



This information is current as of August 12, 2013.

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

J Immunol published online 2 August 2013

<http://www.jimmunol.org/content/early/2013/08/01/jimmunol.1300730>

Supplementary Material <http://www.jimmunol.org/content/suppl/2013/08/06/jimmunol.1300730.DC1.html>

Subscriptions Information about subscribing to *The Journal of Immunology* is online at: <http://jimmunol.org/subscriptions>

Permissions Submit copyright permission requests at: <http://www.aai.org/ji/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: <http://jimmunol.org/cgi/alerts/etoc>



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 I κ B and enhanced inhibitory binding of PPAR γ to NF- κ B. 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.

Asthma is one of the most common chronic inflammatory diseases. It is typically allergen triggered and characterized by overproduction of Ag-specific IgE, excessive immunological responses, and consequent inflammation (1, 2). Asthma pathophysiology involves a variety of complex feedback loops and numerous cell types that serve as both producers and targets of inflammatory mediators. Prominent clinical symptoms include excessive mucus production and bronchospasm. Medications targeting specific, supposedly crucial pathways have shown some usefulness, but the potent, broadly anti-inflammatory glucocorticoids remain standard treatment. However, such glucocorticoids have significant side effects that can cause or exacerbate comorbidities. Furthermore, they fail to suppress significant aspects of asthma pathology and are ineffective in some patients (3–6), so new efficacious and safe therapies are needed.

The nitrated fatty acids (NFAs) are a family of novel endogenous compounds having anti-inflammatory properties potentially relevant to asthma. They are produced by nonenzymatic reactions of NO with unsaturated fatty acids (7), constituting one of the largest pools of biologically active oxides of nitrogen in the bloodstream

(8). Regioisomers of nitro-oleic and nitrolinoleic acids are the most common and most potent NFAs and, therefore, have been the most investigated. NFAs potently activate peroxisome proliferator-activated receptor (PPAR) γ , which has multiple anti-inflammatory effects, including transrepression of the proinflammatory transcription factor NF- κ B; they are also weaker agonists of PPAR α and β/δ (9, 10). NFAs also have known PPAR-independent anti-inflammatory actions, including inhibition of NF- κ B activity via nonenzymatic alkylation of its p65 subunit (11); activation of adenylyl cyclase (12), which has anti-inflammatory effects in neutrophils; and activation of the antioxidant transcription factor Nrf2 via alkylation of its inhibitor, Keap-1 (13). Because NO production is upregulated by inflammation, NFAs might plausibly serve as endogenous modulators of inflammation. However, studies of the in vivo anti-inflammatory effects of NFAs have been limited (14–17), and their role in allergic airway disease is unknown.

To assess whether NFAs might have therapeutic potential in asthma, we tested the effects of the most potent PPAR γ agonist in the class (9), 10-nitro-oleic acid (OA-NO₂), on inflammation and asthma-relevant pathophysiological markers in a murine model of allergic airway disease. We also used normal human bronchial epithelial (NHBE) cells to study the mechanisms of the anti-inflammatory effects of OA-NO₂.

Materials and Methods

Animals

Male C57BL/6 mice, 6–8 wk of age (20–25 g), were obtained from The Jackson Laboratory (Bar Harbor, ME). All studies were performed according to protocols reviewed and approved by the Atlanta Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

Cells

NHBE cells were obtained from Lonza (Walkersville, MD) at passage 3 and used at passages 5–8. Cells were grown and maintained in B-ALI-Growth medium, supplemented with 10% FBS, 0.4% bovine pituitary extract, 0.1% insulin, 0.1% human EGF, 0.1% hydrocortisone, 0.1% GA-1000, 0.1% retinoic acid, 0.1% transferrin, 0.1% triiodothyronine, epinephrine,

Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; and Veterans Affairs Pittsburgh Healthcare System, Pittsburgh, PA 15240

Received for publication March 18, 2013. Accepted for publication July 2, 2013.

This work was supported by National Institutes of Health Grant HL093196 (to R.C.R.).

Address correspondence and reprint requests to Dr. Raju C. Reddy, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh/Veterans Affairs Pittsburgh Healthcare System, Pittsburgh, PA 15240. E-mail address: reddeyrc@upmc.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: AM, alveolar macrophage; BALF, bronchoalveolar lavage fluid; i.t., intratracheal(ly); MIP, macrophage inflammatory protein; NFA, nitrated fatty acid; NHBE, normal human bronchial epithelial; OA-NO₂, 10-nitro-oleic acid; PAS, periodic acid-Schiff; PI, propidium iodide; PMN, polymorphonuclear neutrophil; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species.

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, Québec, 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 $500 \times g$ 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 cytospin 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 $14,000 \times g$ 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 PPAR γ and the p65 subunit of NF- κ B 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- κ B p65, PPAR γ , 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-Ik β and Ik β 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 Imager (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 2 $\times$ 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 (C_t) was determined for each experiment. Relative cDNA levels ($2^{-\Delta\Delta C_t}$) for the genes of interest were determined using the comparative C_t method, which generates $\Delta\Delta C_t$ as the difference between the gene of interest and the housekeeping genes GAPDH and 9s rRNA 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-PPAR γ or anti-IgG (control) Abs (Santa Cruz Biotechnologies) were bound to Dynabeads Protein G, and the Dynabeads–Ab complex was used to precipitate PPAR γ from nuclear extracts. Unbound proteins were washed away, and PPAR γ 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- κ B p65 and PPAR γ 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 (Siemens). The morphology of the cells was examined and imaged by light microscopy (Leica DM4000B; Leica Microsystems, Wetzlar, Germany) with a 100 $\times$ 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

