Nortriptyline Reverses Corticosteroid Insensitivity by Inhibition of Phosphoinositide-3-Kinase- δ ^[S]

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ABSTRACT

Corticosteroid insensitivity represents a major barrier to the treatment of chronic obstructive pulmonary disease (COPD) and severe asthma. It is caused by oxidative stress, leading to reduced histone deacetylase-2 (HDAC2) function through activation of phosphoinositide-3-kinase- δ (PI3K δ). The tricyclic antidepressant nortriptyline has been identified in high-throughput screens as an agent that increases corticosteroid responsiveness. The aim of this study was to identify the molecular mechanism whereby nortriptyline increases corticosteroid sensitivity. Phosphorylation of Akt, a footprint of PI3K activation, and HDAC activity were evaluated by Western blotting and fluorescent activity assay in U937 monocytic cells. Corticosteroid sensitivity was evaluated by the inhibition of tumor necrosis factor α (TNF α)-induced interleukin

8 (IL-8) production by budesonide. Hydrogen peroxide (H₂O₂) or cigarette smoke extract (CSE) increased the level of phosphorylated Akt (pAkt) and reduced HDAC activity. Pretreatment with nortriptyline inhibited pAkt induced by CSE and H₂O₂ as well as restored HDAC activity that had been decreased by H₂O₂ and CSE. In addition, nortriptyline inhibited PI3K δ activity, but had no effect on the PI3K α and PI3K γ isoforms. Although CSE reduced the effects of budesonide on TNF α -induced IL-8 production in U937 cells, nortriptyline reversed CSE-induced corticosteroid insensitivity. Nortriptyline restores corticosteroid sensitivity induced by oxidative stress via direct inhibition of PI3K δ and is a potential treatment for corticosteroid-insensitive diseases such as COPD and severe asthma.

Introduction

Corticosteroids are the most effective therapy for many inflammatory and immune diseases. However, in patients with chronic obstructive pulmonary disease (COPD) and severe asthma or asthmatic patients who smoke, corticosteroids are largely ineffective (Barnes and Adcock, 2009). Corticosteroid insensitivity represents a huge management problem, and novel treatments are urgently needed for the treatment of these diseases. The anti-inflammatory effects of corticosteroids are mediated by the binding to glucocorticoid receptors (GRs) and subsequent nuclear translocation. Activated GR inhibits proinflammatory gene transcription via inhibition of nuclear factor- κ B-associated histone acetylation by both direct inhibitions of CREB-binding protein-associated histone acetyltransferase activity and recruitment of histone deacetylase 2 (HDAC2) to the promoter of actively

transcribed inflammatory genes (Ito et al., 2006a). HDAC2 expression and activity are reduced in bronchial biopsies, bronchoalveolar lavage macrophages, and peripheral lung tissue obtained from patients with COPD, and the reduction correlates with disease severity (Ito et al., 2005). HDAC2 activity has also been shown to be decreased in some severe asthmatics and asthmatics who smoke (Ito and Mercado, 2009). Moreover, knockdown of HDAC2 expression in bronchoalveolar lavage macrophages induces corticosteroid insensitivity, whereas HDAC2 overexpression restores corticosteroid function (Ito et al., 2006b). Reactive oxygen species derived directly by cigarette smoke or indirectly from the inflammatory response to cigarettes can have a marked impact on HDAC2 expression and function and are one of the critical factors in the development of corticosteroid insensitivity (Marwick et al., 2007). For example, HDAC2 is down-regulated by post-translational modifications, such as nitration and oxidation (Barnes, 2009a) after treatment with hydrogen peroxide (H₂O₂), a peroxynitrate generator 3-morpholinolinosydnonimine (Osoata et al., 2009), or cigarette smoke (Adenuga et al., 2009). In these studies, pretreatment with antioxidants, such as *N*-acetylcysteine (NAC) and glutathi-

