

Roflumilast N-oxide reverses corticosteroid resistance in neutrophils from patients with chronic obstructive pulmonary disease

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Background: Glucocorticoid functions are markedly impaired in patients with chronic obstructive pulmonary disease (COPD). The phosphodiesterase 4 inhibitor roflumilast N-oxide (RNO) is the active metabolite of roflumilast approved as a treatment to reduce the risk of exacerbations in patients with severe COPD. **Objective:** We sought to characterize the differential effects of RNO versus corticosteroids and their potential additive/synergistic effect in neutrophils from patients with COPD, thus providing scientific rationale for the combination of roflumilast with corticosteroids in the clinic.

Methods: Peripheral blood neutrophils were isolated from patients with COPD (n = 32), smokers (n = 7), and healthy nonsmokers (n = 25). Levels of IL-8, matrix metalloproteinase 9 (MMP-9), and biomarkers of glucocorticoid resistance were determined by using ELISA and RT-PCR. Neutrophils were incubated with dexamethasone (0.1 nmol/L to 1 μ mol/L), RNO (0.1 nmol/L to 1 μ mol/L), or the combination of 1 nmol/L RNO plus 10 nmol/L DEX and stimulated with LPS (1 μ g/mL) or cigarette smoke extract 5%; levels of IL-8, MMP-9, and other biomarkers were measured at the end of the incubation period. **Results:** Peripheral neutrophils from patients with COPD showed a primed phenotype with an increased basal release of IL-8 and MMP-9 and expressed a corticosteroid resistance molecular profile characterized by an increase in phosphoinositide 3-kinase δ , macrophage migration inhibitory factor, and glucocorticoid receptor β expression and a decrease in HDAC activity and mitogen-activated protein kinase phosphatase 1 expression. RNO demonstrated robust anti-inflammatory effects on neutrophils from patients with COPD, reversing their resistance to corticosteroids. The combination of RNO and dexamethasone showed additive/synergistic effects,

which were consistent with the reversal of corticosteroid-resistant molecular markers by RNO.

Conclusion: RNO reverses corticosteroid resistance and shows strong anti-inflammatory effects alone or in combination with corticosteroids on neutrophils from patients with COPD. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Roflumilast, dexamethasone, corticosteroid resistance, neutrophil, chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a debilitating lung disease and a major cause of morbidity and mortality with a high and increasing prevalence. It is already the fourth leading cause of death worldwide and the third leading cause in the United States. Given its long disease latency, its effect will continue to increase in coming decades.¹ The primary cause of COPD is chronic exposure to cigarette smoke, which leads to airway inflammation and remodeling, thus increasing airflow limitation. The current first-line maintenance treatment for COPD involves the use of bronchodilators, including long-acting muscarinic antagonists and long-acting β -agonists.

Although inhaled corticosteroids have shown good activity in asthmatic patients, they are much less effective in improving lung function and have little or no effect on controlling the underlying chronic inflammation seen in patients with COPD.² The persistent innate immune activation is a characteristic feature of COPD, and current anti-inflammatory therapies based on corticosteroids show poor activity in regulating the innate immune responses, thus limiting their clinical effectiveness.³ Several mechanisms and mediators have been implicated in the development of corticosteroid resistance, including reduced histone deacetylase 2 (HDAC2) expression and activity, impaired corticosteroid activation of the glucocorticoid receptor (GR), and increased proinflammatory signaling pathways in patients with COPD.³

Several new emerging classes of anti-inflammatory drugs are in development for COPD treatment, such as inhibitors of phosphoinositide 3-kinase δ (PI3K δ), phosphodiesterase 4 (PDE4), p38 mitogen-activated protein kinase, and nuclear factor κ B (NF- κ B). Roflumilast is the first PDE4 inhibitor approved for COPD. It is indicated as a treatment to reduce the risk of COPD exacerbations in patients with severe COPD associated with chronic bronchitis and a history of exacerbations.

The PDE4 isoenzymes are the predominant PDEs expressed in leukocytes, including basophils, eosinophils, macrophages, mast cells, monocytes, neutrophils, and T lymphocytes, in which adenosine 3',5'-cyclic monophosphate is known to modulate the responses to proinflammatory agents.⁴ Roflumilast is a potent and selective inhibitor of PDE4 that regulates intracellular adenosine 3',5'-cyclic monophosphate levels.⁵ In preclinical models roflumilast and its active metabolite, roflumilast N-oxide

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Abbreviations used

COPD:	Chronic obstructive pulmonary disease
CSE:	Cigarette smoke extract
DPI:	Diphenylethiodonium
ERK:	Extracellular signal-regulated kinase
fMLP:	<i>N</i> -formyl-Met-Leu-Phe
GOLD:	Global Initiative for Chronic Obstructive Lung Disease
GR:	Glucocorticoid receptor
HDAC2:	Histone deacetylase 2
H ₂ DCF-DA:	2', 7'-Dichlorodihydrofluorescein diacetate
MIF:	Macrophage migration inhibitory factor
MKP-1:	Mitogen-activated protein kinase phosphatase 1
MMP-9:	Matrix metalloproteinase 9
NAC:	<i>N</i> -acetyl-L-cysteine
PDE4:	Phosphodiesterase 4
p-ERK1/2:	Phosphorylated ERK1/2
PI3Kδ:	Phosphoinositide 3-kinase δ
RNO:	Roflumilast N-oxide
ROS:	Reactive oxygen species

(RNO), have been shown to inhibit the production of inflammatory mediators, such as reactive oxygen species (ROS), leukotriene B₄, TNF- α , IL-2, IL-4, IL-5, and IFN- γ , by human neutrophils, eosinophils, monocytes, and lymphocytes after stimulation with different agents.⁵ In clinical studies roflumilast treatment has been shown to reduce neutrophil and eosinophil numbers in sputum from patients with COPD.⁶

The goal of the current study was to characterize the differential effects of RNO versus corticosteroids and their potential additive/synergistic effect in neutrophils from patients with COPD. Neutrophils are thought to play a key role in the pathophysiology of COPD and almost exclusively express PDE4.⁴ Neutrophils secrete a wide range of proinflammatory cytokines and chemokines, as well as tissue-destructive proteases,⁷ and appear to be more resistant than other inflammatory cells to the anti-inflammatory effects of corticosteroids.⁸ PDE4 inhibitors, such as roflumilast, have been shown to inhibit the release of ROS, leukotriene B₄, neutrophil elastase, and matrix metalloproteinase 9 (MMP-9) from human peripheral blood neutrophils and prevent neutrophil tissue infiltration.⁹ The exploration of the differential effects of roflumilast on neutrophils versus corticosteroids could explain the unique effect of roflumilast on patients with COPD. Moreover, a potential additive/synergistic effect between roflumilast and corticosteroids might provide a scientific rationale for the combination of roflumilast with corticosteroids in the clinic.

METHODS

Peripheral neutrophils and whole blood were obtained from patients with mild-to-moderate COPD ($n = 32$) and nonsmoking subjects ($n = 25$). Clinical features of the study subjects are defined in the [Methods](#) section and [Table E1](#) in this article's Online Repository at www.jacionline.org. This project was approved by the local ethics committee of General University Hospital, Valencia, Spain, and written informed consent was obtained from each patient or volunteer before starting blood sampling and lung function testing. See the [Methods](#) section in this article's Online Repository for details.

For information on human neutrophil isolation, preparation of cigarette smoke extract (CSE), HDAC, and PI3K activities, ROS analysis, real-time quantitative RT-PCR (see [Table E2](#) in this article's Online Repository at www.jacionline.org for primer design and characteristics), measurement of IL-8 and MMP-9 by means of ELISA and LUMINEX, antibodies, Western blotting, or

neutrophil experimental conditions, see the [Methods](#) section in this article's Online Repository.

RESULTS**Basal activation and response to bacterial LPSs and cigarette smoke by neutrophils from patients with COPD**

Peripheral blood neutrophils isolated from patients with COPD showed a higher basal IL-8 and MMP-9 release than that of healthy subjects. Furthermore, the bacterial endotoxin LPS (1 μ g/mL) or the oxidant CSE 5% elicited an increase in IL-8 and MMP-9 release that was significantly higher in patients with COPD than in healthy subjects (see [Fig E1](#) in this article's Online Repository at www.jacionline.org).

Because PDE4 enzymes regulate important neutrophil cell functions, we next explored the expression of the 4 different PDE4 isoenzymes. Significantly higher mRNA levels were found for PDE4B and PDE4D, but not for PDE4A or PDE4C, in neutrophils from patients with COPD compared with healthy subjects ([Fig 1, A-D](#)).

Gene expression and activity of molecular markers of corticosteroid resistance at baseline were analyzed in peripheral blood neutrophils from healthy subjects and patients with COPD. Gene expression of HDAC2 was found to be unaltered in neutrophils from patients with COPD compared with that seen in healthy nonsmoking subjects ([Fig 1, E](#)). However, HDAC activity was downregulated in the COPD group ([Fig 1, F](#)). Gene expression of PI3K δ , an upstream regulator of HDAC2, and PI3K activity were increased in neutrophils from patients with COPD compared with that seen in healthy subjects ([Fig 1, G and H](#)). Moreover, the expression of mitogen-activated protein kinase phosphatase 1 (MKP-1) was downregulated, and its natural inhibitor macrophage migration inhibitory factor (MIF) was upregulated in neutrophils from patients with COPD compared with values seen in healthy subjects ([Fig 1, I and J](#)). Low expression of GR in neutrophils from patients with COPD was recently reported as another potential molecular basis for corticosteroid insensitivity.¹⁰ In the current study we observed no difference in GR α expression in neutrophils from healthy subjects and those from patients with COPD. However, GR β expression was significantly increased in neutrophils from patients with COPD compared with that seen in healthy subjects ([Fig 1, K and L](#)). On the other hand, corticosteroids might be exported by the common efflux transporter P-glycoprotein ABCB1 (MDR1), thus providing another possible mechanism of corticosteroid resistance. In this study no difference of ABCB1 transporter expression was observed between the 2 groups ([Fig 1, M](#)).

