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The Nitrated Fatty Acid 10-Nitro-Oleate Attenuates Allergic Airway Disease

Aravind T. Reddy, Sowmya P. Lakshmi, Sireesh Dornadula, Sudheer Pinni, Dileep R. Rampa, and Raju C. Reddy

Asthma is a serious, growing problem worldwide. Inhaled steroids, the current standard therapy, are not always effective in this chronic inflammatory disease and can cause adverse effects. We tested the hypothesis that nitrated fatty acids (NFAs) may provide an effective alternative treatment. NFAs are endogenously produced by nonenzymatic reaction of NO with unsaturated fatty acids and exert anti-inflammatory actions both by activating the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR)γ and via PPAR-independent mechanisms, but whether they might ameliorate allergic airway disease was previously untested. We found that pulmonary delivery of the NFA 10-nitro-oleic acid (OA-NO2) reduced the severity of murine allergic airway disease, as assessed by various pathological and molecular markers. Fluticasone, an inhaled steroid commonly used to treat asthma, produced similar effects on most end points, but only OA-NO2 induced robust apoptosis of neutrophils and their phagocytosis by alveolar macrophages. This suggests that OA-NO2 may be particularly effective in neutrophil-rich, steroid-resistant severe asthma. In primary human bronchial epithelial cells, OA-NO2 blocked phosphorylation and degradation of IκB and enhanced inhibitory binding of PPARγ to NF-κB. Our results indicate that the NFA OA-NO2 is efficacious in preclinical models of allergic airway disease and may have potential for treating asthma patients. The Journal of Immunology, 2013, 191: 000–000.
10,000 U/ml penicillin, and 10,000 µg/ml streptomycin (HyClone, Logan, UT), at 37°C in a humidified atmosphere of 5% CO₂–95% air in T-75 tissue culture flasks, plates, or dishes coated with 2% gelatin. Monolayer cultures at 90% confluence were deprived of serum for 24 h prior to treatment.

**OVA sensitization and challenge, OA-NO₂ administration, and specimen collection**

Allergen-induced asthma was produced in mice through initial sensitization by i.p. injection of 20 µg OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 0.2 ml sterile PBS containing 2 mg aluminum hydroxide (Sigma-Aldrich) on days 0 and 7; mice were challenged by intratracheal (i.t.) injection of 0.1% OVA in 50 µl sterile PBS on even-numbered days 14–22. Thirty minutes after each OVA challenge, mice were injected i.t. with 25 µg OA-NO₂ (Cayman Chemical, Ann Arbor, MI) or fluticasone propionate (Sigma-Aldrich) in 50 µl 10% DMSO or with vehicle. Twenty-four hours following the last challenge and drug delivery, airway hyperresponsiveness to methacholine challenge was measured, the mice were euthanized, bronchoalveolar lavage fluid (BALF) was collected, and the lungs were excised for histopathological examination and measurement of markers of inflammation and oxidative stress. Serum was obtained at the same time.

**Assessment of airway responsiveness**

Airway responsiveness to inhaled methacholine (Sigma-Aldrich) was determined in mice 24 h after the final challenge and drug delivery, using a computer-controlled small-animal ventilator (flexiVent; Scireq, Montreal, Quebec, Canada), as previously described (18). Both the single-compartment model (using the snap-shot method) and the constant-phase model (using the forced oscillation technique method) of respiratory mechanics were applied to assess responses to methacholine. The single-compartment model was used to measure total respiratory system resistance (R) and elastance (E), whereas the constant-phase model was used to measure tissue elastance (H) and tissue damping (G). All data points were determined with flexiVent software (version 5.0) using multiple-linear regression to fit each data point to the single-compartment or the constant-phase model, as appropriate. Following acquisition of baseline data, airway responsiveness to aerosolized PBS and methacholine (6.25, 12.5, 25, and 50 mg/ml saline; delivered by ultrasonic nebulizer) was assessed. Peak responses during each 5-min period were determined, and only values with a coefficient of determination ≥ 0.95 were used.