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ABBREVIATIONS: COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; GR, glucocorticoid receptor; HDAC, histone deacetylase; H₂O₂, hydrogen peroxide; IL-8, interleukin-8; NAC, *N*-acetylcysteine; PI3K, phosphoinositide-3-kinase; TNF α , tumor necrosis factor α ; pAkt, phosphorylated Akt; ELISA, enzyme-linked immunosorbent assay; Nt, nortriptyline; 5HT-2, 5-hydroxytryptamine type 2; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; IC87114, 2-(6-aminopurin-9-ylmethyl)-3-(2-chlorophenyl)-6,7-dimethoxy-3*H*-quinazolin-4-one.

one, prevent the post-translational modification and down-regulation of HDAC2 (Adenuga et al., 2009; Osoata et al., 2009). We have shown that oxidative stress induced phosphorylation and inactivation of HDAC2 through activation of the phosphoinositide-3-kinase (PI3K)/Akt pathway (To et al., 2010).

Nortriptyline is a second-generation tricyclic antidepressant that has also been used to treat nicotine addiction and smoking cessation in patients with COPD and has also shown a marked improvement in certain respiratory symptoms (Borson et al., 1992). High-throughput screening has been used to identify drugs showing synergistic effects with corticosteroids on tumor necrosis factor α (TNF α) production in peripheral blood mononuclear cells (Lehár et al., 2009). Nortriptyline was identified as a drug that unexpectedly increased the anti-inflammatory effect of prednisolone. However, the effects of nortriptyline have not been evaluated in clinical models, and the molecular mechanism of nortriptyline in restoring corticosteroid sensitivity is unknown. The aim of this study was to verify whether nortriptyline could restore corticosteroid sensitivity in an *in vitro* model of corticosteroid insensitivity induced by reactive oxygen species and cigarette smoke and to identify the molecular mechanisms involved.

Materials and Methods

Cell Culture and Stimulation. Human monocytic U937 cells were maintained in continuous cell culture at 37°C and 5% CO₂ in RPMI medium 1640 containing 10% fetal calf serum and 15 mM glutamine. For stimulation with H₂O₂ (Sigma Chemical, Poole, Dorset, UK) or cigarette smoke extract (CSE), U937 cells were seeded (0.5×10^6 cells/ml) using starvation medium RPMI 1640 (phenol red free) with 1% fetal calf serum and 15 mM L-glutamine at 37°C and 5% CO₂.

Preparations of Cigarette Smoke Extract. CSE was prepared using two full-strength Marlboro cigarettes with filters removed (Phillip Morris, Richmond, VA), which were combusted through a modified 60-ml syringe apparatus into 20 ml of RPMI medium 1640 as described previously (Walters et al., 2005). CSE was then passed through a 0.25- μ m filter to sterilize and remove particulate matter and was used immediately. The optical density was measured at 320 nm wavelength, and values were diluted to achieve a value of 0.15 to provide a concentration that stimulated the cells without inducing cell death.

pAkt Assay. U937s were seeded (0.5×10^6 cells/ml) in starvation media and left overnight at 37°C with 5% CO₂. Cells were incubated with nortriptyline (Sigma Chemical; 1, 3.3, 10, and 33 μ M) or a nonselective PI3K inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) (Sigma Chemical; 0.033, 0.1, 0.33, and 1 μ M) for 30 min and then stimulated with H₂O₂ (200 μ M) for 15 min or CSE for 5 min. Phosphatase inhibitor (Active Motif Inc., Rixensar, Belgium) warmed at 37°C was added at 5% as a final concentration to each well to stop the reaction. Whole-cell extraction was performed with lysis buffer from a nuclear extraction kit (Active Motif Inc.), and 40 μ g of protein samples were separated using 10% SDS-polyacrylamide gel electrophoresis/Western blot (Invitrogen, Paisley, UK). Serine 473 phosphorylation of Akt and total Akt1 were detected with mouse monoclonal anti-S473 Akt (Cell Signaling, Hitchin, UK) and rabbit polyclonal anti-Akt1 (Cell Signaling), respectively. Band density of S473Akt was normalized to total Akt1.

HDAC Activity. Cells seeded at 0.5×10^6 cells/ml in starvation media were treated with nortriptyline (1, 0, 3.3, and 10 μ M) for 30 min and then stimulated with H₂O₂ (200 μ M) for 15 min or CSE (100 and 33%) for 2 h. Nuclear extracts were prepared as described

previously (Ito et al., 2002), and HDAC activity was measured in 10- μ l samples from nuclear extracts and normalized to protein concentrations.