Oxidative stress and PI3K activation are required for mediating corticosteroid insensitivity in neutrophils from patients with COPD

To investigate the potential mediators of corticosteroid insensitivity of neutrophils from patients with COPD, we studied the effects of PI3K and ROS activation on IL-8 and MMP-9 releases. The release of IL-8 and MMP-9 after LPS or CSE stimulation was nearly abrogated by the antioxidant *N*-acetyl-L-cysteine (NAC; 1 mmol/L) in neutrophils from patients with COPD ([Fig 2](#)). In addition, the nicotinamide adenine dinucleotide phosphate inhibitor

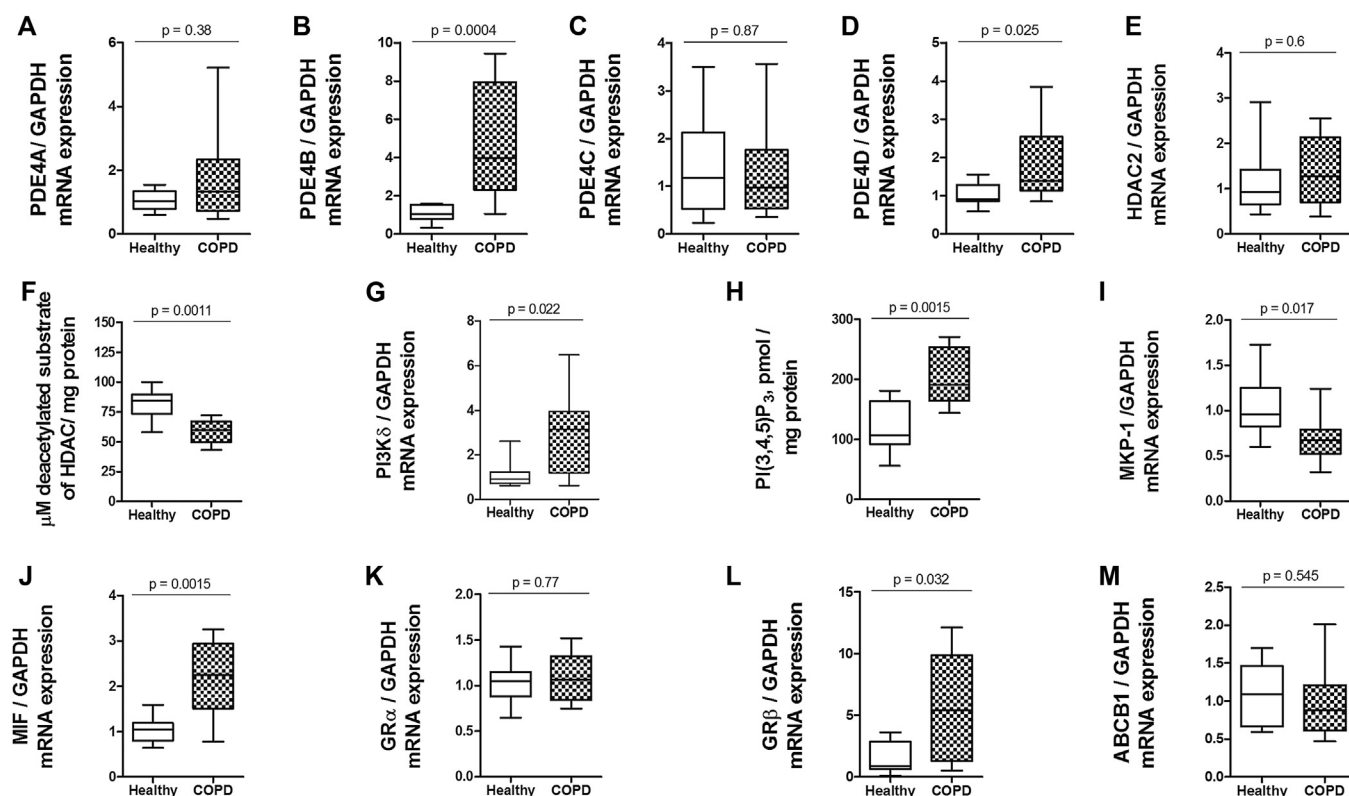


FIG 1. Basal expression of putative corticosteroid-resistant markers in neutrophils from healthy subjects and patients with COPD. Peripheral blood neutrophils from healthy subjects ($n = 15$) and patients with COPD ($n = 15$) are shown. Data are presented as a box and whisker plot with median, interquartile range, and minimum and maximum values. *P* values were obtained by using the Mann-Whitney test. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

diphenyleneiodonium (DPI) at 1 μ M effectively inhibited IL-8 and MMP-9 release after both LPS and CSE stimulation to a level similar to that inhibited by NAC (data not shown). In contrast, dexamethasone (1 μ M) slightly inhibited IL-8 and MMP-9 secretion, and the PI3K paninhibitor LY-294002 (10 μ M) was without effect. Interestingly, the combination of dexamethasone (1 μ M) and LY-294002 (10 μ M) showed a synergistic inhibition of IL-8 and MMP-9 release (Fig 2). By contrast, in neutrophils from healthy subjects, 1 μ M dexamethasone nearly suppressed the IL-8 and MMP-9 release induced by LPS or CSE. The anti-inflammatory effect of dexamethasone was prevented by the GR antagonist RU-486 (1 μ M), confirming the specific effect of dexamethasone (see Fig E2 in this article's Online Repository at www.jacionline.org).

Inhibition of neutrophil response by RNO and dexamethasone

In neutrophils from healthy subjects, RNO (0.1 nmol/L to 1 μ M) and dexamethasone (0.1 nmol/L to 1 μ M) concentration-dependently inhibited LPS-induced (1 μ g/mL) and CSE 5%-induced IL-8 secretion with similar efficacy and potency (Fig 3, A and B; see Table E3 in this article's Online Repository at www.jacionline.org). In neutrophils from patients with COPD, RNO (0.1 nmol/L to 1 μ M) maintained its efficacy and 50% inhibition comparable with values observed in healthy subjects. In contrast, neutrophils from patients with COPD were

refractory to the anti-inflammatory effect of corticosteroids. Dexamethasone (0.1 nmol/L to 1 μ M) only reached a maximal percentage of inhibition of $15\% \pm 4.4\%$ and $20.6\% \pm 12.1\%$ in patients with COPD for the LPS (1 μ g/mL) and CSE 5% stimuli, respectively, which is significantly lower than values observed in neutrophils from healthy subjects (Fig 3, A and B; see Table E3).

Similar results were observed for MMP-9 (Fig 3, C and D; see Table E3). Although both RNO and dexamethasone effectively inhibited MMP-9 release from neutrophils from healthy subjects, only RNO had a strong inhibitory effect on MMP-9 release from neutrophils of patients with COPD.

The number of neutrophils in peripheral blood from patients with COPD might reflect the results. In this respect we did not observe any significant correlation between the number of peripheral neutrophils and the maximal percentage of inhibition of dexamethasone (Spearman $\rho = 0.23$, $P = .1210$).

Effect of RNO plus dexamethasone on neutrophil response

We next explored the anti-inflammatory properties of the combination of RNO (1 nmol/L) and dexamethasone (10 nmol/L), both at suboptimal concentrations, in neutrophils from patients with COPD. RNO (1 nmol/L) or dexamethasone (10 nmol/L) alone showed no inhibitory effect on IL-8 or MMP-9 release from neutrophils of patients with COPD. However, the combination of RNO with dexamethasone showed