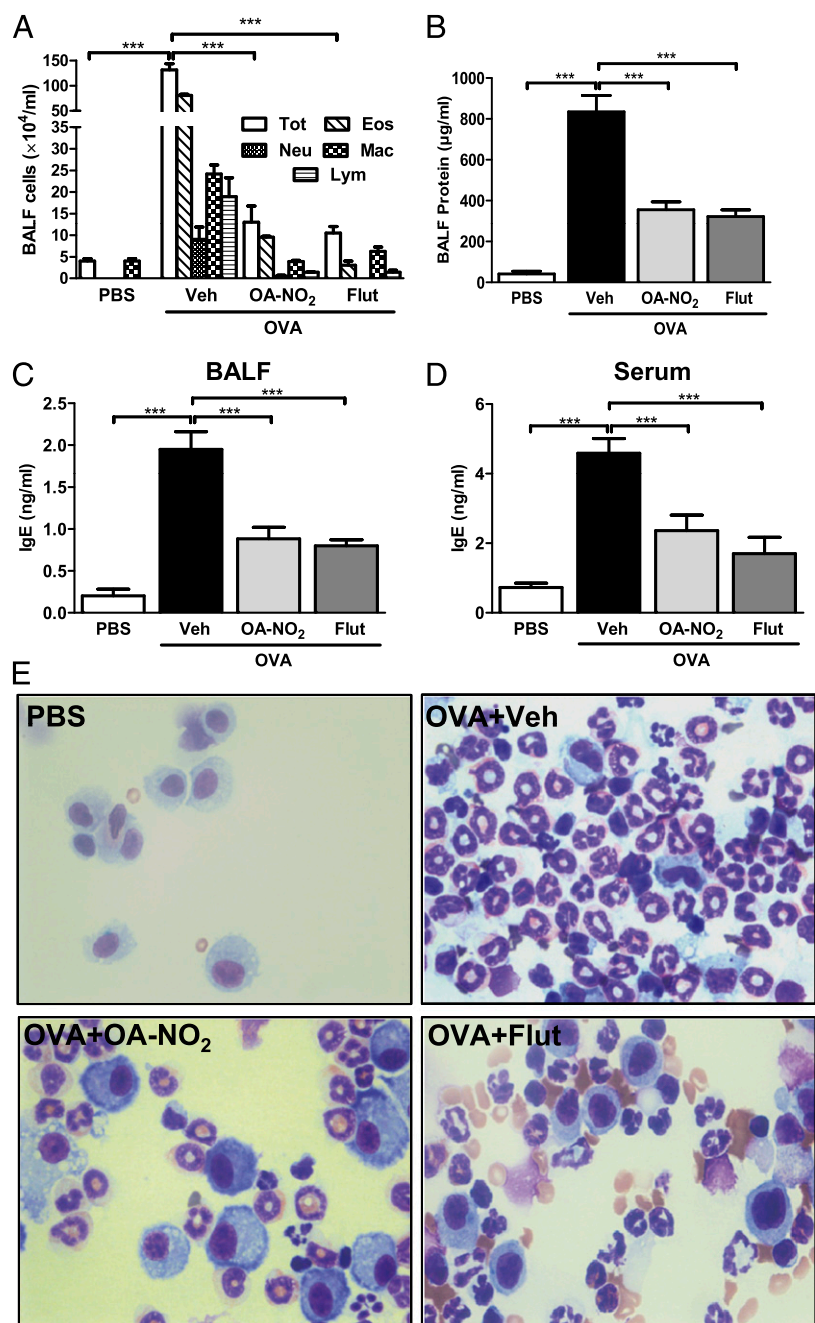
Data are presented as mean ± SD. Differences between groups were analyzed using ANOVA, followed by a Bonferroni multiple-comparison test, with GraphPad Prism 5.03 software (GraphPad Software, La Jolla, CA). A *p* value < 0.05 was considered significant.

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. 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 in-