HDAC2 Expression. Nuclear extracts were resolved by 10% SDS-polyacrylamide gel electrophoresis/Western blot, and HDAC2 was detected using mouse monoclonal anti-HDAC2 (Sigma Chemical). Bands densities were normalized to that of lamin A/C (Santa Cruz Biotechnology, Heidelberg, Germany).

Corticosteroid Sensitivity Assay. U937 cells (seeded at 0.5×10^6 cells/ml) were treated with nortriptyline (Klein et al., 1991) (1 and 3.3 μ M) (Sigma Chemical), NAC (10 mM), LY294002 (Gharbi et al., 2007) (1 μ M) (Sigma Chemical), prazosin (Faridbod et al., 2010) (0.1 μ M) (Sigma Chemical), mepyramine (Kiss et al., 2004) (1 μ M) (Sigma Chemical), and ketanserin (Herndon et al., 1992) (0.1 μ M) (Sigma Chemical) for 30 min before stimulation of CSE (33%) for 2 h. Cells were collected by centrifugation, and pellets were resuspended in starvation media. Cells were seeded in 96-well plates in the presence of budesonide (AstraZeneca Pharmaceuticals, Lund, Sweden; 10^{-12} to 10^{-7} M) for 45 min before overnight stimulation with TNF α (10 ng/ml; R&D Systems Europe Ltd., Abingdon, Oxfordshire, UK). Supernatants were collected, and interleukin-8 (IL-8) expression was measured by ELISA (R&D Systems). The percentage of inhibition of IL-8 by budesonide was calculated, and corticosteroid sensitivity was measured as EC₅₀.

PI3K Activity Assay. PI3K enzyme inhibitory activity was determined by time-resolved fluorescence resonance energy transfer (HTRFPI3K enzyme assay; Millipore, Watford, UK). Nortriptyline or LY294002 was added at the desired final concentrations to a mixture of phosphatidylinositol bisphosphate substrate and recombinant PI3K α , δ , or γ enzymes (Millipore), and the mixture was incubated for 2 h at room temperature. After this incubation period, ATP (20 μ M) was added to the enzyme/compound/phosphatidylinositol bisphosphate substrate mixture, and the resulting mixture was incubated for 30 min at room temperature. The percentage of inhibition of each reaction was calculated relative to vehicle-treated control, and the IC₅₀ value was calculated from the concentration-response curve.

Statistical Analysis. Data are expressed as means $\pm$ S.E.M. Results were analyzed using paired *t* test or one-way analysis of variance for repeated measures with Dunnett post-test for multiple comparisons. EC₅₀ values of budesonide were determined from the concentration-inhibitory response curves of IL-8 production, and the differences of EC₅₀ values of budesonide were assessed using Bonferroni's multiple comparison test. Prism software (GraphPad Software, Inc., San Diego, CA) was used for statistical calculations. Experiments were repeated at least three times. *P* < 0.05 was considered statistically significant. Synergy of two compounds were analyzed by isobologram by the method of Chou and Talalay (1977) using CalcuSyn software (BISOFT, Cambridge, UK).

Results

Nortriptyline Prevents CSE- and H₂O₂-Induced Phosphorylation of Akt. Phosphorylation of Akt at serine 473 was used to measure PI3K activation. S473Akt phosphorylation was increased by H₂O₂ (3.4 ± 0.2 -fold increase versus control; *p* < 0.01) and CSE (3.3 ± 0.2 -fold increase versus control; *p* < 0.01) (Fig. 1). Preincubation with nortriptyline (1–10 μ M; *p* < 0.01) significantly prevented Akt phosphorylation by H₂O₂ (Fig. 1A) and CSE (*p* < 0.01), and the IC₅₀ value was calculated to be 1.67 μ M (Fig. 1B). LY294002 also produced a concentration-dependent inhibition of CSE-induced pAkt with an IC₅₀ at 0.25 μ M (Supplemental Fig. 1A). Thus, nortriptyline was only 6.7-fold weaker than LY294002 in inhibiting CSE-induced PI3K activation. In the *in vitro* enzymatic assay that measures PI3K α , γ , and δ