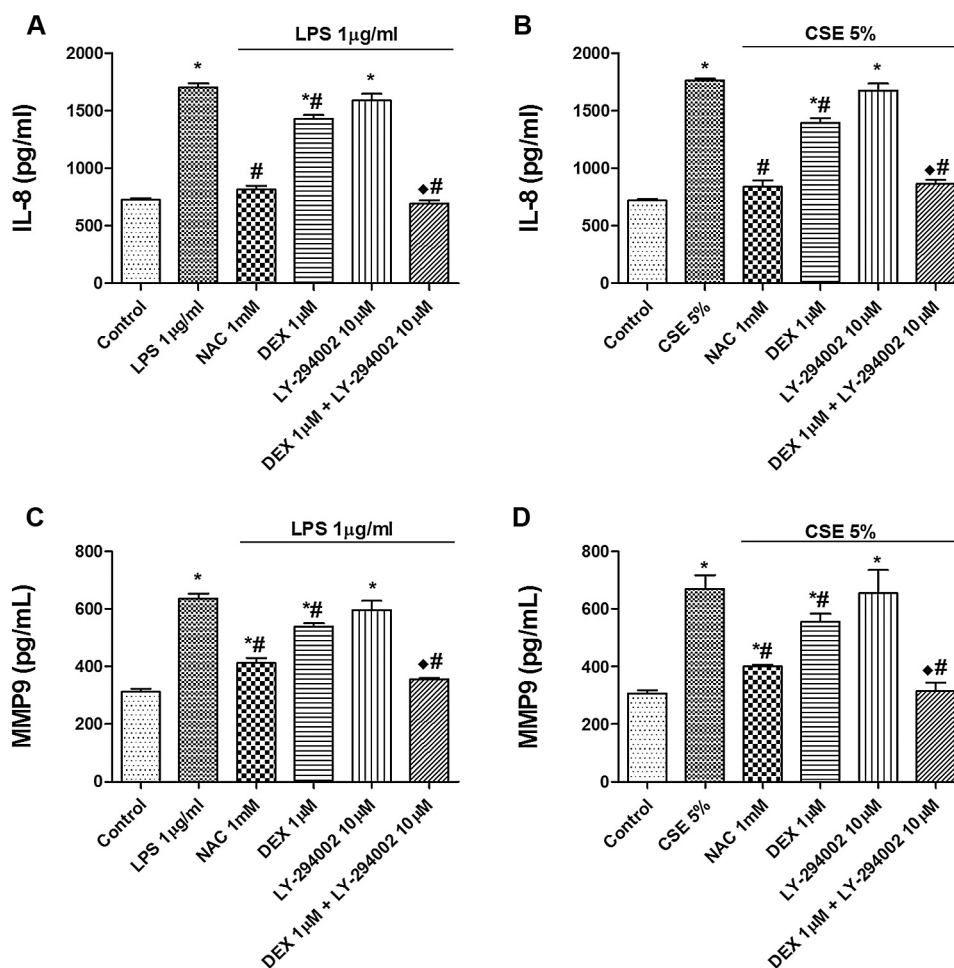


FIG 2. Peripheral blood neutrophils were isolated from patients with COPD and incubated with NAC (1 mmol/L), dexamethasone (DEX; 1 µmol/L), LY-294002 (10 µmol/L), or combinations for 1 hour, followed by stimulation with LPS (1 µg/mL) or CSE 5%. Results are expressed as means $\pm$ SEs of 3 (3 cell population) independent experiments. Two-way ANOVA followed by *post hoc* Bonferroni tests: * $P < .05$ related to control; # $P < .05$ related to LPS or CSE; ♦ $P < .05$ related to drugs alone.

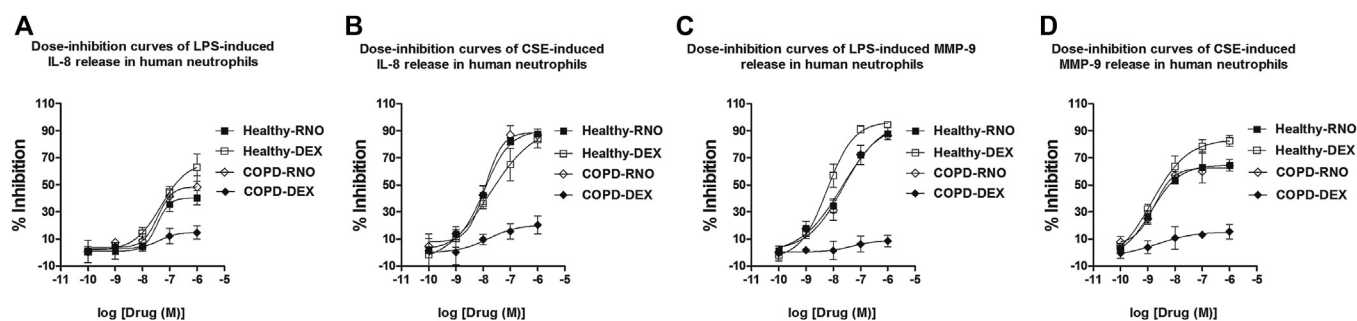


FIG 3. Concentration-dependent inhibition of LPS- or CSE-induced IL-8 or MMP-9 release by RNO or dexamethasone (DEX) from neutrophils of healthy subjects and patients with COPD. Neutrophils were pre-incubated with RNO (0.1 nmol/L to 1 µmol/L) or DEX (0.1 nmol/L to 1 µmol/L) for 1 hour, followed by cell stimulation with LPS (1 µg/mL; A and B and E and F) or CSE 5% (C and D and G and H) for 6 hours. Results are expressed as means $\pm$ SEs of 3 (3 healthy subject and 3 patient with COPD cell populations) independent experiments.

synergistic effects, resulting in nearly 50% inhibition of LPS- and CSE-induced IL-8 release (Fig 4, A and B). Furthermore, these combinations fully suppressed MMP-9 release in response to

both stimuli (Fig 4, C and D). These observations are consistent with the reported clinical benefits for the combination of roflumilast with inhaled corticosteroids.¹¹

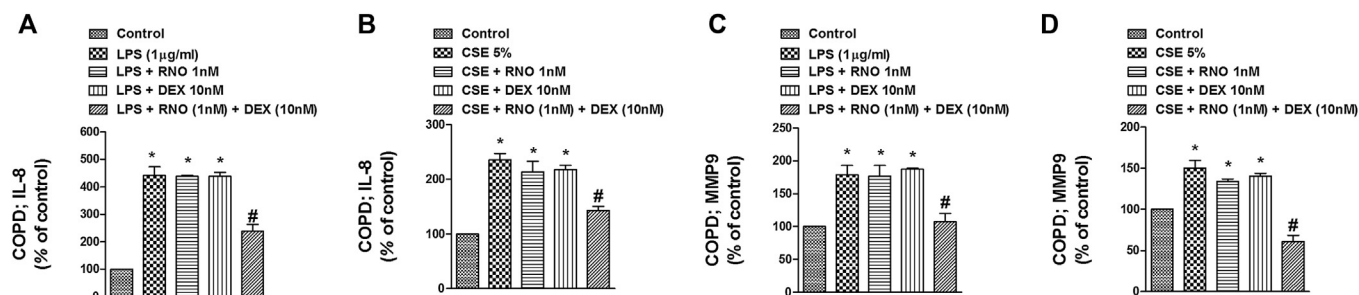


FIG 4. Additive/synergistic effects of RNO with dexamethasone (DEX) on IL-8 and MMP-9 secretion in neutrophils from patients with COPD. Results are expressed as means $\pm$ SEs of 3 (3 cell populations) independent experiments per condition. Two-way ANOVA, followed by *post hoc* Bonferroni tests: * $P < .05$ related to control; # $P < .05$ related to stimulus and single RNO or DEX exposure.

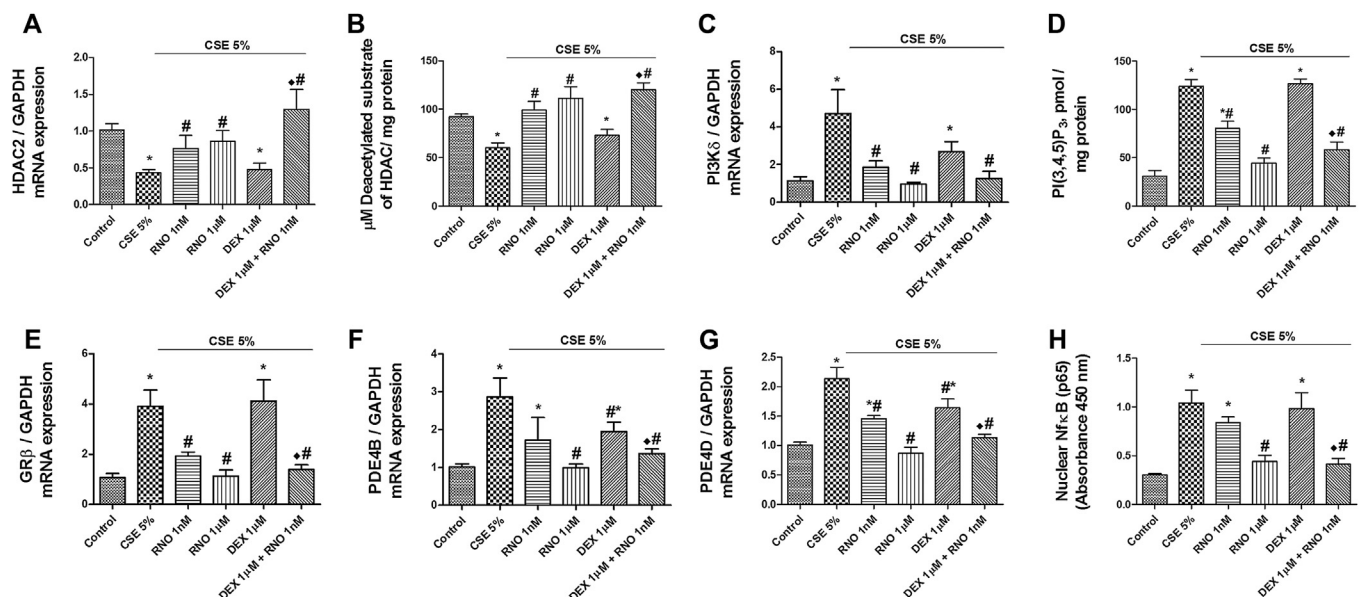


FIG 5. Effects of RNO and dexamethasone (DEX) on CSE-induced corticosteroid-resistant markers. Human peripheral blood neutrophils from patients with COPD were isolated and plated on 24-well plates. Cells were incubated with RNO (1 nmol/L or 1 µmol/L), DEX (1 µmol/L), or their combination for 1 hour and stimulated with CSE. Results are expressed as means $\pm$ SEs in 3 (3 to 6 cell populations) independent experiments. Two-way ANOVA, followed by *post hoc* Bonferroni tests. * $P < .05$ related to control expression; # $P < .05$ related to CSE; ♦ $P < .05$ related to 1 nmol/L RNO or 1 µmol/L DEX alone. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Role of RNO on corticosteroid-resistant markers and PDE4 expression altered by cigarette smoke in neutrophils from patients with COPD

The effects of RNO on molecular markers of corticosteroid resistance in neutrophils from patients with COPD were determined. CSE 5% decreased HDAC2 expression and HDAC activity and increased PI3Kδ expression and activity (Fig 5, A-D). In contrast to dexamethasone at 1 µmol/L, RNO (1 nmol/L and 1 µmol/L) significantly attenuated the effects of CSE on these markers. The combination of 1 nmol/L RNO with 1 µmol/L dexamethasone showed additive effects, inhibiting the CSE-induced PI3Kδ upregulation and HDAC2 downregulation (Fig 5, A-D).