creased (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,

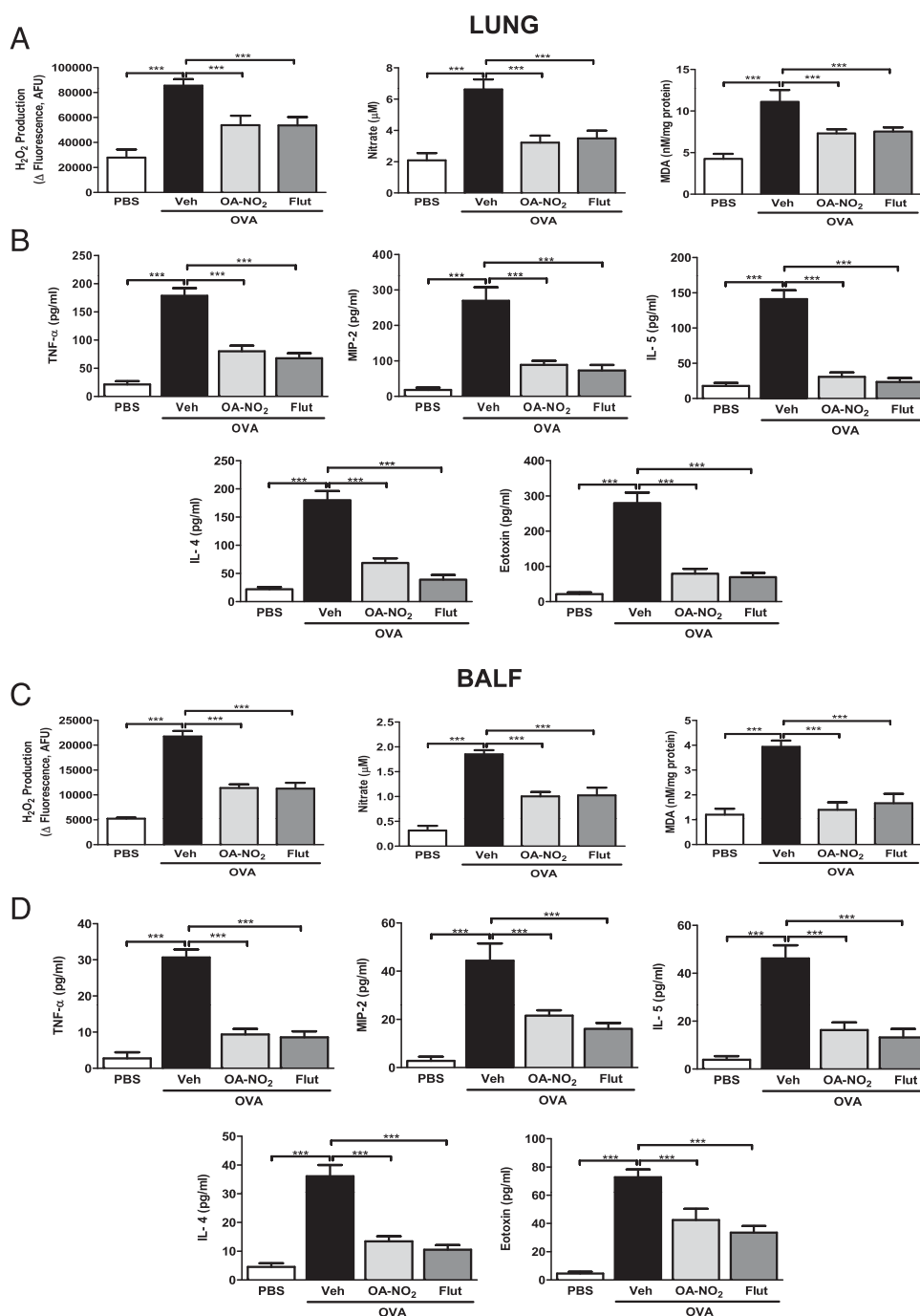


FIGURE 2. OA-NO₂ treatment reduces markers of OVA-induced lung inflammation. Allergic airway disease was induced in mice by OVA sensitization on days 0 and 7 and challenge on even-numbered days 14–22. Each challenge was followed by i.t. administration of OA-NO₂ (25 μg), fluticasone (Flut; 25 μg), or vehicle (Veh; 10% DMSO). Twenty-four hours following the last challenge, lung samples (**A**, **B**) and BALF (**C**, **D**) were obtained, and oxidant stress or cytokine and chemokine levels were measured. (A and C) H₂O₂ production, total nitrate levels, and malondialdehyde/protein ratio. (B and D) TNF- α , MIP-2, IL-5, IL-4, and eotaxin. Data are representative of three independent experiments, with $n = 6$ –8 mice/group. *** $p < 0.001$.

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

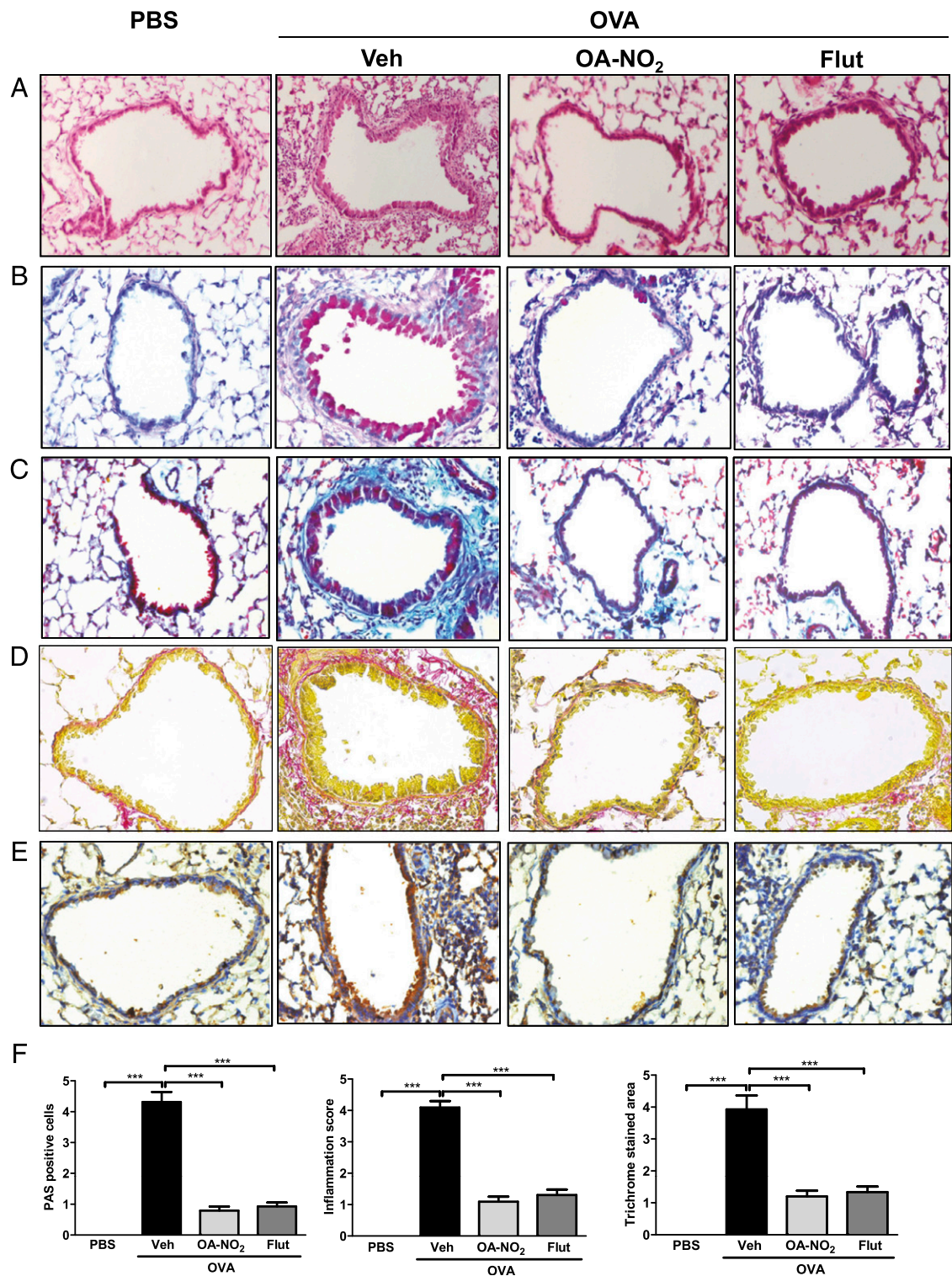


FIGURE 3. Pulmonary delivery of OA-NO₂ reduces OVA-induced inflammatory cell infiltration and remodeling of airways. Allergic airway disease was induced in mice by OVA sensitization on days 0 and 7 and challenge on even-numbered days 14–22. Each challenge was followed by i.t. administration of OA-NO₂ (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 picosirius 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 $\geq 50\%$.

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 $\sim 80\%$) 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- κ B. OVA-challenged mice exhibited a major increase in the NF- κ B 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 $\sim 70\%$ 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