**BALF collection and cell count**

BALF was collected by flushing three times with 1 ml PBS containing 0.1 mM EDTA into the lung via a tracheal cannula. The pooled BALF was centrifuged at 500 × g at 4°C for 5 min. Pelleted cells were then resuspended in 1 ml PBS. Total cell number was counted using a hemocytometer, and a differential cell count was performed using cytopsin preparations stained with Diff-Quik (Siemens, Newark, DE).

**BALF protein**

Total protein concentration in the supernatant following BALF centrifugation was determined using the BCA Protein Assay kit (Pierce, Rockford, IL).

**Measurement of serum and BALF IgE levels**

Serum and BALF levels of allergen-induced IgE were measured using an ELISA kit (MD Bioproducts, St. Paul, MN), according to the manufacturer’s instructions.

**Measurement of lung and BALF cytokine levels**

Frozen lung tissues were thawed, weighed, homogenized, and sonicated on ice in radioimmunoprecipitation assay buffer supplemented with Halt protease inhibitor mixture (Pierce). Extracts were incubated for 20 min at 4°C, followed by centrifugation at 14,000 × g for 15 min. The supernatants were collected and stored at −80°C. Levels of TNF-α, macrophage inflammatory protein (MIP)-2, IL-5, IL-4, and eotaxin in lung homogenates and BALF were measured using ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

**Measurement of oxidant stress**

H₂O₂ production in lung homogenates and BALF was determined using the Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes, Eugene, OR), and the concentrations of nitrate and malondialdehyde were measured using colorimetric assay kits (Cayman Chemical).

**Transcription factor DNA-binding activity assay**

Nuclear proteins were extracted using a nuclear extraction kit (Active Motif, Carlsbad, CA), and total protein concentration was determined using the BCA Protein Assay kit (Pierce). Nuclear extracts were used to quantify DNA-binding activity of PPARγ and the p65 subunit of NF-κB using ELISA-based TransAM kits (40696 and 40096; Active Motif).

**Western blotting**

Total protein extracts were prepared, and Western blotting was performed as described previously (19). Primary Abs against NF-κB p65, PPARγ, lamin B1, VCAM-1, ICAM-1, E-selectin, endothelial NO synthase, inducible NO synthase, NADPH oxidase 4, CD36, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Abs against p-IκBα and IκBα were from Cell Signaling Technology (Beverly, MA). The secondary Abs were donkey anti-mouse IR-680 (red) and goat anti-rabbit IR-780 (green; both from LI-COR, Lincoln, NE). The infrared signal was detected using an Odyssey Infrared Imaging (LI-COR).

**Lung histopathology and immunostaining**

Lungs were inflated and fixed with 10% neutral formalin overnight at room temperature. Lung tissue was dehydrated with increasing ethanol (EtOH) concentrations and then embedded in paraffin. Five-micrometer-thick paraffin sections were stained with H&E, periodic acid-Schiff (PAS), Masson’s trichrome, or picrosirius red. A five-point scoring system was used, as described previously, to determine peribronchial cell counts and extent of inflammatory cell infiltration, extent of mucus-secreting goblet cells, and subepithelial fibrosis (20). The analyses were performed in a blind fashion, and the slides were presented in a random order for each examination. Immunostaining was performed as previously described (21).

**Immunofluorescence staining and confocal imaging**

Immunofluorescence staining of cultured cells, followed by confocal imaging, was carried out as previously described (21).

**RNA isolation and quantitative real-time RT-PCR**

RNA was isolated from different groups of mouse lungs using an RNeasy Mini kit (QIAGEN, Valencia, CA), and CDNA was generated from 100 ng total RNA using MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) and random and oligo-dT primers. Real-time quantitative PCR was performed using 100 ng cDNA with 2X SYBR Green Master Mix (Applied Biosystems) and specific primers for the genes of interest (Supplemental Table I). These experiments were performed on an AB 7500 fast thermal cycler using a three-step protocol and the melting curve method. The average of each gene cycle threshold (Cₜ) was determined for each experiment. Relative cDNA levels (2⁻ADΔCₜ) for the genes of interest were determined using the comparative Cₜ method, which generates ΔΔCₜ, as the difference between the gene of interest and the housekeeping genes GAPDH and 9s RNA for each sample. Each averaged experimental gene-expression sample was compared with the averaged control sample, which was set to 1.