Stimulation of neutrophils from patients with COPD with CSE induced a further increase in GRβ expression. This effect was blocked by RNO (1 nmol/L and 1 µmol/L) but not by 1 µmol/L

dexamethasone. The combination of 1 nmol/L RNO with 1 µmol/L dexamethasone showed additive effects (Fig 5, E).

In other experiments RNO and dexamethasone attenuated the increase in PDE4B and PDE4D levels, and the combination of low doses of 1 nmol/L RNO with 1 µmol/L dexamethasone showed additive effects (Fig 5, F and G). In addition, CSE significantly increased nuclear NF-κB (p65) levels, which were inhibited by 1 µmol/L RNO but not by 1 µmol/L dexamethasone. The combination of RNO at suboptimal doses of 1 nmol/L with 1 µmol/L dexamethasone showed synergistic effects (Fig 5, H).

Neutrophils from patients with COPD stimulated with CSE 5% showed significantly reduced MKP-1 expression associated with increased phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 (Fig 6, A and B). Furthermore, expression of MIF, the endogenous inhibitor of MKP-1, was increased in response to CSE 5% (Fig 6, C). Dexamethasone was unable to

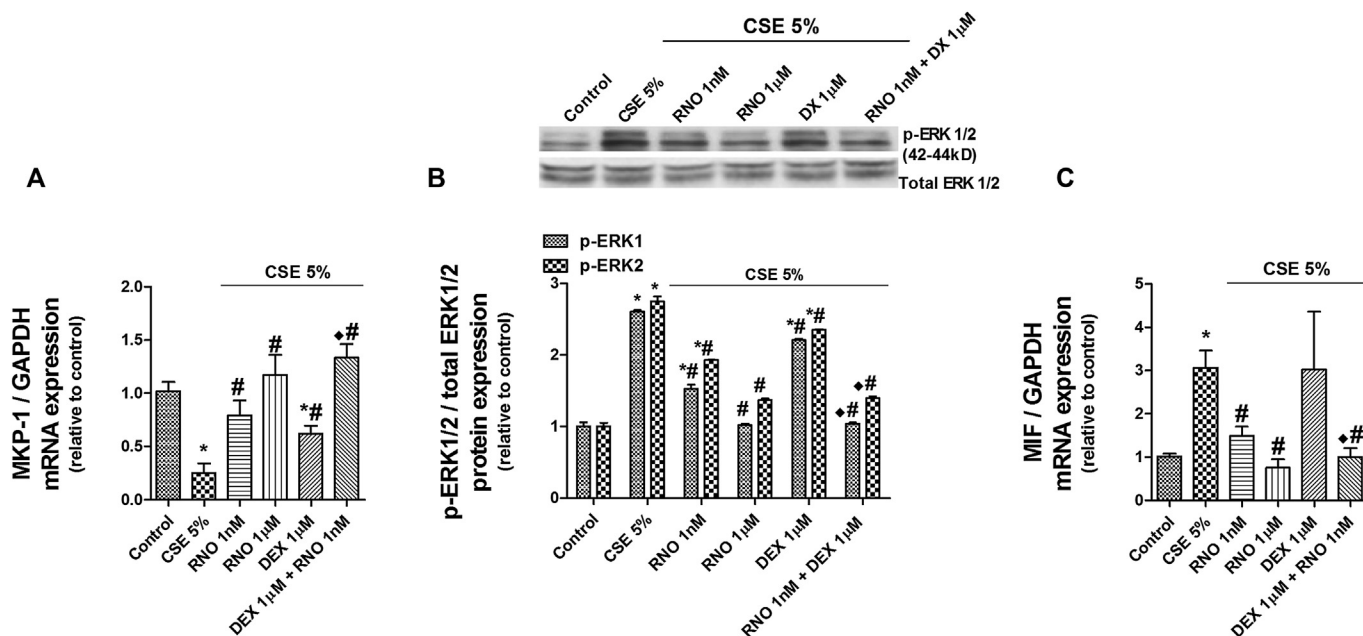


FIG 6. Effects of RNO and dexamethasone (DEX) on CSE-induced markers of corticosteroid resistance. Human peripheral blood neutrophils from patients with COPD were isolated and plated on 24-well plates. Cells were incubated with RNO (1 nmol/L or 1 µmol/L), DEX (1 µmol/L), or their combination for 1 hour and stimulated with CSE. mRNA levels for MKP-1 and MIF were measured, and phosphorylation of ERK1/2 was detected by means of Western blotting. Results are expressed as means $\pm$ SEs of 3 (3 to 6 cell populations) independent experiments. A representative picture for Western blots is shown in Fig 6, B. Two-way ANOVA, followed by *post hoc* Bonferroni tests: * $P < .05$ related to control; # $P < .05$ related to CSE; ♦ $P < .05$ related to 2 nmol/L RNO or 1 µmol/L DEX alone. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

reverse the downregulation of MKP-1 or the increased MIF expression and phosphorylated ERK1/2 (p-ERK1/2) levels induced by CSE (Fig 6, A-C). In contrast, RNO concentration-dependently reversed the CSE-induced effects on MKP-1 and MIF expression and p-ERK1/2 levels (Fig 6, A-C). Interestingly, the combination of low doses of RNO (1 nmol/L) with 1 µmol/L dexamethasone showed additive effects on MKP-1, MIF, and p-ERK1/2 (Fig 6, A-C). Furthermore, RNO reduced ROS levels in neutrophils from patients with COPD after inflammatory and oxidant stimulus. RNO (1 nmol/L to 1 µmol/L) concentration-dependently inhibited *N*-formyl-Met-Leu-Phe (fMLP) and CSE-induced intracellular ROS in neutrophils from patients with COPD, reaching a maximal inhibitory effect comparable with 1 mmol/L NAC (see Fig E3 in this article's Online Repository at www.jacionline.org) or 1 µmol/L DPI (data not shown).

DISCUSSION

The present work provides novel evidence for the mechanistic pathways mediating corticosteroid resistance in neutrophils from patients with COPD. RNO was shown to reverse the corticosteroid resistance, and furthermore, RNO, in combination with dexamethasone, showed additive/synergistic anti-inflammatory properties in neutrophils from patients with COPD. These results might explain the enhanced clinical benefits observed for the combination of inhaled corticosteroids and roflumilast in patients with COPD with chronic bronchitis.¹¹

Neutrophils play a critical role in the pathophysiology of COPD through the release of a number of mediators, including neutrophil elastase, MMP-9, IL-8, IL-1 β , and ROS. In patients

with emphysema, neutrophils shift the balance between protease and antiprotease in the lung in favor of lung destruction. The structural changes that ensue include loss of respiratory bronchioles, alveolar ducts, and alveoli. In patients with chronic bronchitis, inflammation is characterized by mucosal infiltration with neutrophils, macrophages, and lymphocytes, resulting in epithelial disruption, smooth muscle hypertrophy, and fibrosis. Studies have also shown that high-resolution computed tomographic indices of peripheral airways dysfunction correlate with neutrophil counts.¹² In addition, recent studies have reported that neutrophils in the peripheral blood of patients with COPD exhibit characteristics of a primed phenotype, with increased number and size of neutrophils derived from bone marrow stimulation and increased ROS production thus considered as indicators of the progression and severity of COPD.¹³⁻¹⁶ Peripheral neutrophils of patients with COPD have been shown to have enhanced chemotaxis and extracellular proteolysis and produce more ROS than those from nonsmokers and healthy smoking control subjects.^{13,17,18} Furthermore, antioxidant capacity has been shown to be reduced in smokers and patients with COPD compared with that seen in healthy nonsmoking control subjects¹⁹ and further reduced during severe exacerbations of COPD,²⁰ supporting a potential role for dysregulated ROS production in patients with COPD. In fact, a previous report from our group demonstrated significantly lower levels of thiols and higher levels of ROS in peripheral neutrophils from patients with COPD versus those seen in healthy subjects.²¹

In the current study we observed that basal secretion of IL-8 and MMP-9 was higher in blood neutrophils from patients with COPD, which were more primed to the release of IL-8 and

MMP-9 in response to an inflammatory (LPS) or oxidative (CSE) stimulus than neutrophils from healthy subjects. Similar results have been observed previously in peripheral blood neutrophils from patients with COPD,^{21,22} supporting our findings. Therefore therapies targeted to inhibit persistent activation of neutrophils might be of potential value in patients with COPD.