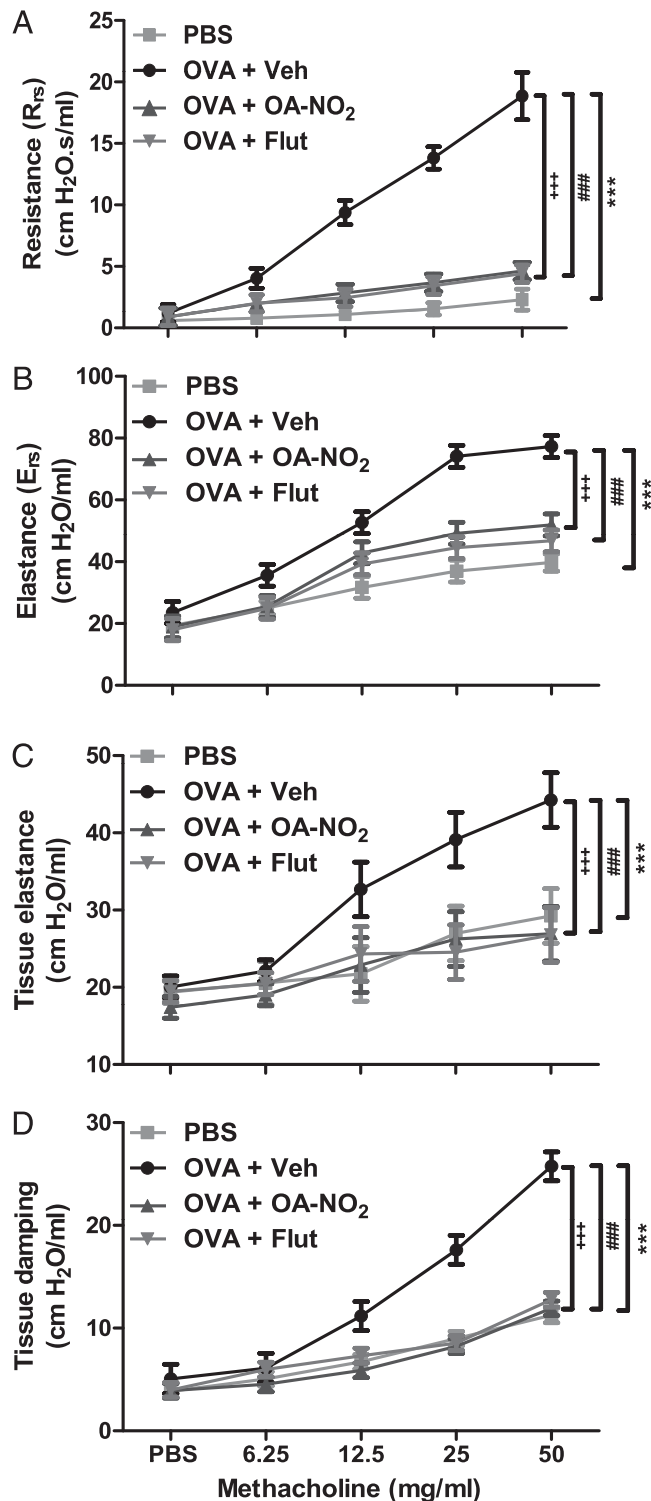


FIGURE 4. OA-NO₂ reduces airway hyperresponsiveness. Allergic airway disease was induced in mice by OVA sensitization on days 0 and 7 and challenge on even-numbered days 14–22. Control mice were similarly treated with PBS. Each challenge was followed by i.t. administration of OA-NO₂ (25 μ g), fluticasone (Flut; 25 μ g), or vehicle (Veh; 10% DMSO). Twenty-four hours later, airway hyperresponsiveness to increasing concentrations of inhaled methacholine was determined. (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, ### $p < 0.001$ versus Flut.

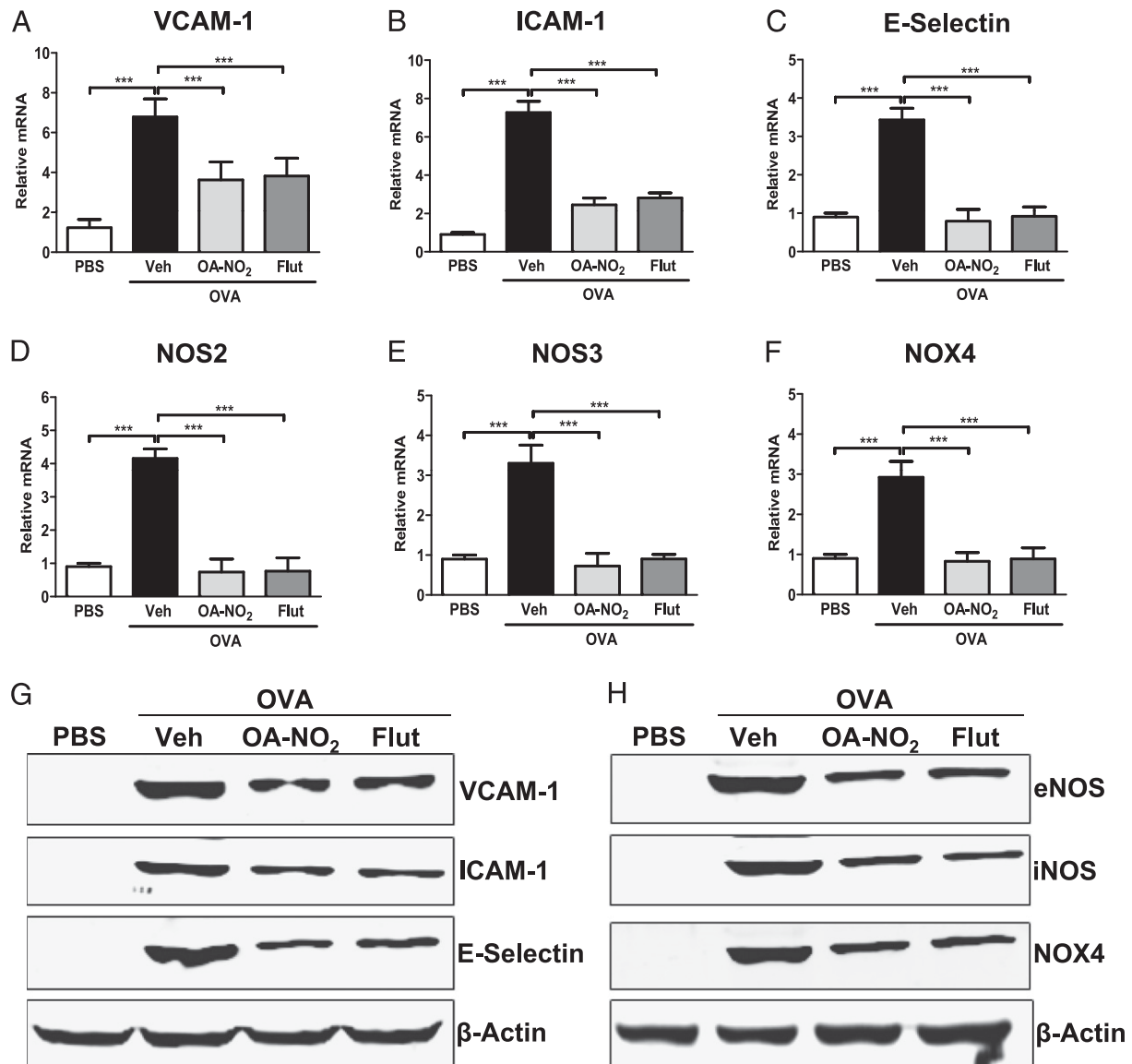


FIGURE 5. OA-NO₂ inhibits OVA-induced upregulation of adhesion molecule and ROS-producing enzyme expression. Allergic airway disease was induced in mice by OVA sensitization on days 0 and 7 and challenge on even-numbered days 14–22. Each challenge was followed by i.t. administration of OA-NO₂ (25 µg), fluticasone (Flut; 25 µg), or vehicle (Veh; 10% DMSO). Twenty-four hours following the last challenge, lung samples were obtained, and RNA and protein were isolated. **(A–F)** Expression of the indicated genes was determined using real-time PCR. Results are normalized to values for the housekeeping genes GAPDH and 9s rRNA. **(G and H)** Expression levels of the indicated proteins were determined by Western blotting. Data are representative of three independent experiments, with $n = 6–8$ mice/group. *** $p < 0.001$.

expression provides an earlier response and indicator than that of the encoded proteins, which persist longer.