Fig. 1. Nortriptyline prevents oxidative stress activation of the PI3K/Akt pathway. A, U937 cells were preincubated with Nt (1–10 μM) for 30 min and treated with H₂O₂ (200 μM) for 15 min. Levels of phosphoserine 473 Akt and total Akt1 were measured by Western blot in whole-cell extracts. B, U937 cells were preincubated with nortriptyline (1–33 μM) and treated with CSE for 5 min. Levels of phosphoserine 473 Akt and total Akt1 were measured by Western blot in whole-cell extracts, and IC₅₀ values were calculated. *, $p < 0.05$; **, $p < 0.01$ compared with controls.

activity, nortriptyline concentration-dependently inhibited PI3Kδ activity (IC₅₀ 0.82 μM), and the efficacy was similar to that of LY294002 (IC₅₀ 0.98 μM), whereas nortriptyline had no effect on PI3Kα or PI3Kγ (Table 1).

Nortriptyline Prevents CSE or H₂O₂ Reduction of HDAC Activity. H₂O₂ significantly reduced HDAC activity (39 ± 12% versus control; $p < 0.05$), and this was completely prevented by nortriptyline at 1 μM ($p < 0.05$) (Fig. 2A). Incubation with CSE also resulted in a significant decrease of HDAC activity (9 ± 3% at 33% CSE and 19 ± 3% at 100% CSE; $p < 0.05$) (Fig. 2B). Preincubation with nortriptyline (1 μM) also completely restored HDAC activity ($p < 0.05$ versus CSE) reduced by CSE (Fig. 2B). Because HDAC2 protein expression was not reduced after CSE (Supplemental Fig. 1B), the decrease in HDAC activity was not explained by a decrease in HDAC2 protein expression.

Nortriptyline Prevents CSE-Induced Corticosteroid Insensitivity. Budesonide concentration-dependently inhibited TNFα-induced IL-8 release in U937 cells with an EC₅₀ value of 9.3×10^{-10} M and $E_{\max}$ of 56%. Pretreatment with CSE (33%) resulted in a decrease in budesonide sensitivity (EC₅₀ 1.2×10^{-9} M; $p < 0.05$ versus Nt) and reduced $E_{\max}$ (24%; $p < 0.05$ versus Nt) (Table 2). This was inhibited by pretreatment with NAC (10 mM) (EC₅₀ 6.1×10^{-10} M with

NAC), suggesting the reduction of budesonide sensitivity was mediated by oxidative stress (Fig. 3A). Nortriptyline also prevented CSE-induced corticosteroid insensitivity. CSE (EC₅₀ 5.9 nM; $E_{\max}$ 24 ± 2%) induced budesonide insensitivity compared with control (EC₅₀ 0.85 nM, $p < 0.01$ versus Nt; $E_{\max}$ 56 ± 5%, $p < 0.05$ versus Nt) (Fig. 3B). Nortriptyline reversed budesonide insensitivity with CSE (EC₅₀ at 1 μM: 0.54 nM, $p < 0.001$ versus CSE; EC₅₀ at 3.3 μM: 1.00 nM, $p < 0.001$ versus CSE). $E_{\max}$ was also reduced by nortriptyline, however, not significantly ($E_{\max}$ at 1 μM: 33 ± 6% and at 3.3 μM: 32 ± 8%). LY294002 (1 μM) also reversed CSE-induced budesonide insensitivity (EC₅₀ of budesonide 1.07 nM with LY294002 in the presence of CSE versus 2.67 nM in CSE control; $p < 0.05$), but did not have any impact on $E_{\max}$ (Fig. 3C).

Nortriptyline is also known to be a potent histamine (H1) receptor antagonist (Taylor and Richelson, 1980) as well as a partial inhibitor of α1 adrenergic receptors (Brown et al., 1980) and 5-HT2 receptors (Sánchez and Hyttel, 1999). Accordingly, we measured the inhibitory effect of antagonists for H1 receptor (mepyramine), 5-HT2 receptor (ketanserin), and α1 adrenergic receptor (prazosin) in our CSE-induced corticosteroid insensitive model. The use of the various antagonists did not have an effect on EC₅₀ or $E_{\max}$ (Supplemental Table 1), whereas nortriptyline was shown to restore EC₅₀.

