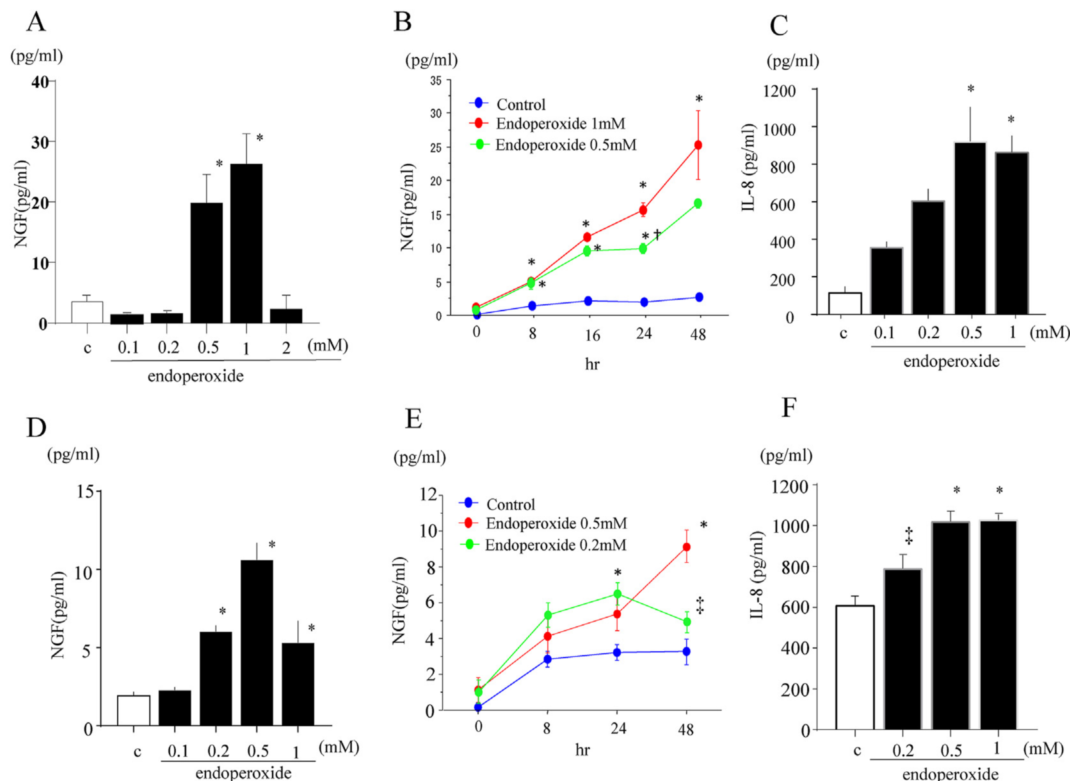

Fig. 6. Effect of singlet oxygen on NGF and IL-8 production by bronchial epithelial cells. NGF and IL-8 production by bronchial epithelial cells in response to different concentrations of endoperoxide, which produces singlet oxygen at 37 °C. **A)** NGF production in a dose dependent manner by BEAS-2B cells. **B)** Time course of NGF production by BEAS-2B cells. **C)** IL-8 production by BEAS-2B cells. **D)** NGF production in a dose dependent manner by Primary cultured normal human bronchial epithelial (NHBE) cells. **E)** Time course of NGF production by NHBE cells. **F)** IL-8 production by NHBE cells. The results are representative of three different experiments and are expressed as the means ± SE from the data obtained from four wells for each group. *P < 0.05 compared to control. †P < 0.05 compared to BEAS-2B cells stimulated by 1 mM of endoperoxide. ‡P < 0.05 compared to NHBE cells stimulated by 0.5 mM of endoperoxide. NGF, nerve growth factor.

were increased in mice prior to the development of diabetes.[33] These reports suggest that 10- and 12-(Z,E)-HODEs may be produced as result of the MPO reaction triggered by hyperglycemia-derived neutrophil recruitment to adipose tissue. In current clinical practice, exhaled nitric oxides and eosinophils and IgE levels are measured as markers of eosinophilic inflammation in type 2 asthma. In addition, Th2-derived periostin may be a marker of type 2 inflammation.[34] Although, various molecules have been explored as biomarkers for non-type 2 asthma, including YKL-40[35] and hydrogen sulfide,[36] these are still not used in clinical applications. Yoshida *et al.* have established a technique to measure linoleic acid-derived lipid oxidants, and proposed 10- and 12- (Z,E)-HODEs as the prominent markers of singlet oxygen.[13] Although further investigation is needed, our results indicate that 10-and 12-(Z,E)-HODE levels constitute a promising biomarker for determining the severities of neutrophilic inflammation AHR in non-type 2 asthma and severe asthma.

Importantly, 10-HODE and 12-HODE isomers selectively activate the nuclear factor-erythroid 2-related factor-2 (Nrf2) transcription factor in human epidermal cell lines and induce the expression of a group of antioxidant and detoxification enzymes including heme oxygenase-1.[37] In Nrf2-deficient asthmatic mice, Nrf2 disruption exacerbates allergic airway inflammation and asthma.[38] The Nrf2-mediated oxidative stress response is critical for maintaining the epithelial barrier integrity upon steroid treatment.[39] Furthermore, Umeno *et al.* reported that 10- and 12-(Z,E)-HODEs show agonist activity against the transcription factor Peroxisome Proliferator-Activated Receptor γ (PPARγ).[40] PPARγ agonists reduce eosinophil survival and chemotaxis,[41] and improve airway allergic inflammation and lung function.[42] Thus, both Nrf2 activity and PPARγ agonist activity improve the pathology of bronchial asthma. The increase in 10- and 12-(Z,E)-HODEs observed in this study may partially be attributed to this kind of adaptive response, although further investigations are needed to confirm this.

In conclusion, we demonstrated that neutrophil-MPO-derived singlet oxygen induces increased NGF production, leading to AHR. Further studies are needed to develop new therapies targeting this axis and to clinically employ 10- and 12- (Z,E)-HODEs levels as new biomarkers for non-type 2 asthma and refractory asthma.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2022.02.005.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

HO, MA, and KT contributed to study conception, and design, data interpretation, and writing of the manuscript. HO and MS performed mouse experiments, histopathological analysis and ELISA. AU, KM, and YY conducted LC-MS/MS experiments, performed the acquisition, analysis, and interpretation of LC-MS/MS data, and contributed to the drafting of the manuscript. YN participated in the experimental design and helped to draft the manuscript. All authors have read and approved the final manuscript.

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