**Immunoprecipitation**

Nuclear extracts were prepared and were precipitated using the Dynabeads Protein G ImmunoPrecipitation kit (Invitrogen, Grand Island, NY). Anti-PPARγ or anti-IgG (control) Abs (Santa Cruz Biotechnologies) were bound to Dynabeads Protein G, and the Dynabeads–Ab complex was used to precipitate PPARγ from nuclear extracts. Unbound proteins were washed away, and PPARγ was eluted. All samples (20 µg/lane) were separated by electrophoresis on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes, and Western blotting was performed with NF-κB p65 and PPARγ Abs.

**Apoptotic polymorphonuclear neutrophils and polymorphonuclear neutrophil–associated macrophages in BALF**

Cells from BALF of different groups were collected, and cytospin preparations were made and then stained with Diff-Quik (Sidmans). The morphology of the cells was examined and imaged by light microscopy (Leica DM4000B; Leica Microsystems, Wetzlar, Germany) with a 100× objective. Apoptotic polymorphonuclear neutrophils (PMNs) were identified by characteristic changes (pyknotic nuclei, condensed chromatin, and simplification of nuclear structure). Apoptotic PMNs and PMN-associated macrophages (an indicator of phagocytosis) were counted. Results are represented as the percentage of total cells counted.

**Quantitation of PMN apoptosis in vitro**

PMNs were isolated from mouse blood, as previously described (22), and cultured in DMEM supplemented with 10% FBS. Cells were treated with drugs for 6 h at 37°C in a humidified atmosphere of 5% CO₂–95% air.
After 6 h of incubation, cells were washed three times with cold PBS and then incubated on ice for 30 min with YO-PRO-1 and propidium iodide (PI) assay buffers (Vybrant Apoptosis Assay Kit #4; Molecular Probes), according to the manufacturer’s instructions. Apoptotic PMNs stain YO-PRO-1+ PI−, live cells stain YO-PRO-1− PI−, and dead or necrotic cells stain YO-PRO-1+ PI+. The number of cells in each class was assessed by flow cytometry within 20 min of the final wash using a BD FACSCalibur flow cytometer and Cell Quest Pro software (BD Biosciences, San Jose, CA).

Quantitation of in vitro phagocytosis of apoptotic PMNs
Alveolar macrophages (AMs) were isolated and cultured in DMEM medium supplemented with 10% FBS, as previously described (14). Cells were treated with drugs for 6 h at 37°C in a humidified atmosphere of 5% CO2–95% air. After 6 h of incubation, CD36 expression was analyzed by Western blotting. Other AMs were labeled with a red fluorescent dye (Cell Tracker Red CMTPX; Invitrogen). Separately, apoptotic PMNs were prepared by culturing them in DMEM medium without FBS for 24 h. Apoptotic neutrophils were labeled with YO-PRO-1 and used as target cells in the following phagocytosis assay: AMs were cocultured with apoptotic neutrophils at a 1:10 ratio in DMEM supplemented with 10% FBS for 90 min at 37°C in a humidified atmosphere of 5% CO2–95% air. Flow cytometric analysis was performed as described in the previous paragraph (Quantitation of PMN apoptosis in vitro).

Statistical analysis
Data are presented as mean ± SD. Differences between groups were analyzed using ANOVA, followed by a Bonferroni multiple-comparison test, with GraphPad Prism 5.03 software (GraphPad Software, La Jolla, CA). A p value < 0.05 was considered significant.

Results
Intrapulmonary OA-NO2 blocks inflammatory responses to OVA challenge as effectively as fluticasone
To test whether intrapulmonary OA-NO2 treatment can block lung inflammation, we used a well-established murine model of allergic airway disease based on OVA sensitization and challenge (18). Some mice received 25 µg OA-NO2 or fluticasone (a fluticasone