Nortriptyline and Budesonide Showed Synergistic Effects. U937 cells were treated with H₂O₂ for 60 min and then stimulated with TNFα. The supernatant was collected 24 h after stimulation, and IL-8 was determined by ELISA. Budesonide and/or nortriptyline were given 30 min before TNFα stimulation. Budesonide and nortriptyline concentration-dependently inhibited IL-8 release. Different combina-

TABLE 1
Inhibitory effects of nortriptyline and LY294002 on PI3K enzyme activity

	IC ₅₀		
	PI3Kδ	PI3Kγ	PI3Kα
	μM		
Nortriptyline	0.82	No effect at 10 μM	No effect at 3.3 μM
LY294002	0.98	12.6	0.63

Fig. 2. Nortriptyline prevents oxidative stress reduction of HDAC activity. A, U937 cells were pretreated with Nt (1 μM) before stimulation with H₂O₂ (200 μM) for 15 min. HDAC activity was measured by fluorometric activity assay in nuclear extracts. B, U937 cells were preincubated with Nt (1 μM) for 30 min before stimulation with CSE (33%) for 2 h, or cells were treated with CSE (100%) for 2 h. HDAC activity was measured by fluorometric activity assay in nuclear extracts. *, $p < 0.05$ compared with controls.

TABLE 2
Inhibitory effects of nortriptyline and LY294002 on EC₅₀ and E_{max} of budesonide

	Effects of Nt				Effects of LY294002		
	Control	CSE	CSE +Nt (3.3 μM)	CSE +Nt (1 μM)	Control	CSE	CSE +LY294002 (1 μM)
EC ₅₀ (nM)	0.85	5.9	1.00	0.54	1.28	2.67	1.07
E _{max} (%)	56 ± 5	24 ± 2	32 ± 8	33 ± 6	49 ± 6	12 ± 3	18 ± 4

tion of budesonide with nortriptyline showed greater inhibitory effects than either compound alone, and isobologram analysis demonstrated that this combination showed synergy (Fig. 4).

Discussion

Oxidative stress, such as H₂O₂ and CSE, may play an important role in the development of corticosteroid insensitivity in COPD and severe asthma (Adcock and Barnes, 2008). As shown in Fig. 3, CSE reduced the effects of budesonide on TNFα-induced IL-8 release in U937 cells as reported previously (Ito et al., 2001; Cosio et al., 2004). Because NAC, an antioxidant, reversed the CSE-induced decrease in budesonide effect, this suggests that oxidative stress directly causes corticosteroid insensitivity. The molecular mechanisms of oxidative stress-induced corticosteroid insensitivity have now been elucidated. H₂O₂ at high concentrations is reported to inhibit nuclear translocation of GR (Okamoto et al., 1999). However, another mechanism is a reduction in activity and expression of HDAC2, which is required for corticosteroids to switch off activated inflammatory genes (Barnes, 2009b). The activity, protein expression, and mRNA expression of HDAC2 are decreased in COPD cells and lung tissue (Ito et al., 2005). Oxidative stress induction of hypoxia-inducible factor 1α has been shown to reduce HDAC2 gene expression at the promoter region (Charron et al., 2009). However, in this study, oxidative stress reduced HDAC activity between 15 min and 2 h without any change in HDAC2 protein expression. Therefore, reduced transcription of HDAC2 is not involved in the model shown in the present study. Instead, post-translational modification is likely to be involved in the reduction of HDAC2 activity. Nitration stress causes nitration of tyrosine residues on HDAC2, resulting in decreased activity (Osoata et al., 2009). The PI3K-dependent Akt pathway has been reported to induce HDAC2 phosphorylation and decrease activity (Adenuga et al., 2009). In fact, both H₂O₂ and CSE induce phosphorylation of Akt at serine 473 (Lahair et al., 2006) (Fig. 1) with concomitant reduction of HDAC activity (Fig. 2) (To et al., 2010). LY294002, a nonselective PI3K inhibitor, concentration-dependently inhibited CSE-induced Akt phosphorylation (Supplemental Fig. 1). We reported previously that LY294002 inhibited Akt phosphorylation and reversed HDAC activity in U937 cells in vitro and in mice exposed to cigarette smoke in vivo (To et al., 2010).