In this work we observed a high maximal percentage of inhibition (efficacy) and median effective concentration (potency) for dexamethasone, inhibiting IL-8 and MMP-9 release in neutrophils from healthy subjects. However, the effects of dexamethasone were dramatically reduced in neutrophils from patients with COPD. In contrast to dexamethasone, the PDE4 inhibitor RNO showed comparable efficacy and potency to reduce LPS- or CSE-induced IL-8 and MMP-9 release from neutrophils of healthy subjects and patients with COPD. PDE4 inhibitors have shown potent anti-inflammatory properties on human neutrophils.²³ Furthermore, roflumilast effectively inhibited cigarette smoke-induced lung emphysema in mice, probably through the inhibition of neutrophil trafficking into the lungs.²⁴ Interestingly, roflumilast also reduced neutrophil numbers in the sputum of patients with COPD.⁶ Therefore roflumilast has shown potent *in vitro* and *in vivo* inhibitory actions on neutrophils.

The combination of a low concentration of RNO (1 nmol/L, which is close to therapeutic plasma levels) with dexamethasone (10 nmol/L) synergistically decrease IL-8 and MMP-9 release from neutrophils from patients with COPD in response to bacterial LPS or CSE exposure, suggesting that RNO might overcome the corticosteroid-resistant mechanisms in patients with COPD. Similar results (although not in corticosteroid-resistant conditions) were recently observed in bronchial epithelial cells.²⁵ In this regard roflumilast and its active metabolite RNO, as well as other PDE4 inhibitors and the β_2 -adrenergic agonist formoterol, showed additive/synergistic properties when they were added to corticosteroids, enhancing glucocorticoid response element transcription, as well as corticosteroid-inducible anti-inflammatory gene expression, thus supporting our findings.

Currently, multiple mechanisms have been implicated in contributing to the loss of the anti-inflammatory properties of corticosteroids.²⁶ One of the most studied mechanisms is the downregulation and loss of HDAC2 activity in lung tissue and alveolar macrophages of patients with COPD.²⁷ Cigarette smoke and the oxidative/nitrative stress of chronic inflammation have been shown to trigger modifications of HDAC2, resulting in gene expression reduction and protein degradation of this enzyme.^{26,27} Mechanistically, HDAC2 is considered to translate some of the anti-inflammatory effects of corticosteroids by regulating the transcription and protein expression of some of the critical anti-inflammatory mediators downstream of corticosteroid receptor signaling.³ Although PDE4 inhibitors might not directly affect HDAC2,²⁸ they reduce ROS generation in various cellular systems, such as neutrophils, and attenuate PI3K/Akt signaling.²⁹ The latter seems to be augmented after CSE exposure and contributes to a specific phosphorylation of HDAC2 and its downregulation.³⁰ We found that the expression and activity of PI3K δ , which is predominantly expressed in leukocytes and regulates immune cell functions, was upregulated and HDAC activity was decreased in neutrophils from patients with COPD. RNO reversed CSE-induced PI3K δ expression and activity, as well as reduction in HDAC2 expression and HDAC activity. Furthermore, RNO reduced ROS levels from neutrophils after bacterial fMLP or

CSE. These findings are consistent with the hypothesis that ROS is the key mediator responsible for the PI3K δ induction and HDAC2 suppression driving corticosteroid insensitivity and that the PDE4 inhibitor exerted its effects and restored corticosteroid sensitivity in this experimental system by reducing ROS levels (see Fig E4 in this article's Online Repository at www.jacionline.org for a more detailed description on mechanistic pathways).

The transcription factor NF- κ B is activated by inflammation or oxidative stress, which translates to the nucleus and promotes the increased expression of a broad number of inflammatory genes. Corticosteroids, after activation of the GR, inhibit the NF- κ B complex with the help HDAC2, thus inhibiting NF- κ B-dependent inflammatory gene expression.

In patients with COPD, HDAC2 activity is reduced, which impairs the inhibitory effect of corticosteroids on NF- κ B, resulting in NF- κ B-dependent inflammation.³¹ In the current study we observed that CSE increased NF- κ B (p65) nuclear binding in neutrophils from patients with COPD, which was significantly inhibited by RNO but not by dexamethasone. Interestingly, RNO at subeffective concentrations enhanced the capacity of dexamethasone to inhibit NF- κ B (p65).

An alternative molecular mechanism for corticosteroid insensitivity is the overexpression of the dominant negative splice variant isoform of GR β .^{32,33} GR β is reported to be increased in corticosteroid-resistant patients with asthma, patients with ulcerative colitis, and patients with rheumatoid arthritis.³⁴⁻³⁶ Furthermore, recent evidence showed that the GR β variant is capable of inhibiting HDAC2 promoter activity, thus controlling cellular levels of HDAC2 in asthmatic patients.³⁷ In patients with COPD, GR α levels are reported to be decreased in bronchial epithelial cells and peripheral blood leukocytes.^{38,39} However, a recent study did not show differences in GR α expression in neutrophils between healthy subjects and patients with COPD.¹⁰ Accordingly, in this study we also did not observe differences in GR α expression in peripheral blood neutrophils between healthy subjects and patients with COPD. However, the expression of GR β was significantly higher in neutrophils from patients with COPD compared with that seen in healthy subjects. Interestingly, in contrast to dexamethasone, RNO concentration-dependently inhibited CSE-induced GR β overexpression in neutrophils from patients with COPD, suggesting a role of GR β expression in corticosteroid insensitivity. In this respect similar results have been observed by our group in pulmonary artery endothelial cells.⁴⁰

In this work we observed increased expression of PDE4B and PDE4D, but not PDE4A or PDE4C, in neutrophils from patients with COPD compared with that seen in healthy subjects, suggesting a potential role of these isoenzymes in neutrophil activation. Accordingly, recent studies showed that PDE4B and PDE4D isoenzymes are necessary for neutrophil recruitment to the lung after endotoxin exposure.⁴¹ Oxidative stress generated by cigarette smoke or inflammatory mediators appears to regulate the induction of PDE4 expression.^{42,43} We observed that expression of PDE4B and PDE4D was increased in neutrophils from patients with COPD stimulated with CSE, which was inhibited by RNO and, to a lesser extent, by dexamethasone. The combination of RNO and dexamethasone showed additive effects, inhibiting both PDE4B and PDE4D expression, which might contribute to the additive/synergistic anti-inflammatory effects of this combination.

Several other molecules, such as MKP-1 and MIF, have been implicated in mediating the anti-inflammatory effects of corticosteroids. MKP-1 is a mitogen-activated protein kinase phosphatase that inhibits the activity of p38 and ERK1/2, thus attenuating inflammatory pathways. For example, in patients with corticosteroid-resistant asthma, ulcerative colitis, or systemic lupus erythematosus, MIF expression is upregulated.⁴⁴⁻⁴⁶ Recent studies have shown that MIF promotes neutrophil trafficking in patients with inflammatory diseases and might inhibit the anti-inflammatory effects of dexamethasone.^{47,48} Prior experimental studies have also revealed mechanisms by which MIF counterregulates the immunosuppressive action of corticosteroids; these mechanisms include suppression of corticosteroid-induced expression of I κ B α , which is a cytosolic inhibitor of NF- κ B,⁴⁹ and suppression of corticosteroid-induced MKP-1,^{50,51} which inactivates the proinflammatory ERK1/2, JNK, and p38 mitogen-activated protein kinase signaling pathways.

In this study we found that MKP-1 was downregulated in neutrophils from patients with COPD compared with that seen in healthy subjects, whereas MIF was upregulated. Additional *in vitro* experiments demonstrated that CSE inhibited MKP-1 and increased MIF expression, as well as ERK1/2 phosphorylation, in neutrophils from patients with COPD. RNO blocked these effects, whereas dexamethasone only showed a weak or no effect. The combination of RNO with dexamethasone potentiated the inhibition of both MIF expression and phosphorylation of ERK1/2 and reversed MKP-1 inhibition.

One limitation of this study is the use of peripheral blood neutrophils because lung tissue or airway neutrophils might have different inflammatory and phenotypic characteristics.⁵² However, a recent study showed that airway neutrophils are also resistant to the anti-inflammatory effects of corticosteroids,¹⁰ which supports our findings in peripheral blood neutrophils.

Corticosteroid resistance is a feature of multiple severe immune and inflammatory diseases and widely described in the literature. In addition to COPD, corticosteroid resistance has been observed in patients with other chronic inflammatory diseases, such as asthma, steroid-resistant inflammatory bowel disease, rheumatoid arthritis, and systemic lupus erythematosus.²⁶ A possible extension of these findings in this study is to investigate the effect of roflumilast and dexamethasone on neutrophils from patients with corticosteroid-resistant asthma, particularly in those situations of smoking and severe asthma in which there is an increase in oxidative stress and loss of corticosteroid sensitivity or in those non-T_H2 asthma phenotypes characterized by increased sputum neutrophilia, with predominant T_H17/IL-8 pathway activation and resistance to corticosteroids.⁵³ In fact, there are similarities between the airway inflammation seen in patients with severe and smoking-related asthma and those with COPD, which might indicate that there are several common mechanisms of corticosteroid resistance between these diseases and other chronic inflammatory pathologies.