![Figure 1](http://www.jimmunol.org/) Pulmonary delivery of OA-NO2 reduces OVA-induced lung inflammation. Allergic airway disease was induced in mice by OVA sensitization on days 0 and 7 and challenge on even-numbered days 14–22. Each challenge was followed by i.t. administration of OA-NO2 (25 µg), fluticasone (Flut; 25 µg), or vehicle (Veh; 10% DMSO). Twenty-four hours following the last challenge, BALF, plasma, and lung samples were obtained. (A) Total and differential cell count in BALF. (B) Protein concentration in BALF. IgE concentration in BALF (C) and serum (D). (E) Photomicrographs (original magnification ×100) of Diff-Quik–stained cells in BALF from the indicated treatment groups. Data are representative of two or three independent experiments, with n = 6–8 mice/group. ***p < 0.001.
dose known to be effective in this model) i.t. 30 min after each OVA challenge. Because a prominent characteristic of allergic airway disease is migration of eosinophils and other inflammatory cells into the airways, from which they can be obtained by bronchoalveolar lavage, we determined the effects of OA-NO2 and fluticasone on the number and type of cells in BALF. Microscopic analysis revealed a significant OVA challenge–induced increase in the numbers of cells in BALF that was markedly reduced by OA-NO2, which was similar to the effect of fluticasone (Fig. 1E). Differential counting showed that the majority of these cells were eosinophils, although the numbers of other cell types were also increased (Fig. 1A). Protein levels in BALF also increased, reflecting the increased presence of inflammatory cells and mediators, and they were similarly inhibited by >50% by both OA-NO2 and fluticasone treatments (Fig. 1B). Allergic airway disease is mediated by elevations in allergen-specific IgE. We found that the increased levels of IgE in both BALF (Fig. 1C) and serum (Fig. 1D) following OVA sensitization and challenge was significantly inhibited by OA-NO2, equivalent to the effects of fluticasone.

Allergen binding to the relevant Ab, followed by receptor interaction, leads through a series of steps, involving multiple cell types, to the production of inflammatory cytokines including TNF-α, IL-4,
and IL-5, and chemokines, such as MIP-2 and eotaxin, which attract inflammatory cells. We found that, in both lung tissue (Fig. 2B) and BALF (Fig. 2D), either OA-NO2 or fluticasone significantly inhibited the OVA-induced increases in these factors. There were no significant differences between the effects of the two treatments.

Pulmonary inflammation involves elevated production of reactive oxygen and nitrogen species by infiltrating inflammatory cells. We measured production of H2O2, a major reactive oxygen species (ROS); the malondialdehyde/protein ratio, a measure of overall oxidative stress; and nitrate, an end product of NO production. NO

FIGURE 3. Pulmonary delivery of OA-NO2 reduces OVA-induced inflammatory cell infiltration and remodeling of airways. Allergic airway disease was induced in mice by OVA sensitization on days 0 and 7 and challenge on even-numbered days 14–22. Each challenge was followed by i.t. administration of OA-NO2 (25 μg), fluticasone (Flut; 25 μg), or vehicle (Veh; 10% DMSO). Lung sections were obtained 24 h following the last challenge and were examined histologically following H&E (A), PAS (B), trichrome (C), and picrosirius red (D) staining, as well as immunohistochemical staining for NF-κB p65 (E). Original magnification ×20. (F) Quantitative scoring of the respective pathological indices shown in (A–C). Data are representative of two independent experiments, with n = 2 sections from three mice/group. **p < 0.001.
is a reactive species produced by pathways distinct from those that produce H₂O₂ and related oxygen species. OVA challenge increased all three of these markers in both lung tissue (Fig. 2A) and BALF (Fig. 2C). Treatment with OA-NO₂ or fluticasone inhibited each of these observed increases by ≥50%.

**OA-NO₂ blocks OVA-induced airway remodeling and hyperresponsiveness**

The pathological features of chronic asthma include airway remodeling and differentiation of mucus-producing goblet cells. Airway remodeling is characterized by thickening of the airway walls, with increases in both muscle tissue and collagen. OVA-challenged mice clearly exhibited such expected increases in airway thickness, seen microscopically in H&E-stained tissue (Fig. 3A). We found large increases in the number of goblet cells by mucopolysaccharide-specific staining (PAS) (Fig. 3B), whereas trichrome staining demonstrated increases in both muscle mass and collagen (Fig. 3C). The increase in collagen deposition was confirmed on staining with picrosirius red (Fig. 3D). All of these prototypical asthma-like responses were markedly attenuated (by >60 to ~80%) by OA-NO₂, as well as by fluticasone, as shown by quantitative analyses of inflammation, numbers of PAS-positive cells, and trichrome-stained area (Fig. 3F).