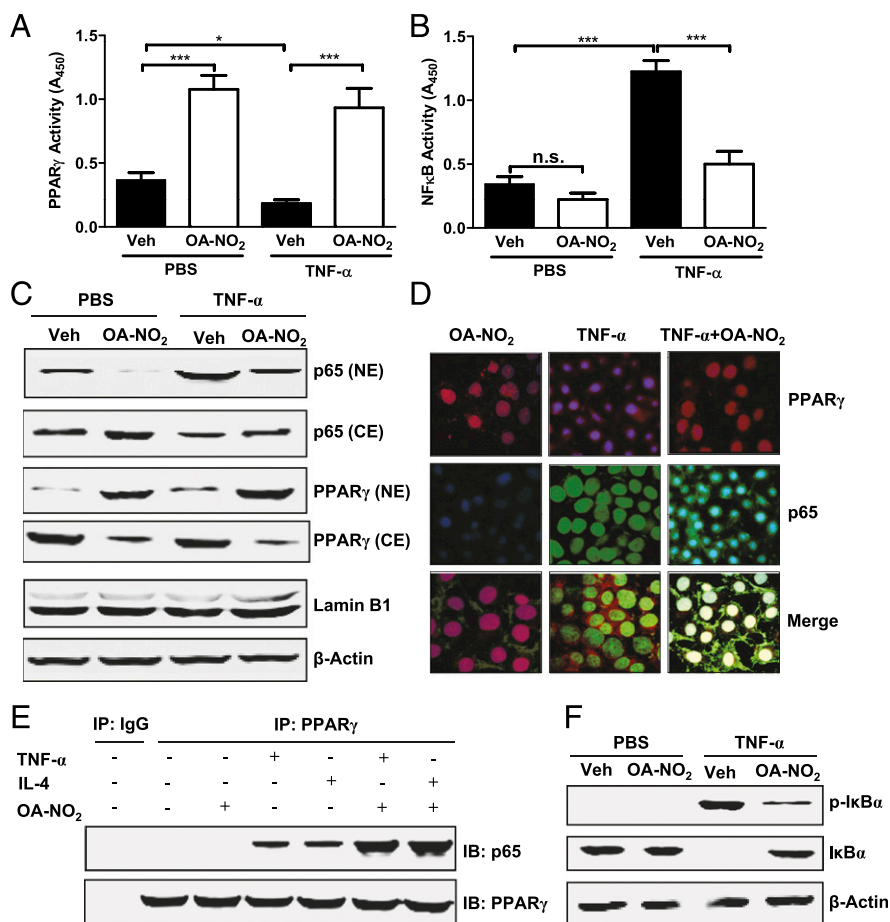
OA-NO₂ upregulates PPAR γ and inhibits NF- κ B activity *in vitro*

OVA-induced inflammation and associated pathological effects reflect upregulation of proinflammatory transcription factors, prominently including NF- κ B (23), accompanied by downregulation of PPAR γ . Activation of either the glucocorticoid receptor (24) or PPAR γ (25, 26) inhibits NF- κ B activity via different mechanisms. Previous evidence suggested that NFAs may inhibit NF- κ B independently of PPAR γ activation (11). We found that treating NHBE cells with the proinflammatory cytokine TNF- α decreased PPAR γ 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- κ B activation contrasted with its effects on PPAR γ : it did not significantly alter NF- κ B activity in the absence

of TNF- α , but it reversed the TNF- α -induced increase in NF- κ B activity (Fig. 6B). Fluticasone reduced NF- κ B activity, but it had no effect on PPAR γ , in agreement with previous results (Supplemental Fig. 1). Treatment with TNF- α induced a shift of the NF- κ B p65 subunit from cytoplasm to nucleus that was opposed by PPAR γ activation (Fig. 6C, first and second rows). Treatment with OA-NO₂ shifted the localization of PPAR γ 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).

PPAR γ activation may inhibit NF- κ B via several mechanisms, some of which involve direct interaction between PPAR γ and NF- κ B (25, 27), whereas another (26) does not. NF- κ B may also be inhibited via direct alkylation by NFAs (11). We found that immunoprecipitation of PPAR γ from TNF- α - and IL-4-treated

FIGURE 6. OA-NO₂ inhibits NF-κB activity by stimulating PPARγ binding. NHBE cells were treated with OA-NO₂ (100 nM) or DMSO (Veh) for 30 min and then with TNF-α (10 ng/ml), IL-4 (10 ng/ml; in some experiments), or PBS for 6 h. Nuclear extracts were prepared, and DNA-binding activities of PPARγ (A) and NF-κB (B) were measured using ELISA-based assays. (C) Nuclear (NE) and cytoplasmic (CE) extracts were prepared and subjected to Western blotting using Abs to p65 and PPARγ. (D) Cells were immunostained with primary Abs to either p65 or PPARγ, followed by rhodamine (PPARγ)- or FITC (p65)-conjugated secondary Abs, and analyzed by confocal microscopy. Original magnification ×60. (E) Nuclear extracts were prepared and precipitated with Dynabeads coated with Ab to PPARγ. Proteins were then separated with SDS-PAGE and subjected to Western blotting. The blots were probed with Abs to p65 and PPARγ. (F) Whole-cell extracts were subjected to Western blotting and probed with Abs to phosphorylated and nonphosphorylated IκB. Data are representative of three independent experiments, with $n = 3-6$. * $p < 0.05$, *** $p < 0.001$.



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 (28). Appropriate signals induce phosphorylation of 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.