Budesonide (nM)	100	75.4	75.0	76.9	83.4	88.2
	33	75.5				87.2
	10	75.1	73.7	73.9	82.0	85.7
	3.3	72.1		73.7		85.5
	1	64.7	67.8	69.2	77.1	81.8
	0.33	50.1		58.5		
	0.1	29.0	31.9	38.7	52.9	61.9
	0	0	1.10	14.7	31.9	45.5
	(% inhibition)	0	1	3.3	10	33
Nortriptyline (μM)						

Fig. 4. Synergistic interaction between nortriptyline and budesonide. U937 cells were treated with H₂O₂ (200 μM) for 60 min and then stimulated with budesonide (10⁻¹⁰ to 10⁻⁷M) and/or Nt (1–33 μM) for 30 min before stimulation with TNFα (10 ng/ml). The supernatant was collected 24 h after treatment, and IL-8 was determined by ELISA. Inhibitory percentages on TNFα-induced IL-8 release are shown.

Fig. 3. Nortriptyline prevents cigarette smoke-induced corticosteroid insensitivity. A, U937 cells were pretreated with NAC (10 mM) for 30 min followed by CSE (33%) for 2 h. Cells were seeded in 96-well plates and treated with budesonide (10⁻¹² to 10⁻⁷M) for 45 min before overnight stimulation with TNFα (10 ng/ml). Supernatants were collected, and IL-8 expression was measured by ELISA. B and C, U937 cells were pretreated with Nt (1 and 3.3 μM) (B) or LY294002 (LY; 1 μM) (C) for 30 min followed by CSE (33%) for 2 h. Cells were collected, seeded in 96-well plates, and treated with budesonide (10⁻¹² to 10⁻⁷M) for 45 min before overnight stimulation with TNFα (10 ng/ml). Supernatants were collected, and IL-8 expression was determined. The percentage of inhibition of IL-8 by budesonide was calculated, and corticosteroid sensitivity was measured by EC₅₀.

onide on TNFα-induced IL-8 release in U937 cells as reported previously (Ito et al., 2001; Cosio et al., 2004). Because NAC, an antioxidant, reversed the CSE-induced decrease in budesonide effect, this suggests that oxidative stress directly causes corticosteroid insensitivity. The molecular mechanisms of oxidative stress-induced corticosteroid insensitivity have now been elucidated. H₂O₂ at high concentrations is reported to inhibit nuclear translocation of GR (Okamoto et al., 1999). However, another mechanism is a reduction in activity and expression of HDAC2, which is required for corticosteroids to switch off activated inflammatory genes (Barnes, 2009b). The activity, protein expression, and mRNA expression of HDAC2 are decreased in COPD cells and lung tissue (Ito et al., 2005). Oxidative stress induction of hypoxia-inducible factor 1α has been shown to reduce HDAC2 gene expression at the promoter region (Charron et al., 2009). However, in this study, oxidative stress reduced HDAC activity between 15 min and 2 h without any change in HDAC2 protein expression. Therefore, reduced transcription of HDAC2 is not involved in the model shown in the present study. Instead, post-translational modification is likely to be involved in the reduction of HDAC2 activity. Nitration stress causes nitration of tyrosine residues on HDAC2, resulting in decreased activity (Osoata et al., 2009). The PI3K-dependent Akt pathway has been reported to induce HDAC2 phosphorylation and decrease activity (Adenuga et al., 2009). In fact, both H₂O₂ and CSE induce phosphorylation of Akt at serine 473 (Lahair et al., 2006) (Fig. 1) with concomitant reduction of HDAC activity (Fig. 2) (To et al., 2010). LY294002, a nonselective PI3K inhibitor, concentration-dependently inhibited CSE-induced Akt phosphorylation (Supplemental Fig. 1). We reported previously that LY294002 inhibited Akt phosphorylation and reversed HDAC activity in U937 cells in vitro and in mice exposed to cigarette smoke in vivo (To et al., 2010).