Such pathological airway changes are mediated, in part, by activity of proinflammatory transcription factors, such as NF-κB. OVA-challenged mice exhibited a major increase in the NF-κB subunit p65 in airway walls. OA-NO₂ inhibited OVA-elicited p65 expression, as did fluticasone (Fig. 3E). We also measured the functional effects of airway inflammation by examining total respiratory resistance (respiratory system resistance to airflow), respiratory system elastance (a measure of the stiffness and rebound of respiratory system tissues), tissue elastance (elastance of peripheral respiratory tissues [terminal bronchi and lung parenchyma]), and tissue damping (energy dissipated by peripheral airflow resistance) (Fig. 4). For each of these measures, OVA challenge greatly increased the response to methacholine. Treatment with OA-NO₂ significantly attenuated the respective increases in methacholine responses, as did fluticasone.

**OA-NO₂ inhibits OVA-induced upregulation of adhesion molecules and expression of ROS-producing enzymes**

Asthma-associated inflammation involves upregulated expression on pulmonary vascular endothelial cells of the adhesion molecules VCAM-1, ICAM-1, and E-selectin, which facilitate migration of eosinophils and other inflammatory cells into the airways. Therefore, we tested the effects of OA-NO₂ on adhesion protein gene expression, finding that OA-NO₂ reduced the OVA challenge–elicited increases in the expression of mRNAs encoding VCAM-1, ICAM-1, and E-selectin within lung tissue to an extent similar to that seen with fluticasone treatment (Fig. 5A–C). Likewise, OA-NO₂ reduced OVA challenge–induced increases in mRNAs encoding the ROS-producing enzymes inducible NO synthase, endothelial NO synthase, and NADPH oxidase 4 (Fig. 5D–F). The increases seen in OVA challenge–induced expression of these six genes ranged between 2.5- and ~8-fold, with OA-NO₂ treatment inhibiting the increase by ~70% or more in all cases and by almost 100% for four of the six target genes analyzed. Also, for each gene studied, the extent to which OA-NO₂ treatments inhibited OVA-induced upregulation was nearly identical to that seen with fluticasone treatment. Western blotting demonstrated that OVA challenge–induced increases in expression levels of the six corresponding proteins were likewise markedly reduced (Fig. 5G, 5H), although less so compared with those of the encoding mRNAs, probably because inhibition of mRNA

![FIGURE 4. OA-NO₂ reduces airway hyperresponsiveness. Allergic airway disease was induced in mice by OVA sensitization on days 0 and 7 and challenge on even-numbered days 14–22. Control mice were similarly treated with PBS. Each challenge was followed by i.t. administration of OA-NO₂ (25 μg), fluticasone (Flut; 25 μg), or vehicle (Veh; 10% DMSO). Twenty-four hours later, airway hyperresponsiveness to increasing concentrations of inhaled methacholine was determined.](http://www.jimmunol.org/)

(A) Total respiratory system resistance. (B) Respiratory system elastance. (C) Tissue elastance. (D) Tissue damping. Data are representative of two independent experiments, with n = 6–8 mice/group. ***p < 0.001 versus PBS. +++p < 0.001 versus OA-NO₂. aaaa p < 0.001 versus Flut.
expression provides an earlier response and indicator than that of the encoded proteins, which persist longer.