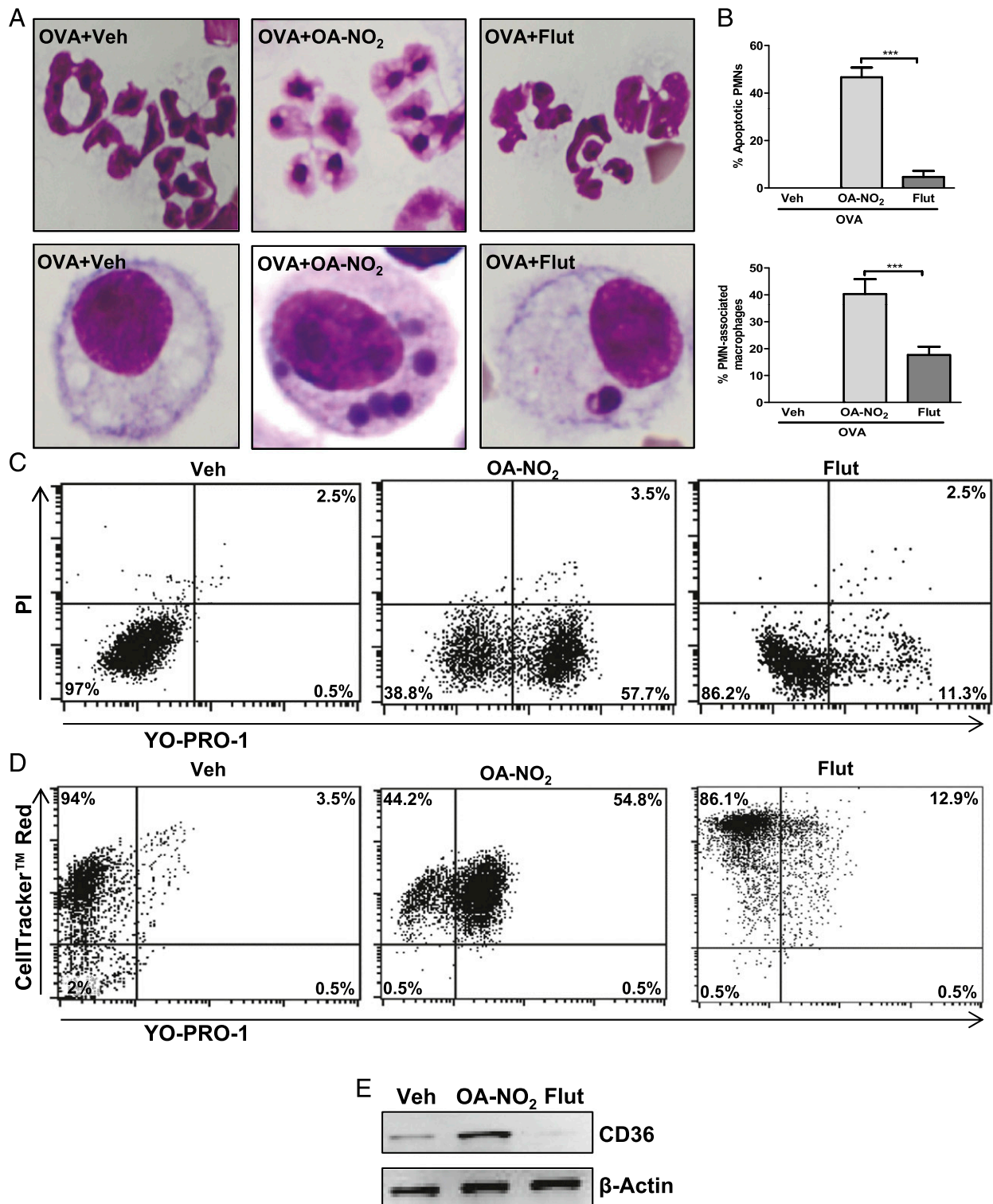


FIGURE 7. OA-NO₂ increases neutrophil apoptosis and phagocytosis by AMs. (**A** and **B**) Allergic airway disease was induced in mice by OVA sensitization on days 0 and 7 and challenge on even-numbered days 14–22. Each challenge was followed by i.t. administration of OA-NO₂ (25 μg), fluticasone (Flut; 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 CMTX 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 PPAR γ -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). PPAR γ 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 SIRP α (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- κ B activity. In the inflammatory bowel disease study, OA-NO₂ upregulated PPAR γ expression and exerted therapeutic effects that were abolished by the PPAR γ antagonist GW9662 (15), supporting a role for PPAR γ in its ameliorative effects.

We found that OA-NO₂ inhibits activation of NF- κ B, a key inflammatory event (42), probably via multiple mechanisms. For example, OA-NO₂ reversed TNF- α -induced phosphorylation of the NF- κ B inhibitory protein I κ B in NHBE cells, which elevated NF- κ B activity by causing ubiquitin-dependent I κ B degradation (28). The mechanism through which OA-NO₂ blocks such I κ B phosphorylation is unknown, but it could be similar to that of other

electrophilic lipids, which inhibit I κ B kinase by alkylating a key cysteine residue (43–45). NFAs can also inhibit NF- κ B by alkylating a specific cysteine thiol within its DNA-binding domain (11).

We find that OA-NO₂ also inhibits NF- κ B via PPAR γ activation, which likewise downregulates NF- κ B activity by multiple mechanisms. Classically, activated PPAR γ competes for the same coactivators required by NF- κ B and other proinflammatory transcription factors (26). In addition, phosphorylated PPAR γ may inhibit NF- κ B by binding to it directly (27), and SUMOylated PPAR γ may prevent dissociation of corepressors from DNA-bound NF- κ B (25). Also, PPAR γ was found to bind via its ligand-binding domain to NF- κ B, triggering ubiquitination and degradation of the NF- κ B p65 subunit, with such binding being enhanced by PPAR γ agonists (46). In agreement, we found that PPAR γ binds to NF- κ B. Moreover, the PPAR γ 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 PPAR γ agonist (10) and to inhibit NF- κ B 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 PPAR γ -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.