In summary, RNO has shown potent anti-inflammatory effects on neutrophils from patients with COPD who are corticosteroid resistant. RNO in combination with dexamethasone demonstrates additive/synergistic anti-inflammatory properties. Furthermore, we have provided novel evidence for the mechanistic pathway or pathways that might mediate the anti-inflammatory properties of roflumilast and corticosteroid resistance, as well as the synergistic anti-inflammatory properties of the combination of RNO and corticosteroids. These

observations might provide a scientific rationale for adding roflumilast to corticosteroids in the clinic.

Clinical implications: Additive/synergistic effects between RNO and corticosteroids provide a scientific rationale for evaluation of the clinical effects of the combination of roflumilast and corticosteroids.

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METHODS

All reagents were obtained from Sigma-Aldrich (St Louis, Mo), unless otherwise stated.

Patients

Peripheral blood neutrophils and whole blood were obtained from patients with COPD and healthy nonsmoking subjects. Clinical characteristics of patients are defined in Table E1. Thirty-two patients with COPD, defined according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2013 guidelines, were enrolled in this study. Patients were aged 68.7 ± 10 years and had an FEV₁ of $67.6\% \pm 18\%$ of predicted value, and 15 were prescribed an inhaled corticosteroid, 24 were prescribed long-acting anticholinergics, and 23 were prescribed long-acting β_2 -agonists. Inhaled corticosteroids, long-acting β_2 -agonists, or anticholinergic medications did not affect neutrophil corticosteroid resistance. All patients with COPD were current smokers and had bronchitis. Patients with COPD in this study were classified as GOLD 1 (mild disease, $n = 11$) and GOLD 2 (moderate disease, $n = 21$). No comorbidities were present in patients with COPD. There were no exacerbations of the disease within 2 weeks before taking blood samples. Twenty-five age-matched nonsmoking healthy control subjects with normal lung function (age, 65 ± 4 years old; FEV₁, $98\% \pm 3\%$ of predicted value) were also recruited as healthy control subjects. Routine lung function tests were performed to evaluate the forced vital capacity, FEV₁, and FEV₁/forced vital capacity ratio by using a Vitalograph alpha III spirometer (Vitalograph, Maids Moreton, United Kingdom). This project was approved by the local ethics committee of General University Hospital, Valencia, Spain, and written informed consent was obtained from each patient or volunteer before starting blood sampling and lung function testing.

Human neutrophil isolation

Neutrophils were isolated from peripheral venous blood, as previously outlined,^{E1} by using Dextran 500 at 3% (in 0.9% saline) in a proportion of 2:1 and Ficoll-Paque Histopaque 1077 (Amersham Pharmacia Biotech, Barcelona, Spain). The preparations were greater than 97% pure in neutrophils, as assessed by means of Giemsa staining, and had a viability of greater than 99%, as measured by using trypan blue exclusion. Neither purity nor viability was affected in the study's different experimental conditions.

Preparation of CSE solutions

CSE was prepared, as previously outlined.^{E2} Briefly, the smoke of a research cigarette (2R4F; Tobacco Health Research, University of Kentucky, Ky) was generated by a respiratory pump (Apparatus Rodent Respirator 680; Harvard, Germany) through a puffing mechanism related to the human smoking pattern (3 puffs per minute; 1 puff, 35 mL; each puff of 2 seconds in duration with 0.5 cm above the filter) and was bubbled into a flask containing 25 mL of prewarmed (37°C) RPMI 1640 culture medium. The resulting CSE solution was considered 100% CSE and was used for experiments within 30 minutes of preparation. CSE 10% corresponds approximately to the exposure associated with smoking of packs per day.^{E3} Isolated neutrophils were treated with CSE concentrations of up to 5% for 6 hours to test for cytotoxicity/apoptosis from CSE. No significant difference in the lactate dehydrogenase supernatant level (lactate dehydrogenase cytotoxicity assay; Cayman, Madrid, Spain) or annexin V–fluorescein isothiocyanate was observed in comparison with values seen in the control group (data not shown).

Cell stimulations and IL-8 and MMP-9 assays

Peripheral blood neutrophils were adjusted to 500×10^3 cells per well in 24-well plates and incubated in RPMI 1640 culture medium for 1 hour at 37°C in a 5% CO₂ atmosphere. Cells were then treated in the presence or absence of RNO (0.1 nmol/L to 1 μ mol/L; Forest Research Institute, Jersey City, NJ), dexamethasone (0.1 nmol/L to 1 μ mol/L), the antioxidant NAC (1 mmol/L), the nicotinamide adenine dinucleotide phosphate oxidase inhibitor DPI (1 μ mol/L), or the PI3K paninhibitor LY-294002 (10 μ mol/L) for 1 hour before stimulation with LPS (1 μ g/mL) or CSE 5%. We selected an

LPS concentration of 1 μ g/mL because it is widely used in the literature for this type of study with neutrophils and time exposures^{E4-E6} and because we obtained an approximately 80% of maximal response of IL-8 and MMP-9 release from neutrophils at 1 μ g/mL LPS concentration in preliminary studies.

Both the stimuli and drugs remained together for 6 hours. Supernatants were collected and centrifuged at 120g for 5 minutes, and the cell-free supernatant was used to measure IL-8 and MMP-9 levels. Cellular extracts were used to measure mRNA expression after 6 hours of stimulation. IL-8 was determined by using a commercially available ELISA kit for IL-8 (R&D Systems, Nottingham, United Kingdom), according to the manufacturer's protocol. MMP-9 was measured by using LUMINEX technology, according to the manufacturer's protocol.

Real-time RT-PCR

Total RNA was obtained from isolated neutrophils under basal conditions or after different drug and stimulation periods defined above by using TriPure Isolation Reagent (Roche, Indianapolis, Ind). The integrity of the extracted RNA was confirmed with Bioanalyzer (Agilent, Palo Alto, Calif). Reverse transcription was performed in 300 ng of total RNA with the TaqMan reverse transcription reagents kit (Applied Biosystems, Perkin-Elmer Corporation, Foster City, Calif). cDNA was amplified by using assay-on-demand specific primers predesigned by Applied Biosystems for MIF (catalog no. Hs00236988), MKP-1 (catalog no. Hs00610256), PI3K- δ (catalog no. Hs00192399), HDAC2 (catalog no. Hs00231032), and P-glycoprotein (ABCB1, catalog no. Hs00184500) genes in a 7900HT Fast Real-Time PCR System (Applied Biosystems) by using Universal Master Mix (Applied Biosystems). PDE4A, PDE4B, PDE4C, and PDE4D subtypes and GR α and GR β were quantified by using specific primers (Table E2) and SYBR Green real-time PCR, as previously outlined.^{E7}

Western blot

Western blot analysis was used to detect changes in p-ERK1/2 levels (42–44 kDa). Neutrophils were incubated in RPMI 1640 basal culture medium and treated with RNO, dexamethasone, or their combination for 1 hour and stimulated with CSE 5% for 20 minutes. Cells were then centrifuged, and the total protein was extracted, as previously outlined.^{E8}

Briefly, 20 μ g of proteins (denatured) along with a molecular weight protein marker (Bio-Rad Kaleidoscope marker; Bio-Rad Laboratories) were loaded onto an acrylamide gel consisting of a 5% acrylamide stacking gel stacked on top of a 10% acrylamide resolving gel and run through the gel by application of 100 V for 1 hour. Proteins were transferred from the gel to a polyvinylidene difluoride membrane by using a wet blotting method. The membrane was blocked with 5% Marvel in PBS containing 0.1% Tween 20 and then probed with a rabbit anti-human p-ERK1/2 (1:1,000) antibody (mAb; Cell Signaling, Boston, Mass; catalog no. 4376S) and normalized to total rabbit anti-human ERK1/2 (1:1000) antibody (mAb; Cell Signaling, catalog no. 4695). The enhanced chemiluminescence method of protein detection using enhanced chemiluminescence reagents (ECL plus; Amersham GE Healthcare, Buckinghamshire, United Kingdom) was used to detect labeled proteins. Quantification of protein expression was performed by using densitometry relative to total ERK1/2 expression with the software GeneSnap version 6.08. Results were expressed as ratios of the endogenous control's total ERK1/2 as appropriate and normalized to the control group.

HDAC activity

Neutrophils from patients with COPD were incubated in the presence or absence of RNO (1 nmol/L and 1 μ mol/L), dexamethasone (1 μ mol/L) or the combination of 1 nmol/L RNO plus 1 μ mol/L dexamethasone for 1 hour and stimulated with CSE 5% for 1 hour to measure HDAC activity. Cells were then washed and centrifuged to extract the nuclear protein. Nuclear protein extraction was performed with the extraction kit (Active Motif Europe, Rixensart, Belgium). HDAC activity was measured by using a colorimetric assay system (AK-501, Biomol International, Madrid, Spain) according to the manufacturer's protocol, as previously outlined.^{E7}

PI3K activity

To measure PI3K δ activity, neutrophils from patients with COPD were isolated and incubated with RNO (1 nmol/L or 1 μ mol/L), dexamethasone (1 μ mol/L), or the combination of 1 nmol/L RNO plus 1 μ mol/L dexamethasone for 1 hour. Then cells were stimulated with CSE 5% for 30 minutes. After cell stimulation, neutrophils were centrifuged, and total protein was extracted from neutrophils. The total protein amount was measured with the Bio-Rad assay (Bio-Rad Laboratories) to ensure an equal amount of 500 μ g and immunoprecipitated with anti-PI3K δ antibody (p110 δ ; ab32401; Abcam, Cambridge, United Kingdom). PI3K activity was measured with the PI3-Kinase Activity ELISA: Pico (catalog no. k-1000s; Echelon Bioscience, Salt Lake City, Utah), according to the manufacturer's protocol. In brief, PI3K reactions were run with the class I PI3K physiologic substrate PI(4,5)P₂. The enzyme reactions, phosphatidylinositol (3,4,5)-triphosphate (PIP₃) standards, and controls were then mixed and incubated with PIP₃ binding protein that is highly specific and sensitive to PIP₃. This mixture was then transferred to a PIP₃-coated microplate for competitive binding. Afterward, a peroxidase-linked secondary detector and colorimetric detection was used to detect the amount of PIP₃ produced by PI3K through comparing the enzyme reactions with a PIP₃ standard curve.