OA-NO₂ upregulates PPARγ and inhibits NF-κB activity

OA-NO₂ upregulated PPARγ and inhibits NF-κB activity in vitro

OVA-induced inflammation and associated pathological effects reflect upregulation of proinflammatory transcription factors, prominently including NF-κB (23), accompanied by downregulation of PPARγ. Activation of either the glucocorticoid receptor (24) or PPARγ (25, 26) inhibits NF-κB activity via different mechanisms. Previous evidence suggested that NFAs may inhibit NF-κB independently of PPARγ activation (11). We found that treating NHBE cells with the proinflammatory cytokine TNF-α decreased PPARγ activity, whereas OA-NO₂ greatly increased its activity in both the presence and absence of TNF-α (Fig. 6A). The effects of OA-NO₂ on NF-κB activation contrasted with its effects on PPARγ; it did not significantly alter NF-κB activity in the absence of TNF-α, but it reversed the TNF-α–induced increase in NF-κB activity (Fig. 6B). Fluticasone reduced NF-κB activity, but it had no effect on PPARγ, in agreement with previous results (Supplemental Fig. 1). Treatment with TNF-α induced a shift of the NF-κB p65 subunit from cytoplasm to nucleus that was opposed by PPARγ activation (Fig. 6C, first and second rows). Treatment with OA-NO₂ shifted the localization of PPARγ from predominantly cytoplasmic to nuclear, as seen by Western blotting of nuclear and cytoplasmic fractions (Fig. 6C, third and fourth rows), consistent with increased DNA binding. Immunostaining, followed by confocal imaging, confirmed these TNF-α–and OA-NO₂–induced changes in intracellular localization (Fig. 6D).

PPARγ activation may inhibit NF-κB via several mechanisms, some of which involve direct interaction between PPARγ and NF-κB (25, 27), whereas another (26) does not. NF-κB may also be inhibited via direct alkylation by NFAs (11). We found that immunoprecipitation of PPARγ from TNF-α– and IL-4–treated
NHBE cells, but not from untreated cells, also precipitated bound p65. Treatment with OA-NO2 further increased the extent of this binding (Fig. 6E). Another key mechanism of NF-κB regulation is degradation of the inhibitor IκB, causing its subsequent ubiquitination and degradation. This allows translocation of the DNA-binding NF-κB p65 subunit into the nucleus. As expected, Western blotting demonstrated that TNF-α induced a major increase in phosphorylated IκB and a corresponding decrease in nonphosphorylated IκB. OA-NO2 treatment blocked these TNF-α–induced events (Fig. 6F). Thus, our results support the possibility that OA-NO2 may downregulate NF-κB activity in primary human airway cells by at least two mechanisms: one is PPARγ dependent, whereas the other may not be.

OA-NO2, but not fluticasone, robustly stimulates neutrophil apoptosis and their phagocytosis

Although asthma is described as an eosinophil-rich inflammation, it can also involve infiltration of neutrophils into the lung. Neutrophil apoptosis and their phagocytosis by AMs are prominent mechanisms for the resolution of inflammation. Because PPARγ is expressed in neutrophils (29), we investigated the effects of OA-NO2 and fluticasone on these processes in vivo. We found that OA-NO2 stimulated apoptosis following OVA challenge (Fig. 7A, upper middle panel). Apoptosis was undetectable following vehicle treatment (Fig. 7A, upper left panel) and was quite modest following fluticasone treatment (Fig. 7A, upper right panel). OA-NO2 also stimulated phagocytic association of neutrophils with AMs (Fig. 7A, lower middle panel), whereas the effects of fluticasone were much smaller (Fig. 7A, lower right panel). We quantitated these results by microscopic counting of apoptotic (Fig. 7B, upper panel) and phagocytosed (Fig. 7B, lower panel) neutrophils.

These results were further validated by ex vivo studies. Isolated neutrophils were treated with OA-NO2, fluticasone, or vehicle, followed by apoptosis-specific staining and flow cytometric analysis (Fig. 7C). We also objectively assessed phagocytosis by coculturing macrophages with specifically stained apoptotic neutrophils and analyzing cell association by flow cytometry (Fig. 7D). Both assays demonstrated major effects of OA-NO2 on these parameters, together with much smaller effects of fluticasone. Phagocytosis is triggered by interaction with the scavenger receptor CD36, the expression of which is upregulated by PPARγ activation (30). OA-NO2 treatment significantly upregulated CD36 expression by AMs in vitro (Fig. 7E), as predicted by its observed effect on PPARγ activity. In contrast, fluticasone treatment essentially abolished CD36 expression.