References

- Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyper-responsiveness. *Annu. Rev. Immunol.* 17: 255–281.
- Ying, S., G. Zhang, S. Gu, and J. Zhao. 2006. How much do we know about atopic asthma: where are we now? *Cell. Mol. Immunol.* 3: 321–332.
- Adcock, I. M., and K. Ito. 2004. Steroid resistance in asthma: a major problem requiring novel solutions or a non-issue? *Curr. Opin. Pharmacol.* 4: 257–262.
- Barnes, P. J. 2006. Corticosteroids: the drugs to beat. *Eur. J. Pharmacol.* 533: 2–14.
- Chanez, P., S. E. Wenzel, G. P. Anderson, J. M. Anto, E. H. Bel, L. P. Boulet, C. E. Brightling, W. W. Busse, M. Castro, B. Dahlen, et al. 2007. Severe asthma in adults: what are the important questions? *J. Allergy Clin. Immunol.* 119: 1337–1348.
- Reddy, R. C. 2008. Severe asthma: approach and management. *Postgrad. Med. J.* 84: 115–120.
- O'Donnell, V. B., J. P. Eiserich, A. Bloodsworth, P. H. Chumley, M. Kirk, S. Barnes, V. M. Darley-Usmar, and B. A. Freeman. 1999. Nitration of unsaturated fatty acids by nitric oxide-derived reactive species. *Methods Enzymol.* 301: 454–470.
- Baker, P. R. S., F. J. Schopfer, S. Sweeney, and B. A. Freeman. 2004. Red cell membrane and plasma linoleic acid nitration products: synthesis, clinical identification, and quantitation. *Proc. Natl. Acad. Sci. USA* 101: 11577–11582.
- Baker, P. R., Y. Lin, F. J. Schopfer, S. R. Woodcock, A. L. Groeger, C. Batthyany, S. Sweeney, M. H. Long, K. E. Iles, L. M. Baker, et al. 2005. Fatty acid transduction of nitric oxide signaling: multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands. *J. Biol. Chem.* 280: 42464–42475.
- Schopfer, F. J., Y. Lin, P. R. S. Baker, T. Cui, M. Garcia-Barrio, J. Zhang, K. Chen, Y. E. Chen, and B. A. Freeman. 2005. Nitrolinoleic acid: an endoge-

- nous peroxisome proliferator-activated receptor γ ligand. *Proc. Natl. Acad. Sci. USA* 102: 2340–2345.
11. Cui, T., F. J. Schopfer, J. Zhang, K. Chen, T. Ichikawa, P. R. Baker, C. Batthyany, B. K. Chacko, X. Feng, R. P. Patel, et al. 2006. Nitrated fatty acids: Endogenous anti-inflammatory signaling mediators. *J. Biol. Chem.* 281: 35686–35698.
 12. Coles, B., A. Bloodsworth, S. R. Clark, M. J. Lewis, A. R. Cross, B. A. Freeman, and V. B. O'Donnell. 2002. Nitrooleate inhibits superoxide generation, degranulation, and integrin expression by human neutrophils: novel anti-inflammatory properties of nitric oxide-derived reactive species in vascular cells. *Circ. Res.* 91: 375–381.
 13. Kansanen, E., G. Bonacci, F. J. Schopfer, S. M. Kuosmanen, K. I. Tong, H. Leinonen, S. R. Woodcock, M. Yamamoto, C. Carlberg, S. Ylä-Herttuala, et al. 2011. Electrophilic nitro-fatty acids activate NRF2 by a KEAP1 cysteine 151-independent mechanism. *J. Biol. Chem.* 286: 14019–14027.
 14. Reddy, A. T., S. P. Lakshmi, and R. C. Reddy. 2012. The Nitrated Fatty Acid 10-Nitro-oleate Diminishes Severity of LPS-Induced Acute Lung Injury in Mice. *PPAR Res.* 2012: 617063.
 15. Borniquel, S., E. A. Jansson, M. P. Cole, B. A. Freeman, and J. O. Lundberg. 2010. Nitrated oleic acid up-regulates PPARgamma and attenuates experimental inflammatory bowel disease. *Free Radic. Biol. Med.* 48: 499–505.
 16. Rudolph, V., T. K. Rudolph, G. Bonacci, S. R. Woodcock, M. P. Cole, P. R. Baker, R. Ramani, and B. A. Freeman. 2010. Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion. *Cardiovasc. Res.* 85: 155–166.
 17. Liu, H., Z. Jia, S. Soodvilai, G. Guan, M. H. Wang, Z. Dong, J. D. Symons, and T. Yang. 2008. Nitro-oleic acid protects the mouse kidney from ischemia and reperfusion injury. *Am. J. Physiol. Renal Physiol.* 295: F942–F949.
 18. Reddy, A. T., S. P. Lakshmi, and R. C. Reddy. 2012. Murine model of allergen induced asthma. *J. Vis. Exp.* (63): e3771.
 19. Reddy, A. T., S. P. Lakshmi, J. M. Kleinhenz, R. L. Sutliff, C. M. Hart, and R. C. Reddy. 2012. Endothelial cell peroxisome proliferator-activated receptor γ reduces endotoxemic pulmonary inflammation and injury. *J. Immunol.* 189: 5411–5420.
 20. Xu, L., X. W. Dong, L. L. Shen, F. F. Li, J. X. Jiang, R. Cao, H. Y. Yao, H. J. Shen, Y. Sun, and Q. M. Xie. 2012. Simvastatin delivery via inhalation attenuates airway inflammation in a murine model of asthma. *Int. Immunopharmacol.* 12: 556–564.
 21. Lakshmi, S. P., A. T. Reddy, M. U. Naik, U. P. Naik, and R. C. Reddy. 2012. Effects of JAM-A deficiency or blocking antibodies on neutrophil migration and lung injury in a murine model of ALL. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 303: L758–L766.
 22. Luo, Y., and M. E. Dorf. 2001. Isolation of mouse neutrophils. *Curr. Protoc. Immunol.* Chapter 3: Unit 3.20.
 23. Barnes, P. J. 2006. Transcription factors in airway diseases. *Lab. Invest.* 86: 867–872.
 24. Barnes, P. J., and I. M. Adcock. 2003. How do corticosteroids work in asthma? *Ann. Intern. Med.* 139: 359–370.
 25. Pascual, G., A. L. Fong, S. Ogawa, A. Gamliel, A. C. Li, V. Perissi, D. W. Rose, T. M. Willson, M. G. Rosenfeld, and C. K. Glass. 2005. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* 437: 759–763.
 26. Li, M., G. Pascual, and C. K. Glass. 2000. Peroxisome proliferator-activated receptor gamma-dependent repression of the inducible nitric oxide synthase gene. *Mol. Cell. Biol.* 20: 4699–4707.
 27. Chen, F., M. Wang, J. P. O'Connor, M. He, T. Tripathi, and L. E. Harrison. 2003. Phosphorylation of PPARgamma via active ERK1/2 leads to its physical association with p65 and inhibition of NF-kappaB. *J. Cell. Biochem.* 90: 732–744.
 28. Karin, M., and Y. Ben-Neriah. 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu. Rev. Immunol.* 18: 621–663.
 29. Reddy, R. C., V. R. Narala, V. G. Keshamouni, J. E. Milam, M. W. Newstead, and T. J. Standiford. 2008. Sepsis-induced inhibition of neutrophil chemotaxis is mediated by activation of peroxisome proliferator-activated receptor-gamma. *Blood* 112: 4250–4258.
 30. Majai, G., Z. Sarang, K. Csomós, G. Zahuczky, and L. Fésüs. 2007. PPARgamma-dependent regulation of human macrophages in phagocytosis of apoptotic cells. *Eur. J. Immunol.* 37: 1343–1354.
 31. Bratton, D. L., and P. M. Henson. 2011. Neutrophil clearance: when the party is over, clean-up begins. *Trends Immunol.* 32: 350–357.
 32. Birrell, M. A., H. J. Patel, K. McCluskie, S. Wong, T. Leonard, M. H. Yacoub, and M. G. Belvisi. 2004. PPAR-gamma agonists as therapy for diseases involving airway neutrophilia. *Eur. Respir. J.* 24: 18–23.
 33. Asada, K., S. Sasaki, T. Suda, K. Chida, and H. Nakamura. 2004. Anti-inflammatory roles of peroxisome proliferator-activated receptor γ in human alveolar macrophages. *Am. J. Respir. Crit. Care Med.* 169: 195–200.
 34. Reddy, R. C. 2008. Immunomodulatory role of PPAR-gamma in alveolar macrophages. *J. Investig. Med.* 56: 522–527.
 35. Cox, G. 1995. Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *J. Immunol.* 154: 4719–4725.
 36. Sun, L., A. C. Nicholson, D. P. Hajjar, A. M. Gotto, Jr., and J. Han. 2003. Adipogenic differentiating agents regulate expression of fatty acid binding protein and CD36 in the J774 macrophage cell line. *J. Lipid Res.* 44: 1877–1886.
 37. McCubrey, A. L., J. Sonstein, T. M. Ames, C. M. Freeman, and J. L. Curtis. 2012. Glucocorticoids relieve collectin-driven suppression of apoptotic cell uptake in murine alveolar macrophages through downregulation of SIRP α . *J. Immunol.* 189: 112–119.
 38. European Network for Understanding Mechanisms of Severe Asthma. 2003. The ENFUMOSA cross-sectional European multicentre study of the clinical phenotype of chronic severe asthma. *Eur. Respir. J.* 22: 470–477.
 39. Wenzel, S. E., S. J. Szefer, D. Y. Leung, S. I. Sloan, M. D. Rex, and R. J. Martin. 1997. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. *Am. J. Respir. Crit. Care Med.* 156: 737–743.
 40. Jatakanon, A., C. Uasuf, W. Maziak, S. Lim, K. F. Chung, and P. J. Barnes. 1999. Neutrophilic inflammation in severe persistent asthma. *Am. J. Respir. Crit. Care Med.* 160: 1532–1539.
 41. ten Brinke, A., A. H. Zwinderman, P. J. Sterk, K. F. Rabe, and E. H. Bel. 2004. “Refractory” eosinophilic airway inflammation in severe asthma: effect of parenteral corticosteroids. *Am. J. Respir. Crit. Care Med.* 170: 601–605.
 42. Barnes, P. J., and M. Karin. 1997. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* 336: 1066–1071.
 43. Rossi, A., P. Kapahi, G. Natoli, T. Takahashi, Y. Chen, M. Karin, and M. G. Santoro. 2000. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. *Nature* 403: 103–108.
 44. Ji, C., K. R. Kozak, and L. J. Marnett. 2001. IkappaB kinase, a molecular target for inhibition by 4-hydroxy-2-nonenal. *J. Biol. Chem.* 276: 18223–18228.
 45. Musiek, E. S., J. D. Brooks, M. Joo, E. Brunoldi, A. Porta, G. Zanon, G. Vidari, T. S. Blackwell, T. J. Montine, G. L. Milne, et al. 2008. Electrophilic cyclopentenone neuroprostanes are anti-inflammatory mediators formed from the peroxidation of the omega-3 polyunsaturated fatty acid docosahexaenoic acid. *J. Biol. Chem.* 283: 19927–19935.
 46. Hou, Y., F. Moreau, and K. Chadee. 2012. PPARgamma is an E3 ligase that induces the degradation of NFkappaB/p65. *Nat. Commun.* 3: 1300.