NF- κ B (p65) nuclear transcription factor measures

Neutrophils from patients with COPD were incubated in the presence or absence of RNO (1 nmol/L or 1 μ mol/L), dexamethasone (1 μ mol/L), or the combination of 1 nmol/L RNO plus 1 μ mol/L dexamethasone for 1 hour and stimulated with CSE 5% for 1 hour. Cells were then washed and centrifuged to extract the nuclear protein, as described above. Measurement of nuclear NF- κ B (p65) transcription factor expression was performed with a commercially available NF- κ B (p65) transcription factor assay kit (Cayman Chemical, catalog no. 10007889), according to the manufacturer's protocol.

Measurement of intracellular ROS levels

Intracellular ROS was measured by using a flow cytometric assay with 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA; Molecular Probes, Paisley, United Kingdom), as previously outlined.^{E1} H₂DCF-DA was added for 15 minutes before stimulus to a final concentration of 5 μ mol/L to measure intracellular ROS levels. The bacterial peptide fMLP was added as stimulus at 1 μ mol/L for 30 minutes to generate intracellular ROS.^{E9} In other experiments CSE 5% was added to generate intracellular ROS to reproduce *in vivo* conditions. We measured intracellular ROS levels using fresh whole blood from patients with COPD. Blood neutrophils were selected by using CD16–Alexa Fluor 647 antibody (Molecular Probes) as a neutrophil marker in an Epics Profile II flow cytometer. A total of 1 mL of whole blood was preincubated at 37°C for 30 minutes with RNO (1 nmol/L to 1 μ mol/L), NAC (1 mmol/L), DPI (1 μ mol/L), and H₂DCF-DA, followed by 1 μ mol/L fMLP or CSE 5% stimulation for another 1 hour. Then erythrocytes were lysed with a commercial erythrocyte lysis buffer (BioLegend, Manchester, United Kingdom) for 10

minutes. Fluorescence intensity was measured in the polymorphonuclear region of whole blood in an Epics Profile II flow cytometer. Results were expressed as DCF fluorescence in relative fluorescence units.

Analysis of results

Statistical analysis of results was carried out by using parametric or nonparametric analysis, as appropriate. A *P* value of less than .05 was considered statistically significant. Parametric data were expressed as means $\pm$ SEs of *n* experiments by using the Student *t* test or 2-way ANOVA, followed by the Bonferroni *post hoc* test. In nonparametric tests data were displayed as medians, interquartile ranges, and minimum and maximum values and analyzed with the Mann-Whitney test. The concentrations of RNO or dexamethasone producing 50% inhibition were calculated from concentration-response curves by using nonlinear regression in neutrophils from healthy subjects, smokers, and patients with COPD. The Spearman ρ correlation test was used to evaluate the correlation between total peripheral blood neutrophil counts and corticosteroid resistance degree.

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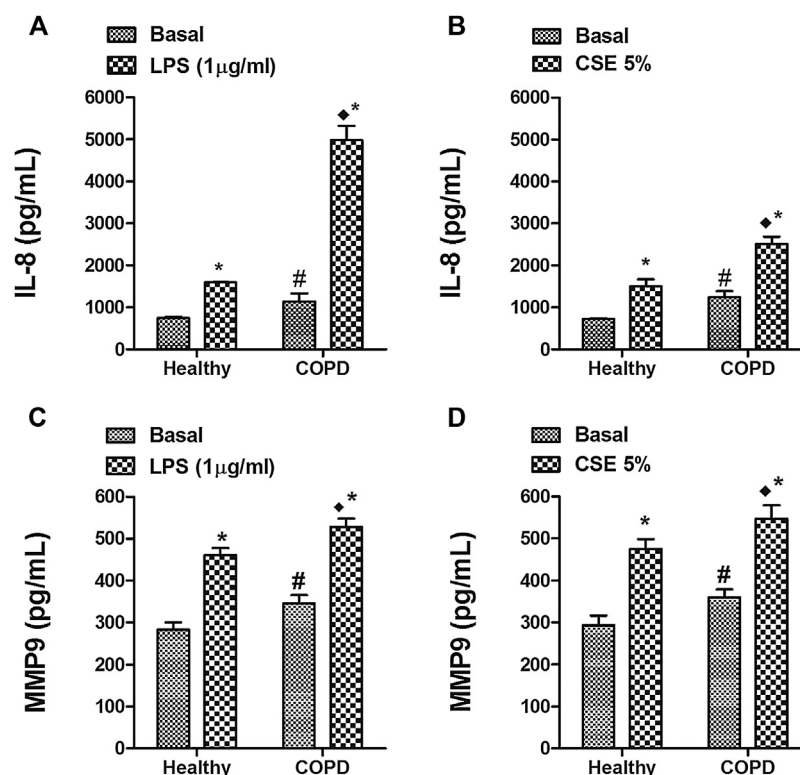


FIG E1. IL-8 and MMP-9 release in human neutrophils from healthy subjects and patients with COPD. Peripheral blood neutrophils were isolated from healthy subjects and patients with COPD and stimulated with LPS (1 μ g/mL; **A** and **C**) or CSE (5%; **B** and **D**) for 6 hours. Results are expressed as means $\pm$ SEs of 3 (3 healthy and 3 COPD cell populations) independent experiments. Two-way ANOVA, followed by *post hoc* Bonferroni tests: * P < .05 related to basal release in the same subject group; # P < .05 related to basal release in healthy group; ♦ P < .05 related to LPS- or CSE-stimulated release in healthy group.

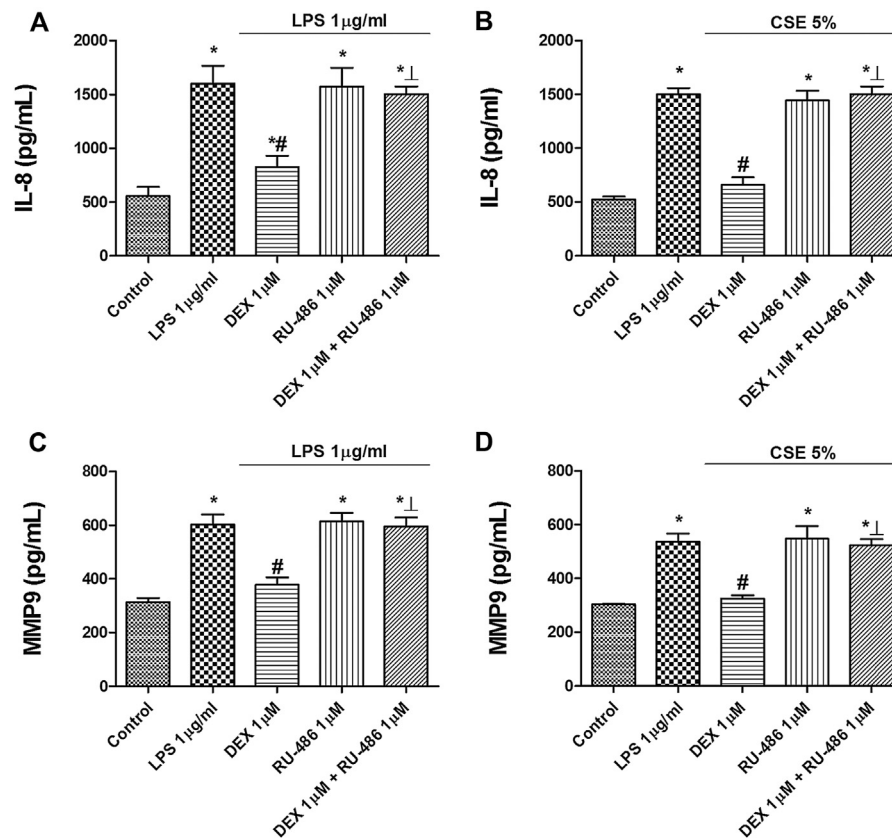


FIG E2. Peripheral blood neutrophils were isolated from healthy subjects and incubated with 1 µmol/L RU-486, 1 µmol/L dexamethasone (DEX), or 1 µmol/L RU-486 1 hour before 1 µmol/L DEX (1 hour of incubation), followed by stimulation with LPS (1 µg/mL) or CSE 5% for 6 hours. Results are expressed as means $\pm$ SEs of 3 (3 cell population) independent experiments. Two-way ANOVA, followed by *post hoc* Bonferroni tests: * P < .05 related to control; # P < .05 related to LPS or CSE; † P < .05 related to DEX.