Discussion

Our findings show that the NFA OA-NO2, an endogenous compound, effectively diminishes disease severity in a murine model of allergic airway disease while suppressing markers of pathogenic inflammation, airway remodeling, and all major components of airway hyperresponsiveness. OA-NO2 suppressed infiltration of inflammatory cells into the airways. It also reduced airway remodeling indices, including airway wall thickening, collagen deposition, and differentiation of mucus-producing cells. Likewise, OA-NO2 reduced mechanistic allergic airway disease markers, including production of proinflammatory cytokines, chemokines, adhesion molecules, and ROS-generating enzymes. We found that the NFA OA-NO2 was at least as effective in all of these respects as fluticasone, which we used for comparison because such glucocorticoids, delivered by inhalation, are generally efficacious and are the most common treatment used for asthma patients.
FIGURE 7. OA-NO₂ increases neutrophil apoptosis and phagocytosis by AMs. (A and B) Allergic airway disease was induced in mice by OVA sensitization on days 0 and 7 and challenge on even-numbered days 14–22. Each challenge was followed by i.t. administration of OA-NO₂ (25 μg), fluticasone (Flut; 25 μg), or vehicle (Veh; 10% DMSO). Twenty-four hours following the last challenge, cells from BALF were collected and stained with Diff-Quik and Hoechst apoptotic DNA stains. (A) Photomicrographs (original magnification ×100) showing neutrophils (top panels) and AMs with or without apoptotic bodies (bottom panels) from the indicated treatment groups. (B) Apoptotic PMNs and PMN-associated AMs (as an indicator of phagocytosis) were counted. Results are presented as the percentage of total cells counted. (C) Dot plots showing flow cytometric analysis of isolated PMNs treated with OA-NO₂ (1 μM), fluticasone (Flut; 1 μM), or vehicle (DMSO) for 6 h and then stained with YO-PRO-1 and PI. Apoptotic cells are YO-PRO-1⁺ PI⁻ (lower right quadrant). The number in each quadrant represents the percentage of total cells. (D) Dot plots showing flow cytometric analysis of AMs stained with Cell Tracker Red CMTPX and then cocultured during 6 h of treatment with OA-NO₂ (1 μM), fluticasone (Flut; 1 μM), or vehicle (DMSO), with serum-starved apoptotic PMNs stained with YO-PRO-1. AMs with associated apoptotic PMNs are in the upper right quadrant. The number in each quadrant represents the percentage of total cells. (E) Western blot of CD36 expression by AMs following 6 h of incubation with OA-NO₂ (1 μM), fluticasone (Flut; 1 μM), or vehicle (DMSO). Data are representative of two or three independent experiments with n = 3–6. ***p < 0.001.
Overall, these results are in line with our prior findings that OA-NO₂ PPARγ-dependently inhibited endotoxin-induced upregulation of endothelial adhesion molecules and trans-endothelial migration of neutrophils (19), but they extend the scope of NFA anti-inflammatory actions qualitatively to a pulmonary airway disease model that encompasses virtually all major relevant pathophysiological aspects. Considering that OA-NO₂ is an endogenous compound, for which we and other investigators found no evidence of acute toxicity at the doses used, these results support the potential applicability of OA-NO₂ as a new therapeutic approach for asthma therapy.

Although typically less prominent than eosinophils, neutrophils contribute significantly to inflammation in some asthma patients. Apoptosis of neutrophils and phagocytosis of such apoptotic neutrophils by AMs are crucial components in the resolution of pulmonary inflammation (31). PPARγ activation induces such neutrophil apoptosis (32) and increases expression of the phagocytosis-mediating macrophage CD36 scavenger receptor (33, 34). We found that OA-NO₂ induced apoptosis and macrophage phagocytosis of pulmonary neutrophils. These actions of OA-NO₂ proved selective and distinct from those of fluticasone, because fluticasone failed to elicit similar apoptotic and phagocytic responses, whereas we found it as effective as OA-NO₂ in inhibiting all of the other inflammatory processes and markers that we tested. Differential effects on phagocytosis reflect contrasting upregulation of the phagocytosis-triggering scavenger receptor CD36 by OA-NO₂ and its downregulation by fluticasone. In accord with our findings, anti-inflammatory glucocorticoids are known to inhibit neutrophil apoptosis in vitro (35) and to suppress macrophage CD36 (36). Fluticasone’s modest ability to stimulate phagocytosis, despite nearly complete suppression of CD36, presumably reflects downregulation of the phagocytosis-inhibiting protein SIRPα (37).