Supplemental Figure 1. Fluticasone Inhibits TNF- α -induced Upregulation of NF- κ B

Activity But Has No Effect on PPAR γ . NHBE cells were treated with OA-NO₂ (100 nM) or

DMSO (Veh) for 30 min and then exposed to TNF- α (10 ng/ml) or PBS as a control for 6 h.

Cells were then harvested and DNA-binding activity of (A) PPAR γ and (B) NF- κ B was

measured. (C) Nuclear (NE) and cytoplasmic (CE) extracts of treated cells were prepared and the

expression of PPAR γ and NF- κ B p65 were determined by Western blotting. Data are

representative of 2 independent experiments with $n = 3$; * $P < 0.05$; *** $P < 0.001$.

Supplemental table 1: Oligonucleotide primers employed.

Gene		Primer sequence	Tm	Amplicon Size bp
9s rRNA	F	5'-ATCCGCCAACGTCACATT-3'	57.6	115
	R	5'-CCGCCGCCATAAGGAGAAC-3'	64.5	
E-Selectin	F	5'-ATGCCTCGCGCTTTCTCTC-3'	62.4	128
	R	5'-GTAGTCCCGCTGACAGTATGC-3'	62.2	
GAPDH	F	5'-GGACGCATTGGTCGTCTGG-3'	63	204
	R	5'-TTTGCACTGGTACGTGTTGAT-3'	60.2	
ICAM-1	F	5'-GCTACCATCACCGTGTATTCG-3'	60.6	223
	R	5'-TAGCCAGCACCGTGAATGTG-3'	62.5	
NOS2	F	5'-ACATCGACCCGTCCACAGTAT-3'	62.7	177
	R	5'-CAGAGGGGTAGGCTTGTCTC-3'	61	
NOS3	F	5'-CGAAGCGTGTGAAGGCAAC-3'	62	250
	R	5'-TTGTACGGGCCTGACATTTCC-3'	62.4	
NOX 4	F	5'-TGCCTGCTCATTTGGCTGT-3'	62.2	180
	R	5'-CCGGCACATAGGTAAAAGGATG-3'	60.8	
VCAM-1	F	5'-TTGGGAGCCTCAACGGTACT-3'	62.7	86
	R	5'-GCAATCGTTTTGTATTCAGGGGA-3'	61.1	

Supplemental Figure 1