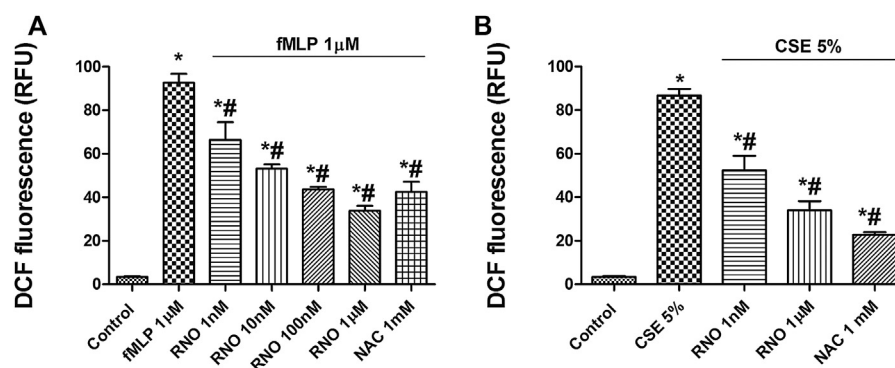


FIG E3. RNO inhibits ROS in neutrophils from patients with COPD. Peripheral whole blood neutrophils from patients with COPD were identified by using CD16-Alexa Fluor 645 by means of flow cytometry. Whole blood was incubated with RNO (1 nmol/L to 1 μ mol/L) or the antioxidant NAC (1 mmol/L) for 1 hour and stimulated with fMLP (1 μ mol/L) or CSE for 30 minutes. Intracellular ROS levels were measured in neutrophil populations by means of fluorescence H_2DCF -DA dye with flow cytometry. Results are expressed as means $\pm$ SEs in 3 (4 cell populations). Two-way ANOVA, followed by *post hoc* Bonferroni tests: * $P < .05$ related to control; # $P < .05$ related to fMLP or CSE. RFU, Relative fluorescence units.

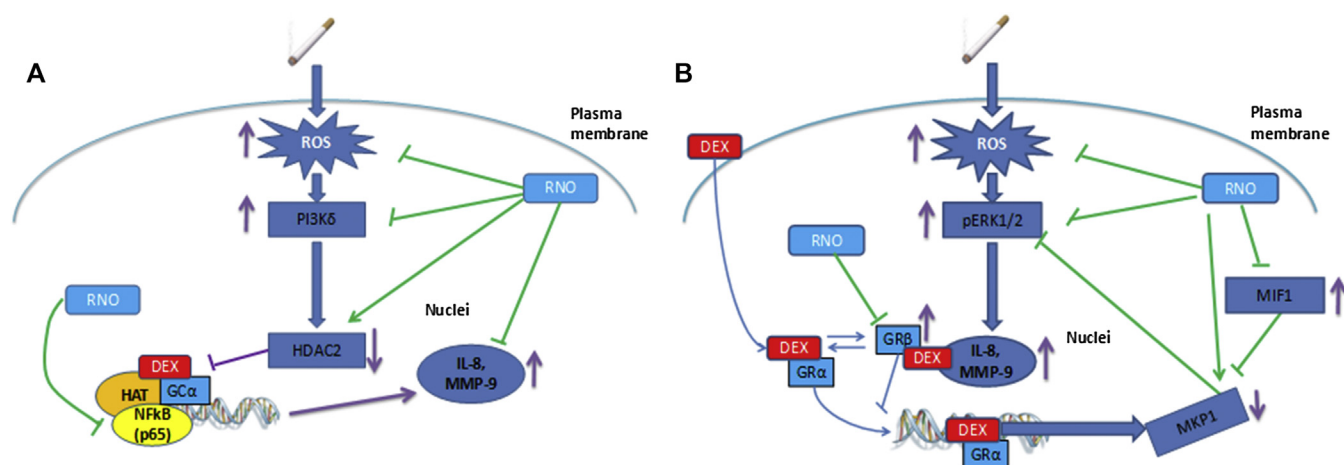


FIG E4. RNO improves corticosteroid resistance in neutrophils from patients with COPD: mechanisms implicated. **A**, Cigarette smoke increases ROS levels in neutrophils from patients with COPD. The increase in ROS levels activates and increases the expression of PI3K δ , which reduces the activity of HDAC2. Furthermore, cigarette smoke induces the NF- κ B (p65) nuclear translocation switching on histone acetyltransferase, leading to histone acetylation and subsequently to transcription of genes encoding inflammatory proteins, such as IL-8 or MMP-9. In normal conditions corticosteroids reverse this by binding to GRs and recruiting HDAC2. This reverses the histone acetylation induced by NF- κ B and switches off the activated inflammatory genes. However, neutrophils from patients with COPD generate oxidative stress, which impairs the activity of HDAC2. This amplifies the inflammatory response to NF- κ B activation but also reduces the anti-inflammatory effect of glucocorticoids because HDAC2 is now unable to reverse histone acetylation. RNO reduces ROS formation after cigarette smoke exposure and consequently inhibits PI3K δ activation and increases HDAC2 activity. Furthermore, RNO inhibits NF- κ B (p65) nuclear translocation. Effects showed by RNO allow corticosteroids to bind HDAC2 to reverse the histone acetylation induced by NF- κ B and switching off the activated inflammatory genes. **B**, ROS formation by cigarette smoke promotes phosphorylation of ERK1/2 and subsequently IL-8 or MMP-9 formation. Furthermore, ROS downregulates MKP1 expression (an inhibitor of ERK1/2) and increases MIF1 expression (an inhibitor of MKP1) and GR β expression, increasing inflammation. In neutrophils from patients with COPD exposed to cigarette smoke, dexamethasone (DEX) is unable to reduce ERK1/2, MIF1, or GR β expression or increase MKP1 expression. RNO reduces GR β expression, allowing DEX to join GR α to translocate to the nucleus. Furthermore, RNO reduces MIF1 expression, which allows DEX to increase MKP1, reducing ERK1/2 phosphorylation and IL-8 and MMP-9 secretion. *Violet lines and arrows* represent the effect of cigarette smoke on neutrophils from patients with COPD. *Green lines and arrows* represent the effects of RNO.

TABLE E1. Clinical features

	Healthy subjects (n = 25)	Patients with COPD (n = 32)
Age (y), mean (SD)	65 ± 4	68.7 ± 10
Sex (M/F)	15/10	21/11
Tobacco consumption (pack-years)	0	32.5 ± 15
FEV ₁ (% predicted)	98 ± 3	67.6 ± 18*
FVC (% predicted)	89 ± 6	92.5 ± 14
FEV ₁ /FVC ratio (%)	94 ± 5	69.2 ± 11*
GOLD 1 (mild) patients (n)	0	11
GOLD 2 (moderate) patients (n)	0	21
GOLD 3 (severe) patients (n)	0	0
GOLD 4 (very severe) patients (n)	0	0
Receiving inhaled steroids (n)	0	15
Receiving theophyllines (n)	0	0
Receiving long-acting β ₂ -agonist (n)	0	23
Receiving anticholinergics (n)	0	24
Total peripheral blood neutrophils (×10 ⁹ /L)	4.1 ± 0.4	9.2 ± 1.2*

Data are presented as means ± SEs.

F, Female; FVC, forced vital capacity; M, male.

**P* < .05 related to healthy subjects.

TABLE E2. Primers for SYBR Green real-time quantitative RT-PCR

Name	Sequence (5'-3')	Product size (bp)
PDE4A forward	TCATGGCCGAGTTCTTCCAG	193
PDE4A reverse	TCCAAAAGTGTCCAAGATCTCCTG	
PDE4B forward	ATTGTATCGGCAATGGACAGAC	213
PDE4B reverse	GTATCGAGAATGTCCTGAGCATC	
PDE4C forward	ATTGTATCGGCAATGGACAGAC	213
PDE4C reverse	GTATCGAGAATGTCCTGAGCATC	
PDE4D forward	AGTTCTTCCAGCAGGGAGACC	196
PDE4D reverse	TCTCGATTGTCCTCCAGCG	
GR α forward	AGCCATTGTCAAGAGGGAAG	146
GR α reverse	AGCAATAGTTAAGGAGATTTTCAACC	
GR β forward	AGCCATTGTCAAGAGGGAAG	108
GR β reverse	TCTGGTTTTTAACCACATAACATTTTC	

TABLE E3. Maximal percentage of inhibition of IL-8 and MMP-9 release from isolated peripheral blood neutrophils from healthy subjects and patients with COPD

	Stimulus	Treatment	Maximal % inhibition	
			Healthy subjects	Patients with COPD
IL-8	LPS	RNO	40.5 ± 4.6	48.7 ± 5.2
		DEX	67.1 ± 11.4	15.0 ± 4.4*
	CSE	RNO	90.4 ± 5.4	88.9 ± 5.6
		DEX	94.3 ± 21.7	20.6 ± 12.1*
MMP-9	LPS	RNO	98.9 ± 12.6	94.6 ± 11.8
		DEX	96.8 ± 5.1	8.8 ± 2.5*
	CSE	RNO	64.8 ± 5.6	62.6 ± 2.3
		DEX	83.9 ± 5.8	15.4 ± 7.0*

Neutrophils were incubated with RNO (0.1 nmol/L to 1 μ mol/L) or dexamethasone (DEX; 0.1 nmol/L to 1 μ mol/L) in response to LPS (1 μ g/mL) or CSE 5%. Levels of IL-8 and MMP-9 in the cell supernatant were determined, and the maximal percentage of inhibition of IL-8 and MMP-9 release by RNO and DEX was calculated. Values are means $\pm$ SEs of 3 independent experiments run in triplicate.

* $P < .05$ versus healthy subjects.