Many patients with severe asthma have large numbers of neutrophils in their airways (38–40). This neutrophilia may be associated with the steroid resistance often seen in severe asthma, because the minority of severe asthma patients with eosinophil-rich inflammation often respond to an additional increase in their steroid dose (41). Our results may explain, in part, the steroid resistance in patients with neutrophil-rich severe asthma and suggest that NFAs might serve as an effective means of treating such patients, for whom alternative treatments are unavailable.

To assess OA-NO₂ efficacy, we delivered it directly to the pulmonary system via i.t. injection. Pulmonary delivery is the preferred method of drug administration for lung diseases, such as asthma, because it maximizes the dose fraction reaching the target organ while minimizing systemic availability and resulting adverse effects. Previously, we found that direct pulmonary delivery of OA-NO₂ reduces the severity of LPS-induced acute lung injury in mice (14). Systemic administration was found to reduce the severity of extrapulmonary diseases, including an inflammatory bowel disease model in mice (15), and cardiac (16) and renal (17) ischemia/reperfusion injury models, in all cases decreasing inflammatory responses and NF-κB activity. In the inflammatory bowel disease study, OA-NO₂ upregulated PPARγ expression and exerted therapeutic effects that were abolished by the PPARγ antagonist GW9662 (15), supporting a role for PPARγ in its ameliorative effects.

We found that OA-NO₂ inhibits activation of NF-κB, a key inflammatory event (42), probably via multiple mechanisms. For example, OA-NO₂ reversed TNF-α-induced phosphorylation of the NF-κB inhibitory protein IκB in NHBE cells, which elevated NF-κB activity by causing ubiquitin-dependent IκB degradation (28). The mechanism through which OA-NO₂ blocks such IκB phosphorylation is unknown, but it could be similar to that of other electrophilic lipids, which inhibit IκB kinase by alkylating a key cysteine residue (43–45). NFAs can also inhibit NF-κB by alkylating a specific cysteine thiol within its DNA-binding domain (11).

We find that OA-NO₂ also inhibits NF-κB via PPARγ activation, which likewise downregulates NF-κB activity by multiple mechanisms. Classically, activated PPARγ competes for the same coactivators required by NF-κB and other proinflammatory transcription factors (26). In addition, phosphorylated PPARγ may inhibit NF-κB by binding to it directly (27), and SUMOylated PPARγ may prevent dissociation of corepressors from DNA-bound NF-κB (25). Also, PPARγ was found to bind via its ligand-binding domain to NF-κB, triggering ubiquitination and degradation of the NF-κB p65 subunit, with such binding being enhanced by PPARγ agonists (46). In agreement, we found that PPARγ binds to NF-κB. Moreover, the PPARγ agonist OA-NO₂ increases this binding, but only following inflammatory stimuli.

All of our findings concur in suggesting that the endogenous compound OA-NO₂ is highly efficacious for reducing the severity of this murine model of allergic asthma. Another endogenous NFA, nitrolinoleic acid, was likewise shown to be a PPARγ agonist (10) and to inhibit NF-κB by direct alkylation (11). Thus, NFAs, including OA-NO₂ and others, may function as endogenous inhibitory modulators of inflammatory responses, but such physiological roles await rigorous investigation. Although we investigated only OA-NO₂, we believe that nitrolinoleic acid and similar NFAs would likewise offer robust protection against asthma exacerbations. In the present model, protective effects of administered OA-NO₂ were at least equivalent to those provided by a commonly used therapeutic glucocorticoid, but only OA-NO₂ promoted neutrophil apoptosis and clearance. This effect on neutrophils may be beneficial in many severe asthma cases. Anti-inflammatory effects of OA-NO₂ were exerted via both PPARγ-dependent and -independent mechanisms, providing redundancy that might favor robust effects under varying conditions. Together, these findings provide strong evidence for mechanistically broad-based efficacy of OA-NO₂ in standard preclinical models of human asthma, warranting further investigation of its efficacy and mechanisms of action, as well as those of related NFAs.

Disclosures

The authors have no financial conflicts of interest.

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