Four different isoforms of PI3K have been identified, α, β, γ, and δ (Ito et al., 2007). Studies have found that pAkt is increased in COPD lung tissue and cells, which is caused by

increased activation of the PI3K δ isoform (To et al., 2010). Another study found that PI3K δ (-/-) null mice were protected from cigarette smoke-induced corticosteroid resistance and down-regulation of HDAC2 activity (Marwick et al., 2009). Furthermore, 2-(6-aminopurin-9-ylmethyl)-3-(2-chlorophenyl)-6,7-dimethoxy-3H-quinazolin-4-one (IC87114), a selective PI3K δ inhibitor (Sadhu et al., 2003), increased the effects of a corticosteroid in mice exposed to smoking (To et al., 2010). In addition, cells with knocked-down PI3K δ by RNA interference did not develop corticosteroid insensitivity in response to H₂O₂ (To et al., 2010). Thus, PI3K δ seems to be crucial in mediating corticosteroid insensitivity after oxidative stress via decreased activity of HDAC2.

Nortriptyline is a tricyclic drug that is the major metabolite of amitriptyline and has been used for a long time in the treatment of depression and nicotine addiction (Wagena et al., 2005). High-throughput screening has been used to identify drugs that had synergistic effects with the anti-inflammatory effects of corticosteroids in suppressing TNF α release (Lehár et al., 2009). Isobologram analysis has demonstrated the synergistic anti-inflammatory effects of nortriptyline and prednisolone. We confirmed the synergy between budesonide and nortriptyline in suppressing TNF α -induced IL-8 release in U937 cells exposed to H₂O₂ for 20 min using isobologram analysis (Fig. 4). In this study, nortriptyline also selectively inhibited both CSE- and H₂O₂-induced Akt phosphorylation and selectively inhibited PI3K δ enzyme activity (Table 1). At lower concentrations nortriptyline also restored the reduced levels of HDAC activity after both H₂O₂ and CSE exposure. Thus, nortriptyline was able to restore corticosteroid insensitivity by inhibiting PI3K δ enzyme activated by oxidative stress.

As shown in Table 2 and Fig. 3, although nortriptyline reversed budesonide insensitivity under conditions of oxidative stress, it did not have a significant impact on $E_{\max}$. LY294002 also failed to reverse the reduced $E_{\max}$ after CSE, suggesting that the reduction of $E_{\max}$ was not PI3K-dependent. We have reported previously that LY294002 modified EC₅₀ but not $E_{\max}$ in peripheral blood mononuclear cells obtained from patients with COPD (To et al., 2010). Nortriptyline is also known to inhibit histamine (H1) (Taylor and Richelson, 1980), α 1 adrenergic (Brown et al., 1980), and 5-HT₂ receptors (Sánchez and Hyttel, 1999). The use of antagonist for these receptors did not have any impact on EC₅₀ or $E_{\max}$, suggesting that nortriptyline's restoration of corticosteroid sensitivity is independent of these pathways.

Another widely used drug, theophylline, is also able to restore corticosteroid responsiveness under conditions of oxidative stress by increasing HDAC2 through selective inhibition of PI3K δ (Ito et al., 2002; Cosio et al., 2004; To et al., 2010). The inhibitory effect of theophylline on PI3K δ is markedly enhanced by oxidative stress, suggesting some allosteric effect on the enzyme, whereas nortriptyline seems to act directly in the enzyme, suggesting that it may act at a different site than theophylline.

In conclusion, nortriptyline was found to be a direct PI3K δ inhibitor and thereby able to reverse corticosteroid insensitivity induced by oxidative stress via restoration of HDAC activity. Thus, the combination therapy of nortriptyline and corticosteroids may be a useful treatment of corticosteroid-insensitive diseases, such as severe asthma and COPD. Because many patients with COPD and severe asthma suffer

from clinical depression (Hill et al., 2008; Ng et al., 2009) this may be a useful therapeutic combination. Clinical trials of nortriptyline in patients with COPD are now indicated, because the inhibitory effect of this drug is within the range of drug concentrations currently used in the treatment of depression.

Authorship Contributions

Participated in research design: Mercado, Ito, and Barnes.

Conducted experiments: Mercado and To.

Performed data analysis: Mercado and To.

Wrote or contributed to the writing of the manuscript: Mercado, Ito, and Barnes.

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