Nortriptyline Reverses Corticosteroid Insensitivity by Inhibition of Phosphoinositide-3-Kinase-δ

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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 (PI3Kδ). 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 (TNFα)-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 PI3Kδ activity, but had no effect on the PI3Kα and PI3Kγ isoforms. Although CSE reduced the effects of budesonide on TNFα-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 PI3Kδ 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 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 downregulated by post-translational modifications, such as nitration and oxidation (Barnes, 2009a) after treatment with hydrogen peroxide (H2O2), a peroxynitrate generator 3-morpholinosydnonimine (Osato et al., 2009), or cigarette smoke (Adenuga et al., 2009). In these studies, pretreatment with antioxidants, such as N-acetylcysteine (NAC) and glutathione, was found to restore HDAC activity and corticosteroid responsiveness.

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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; TNFα, tumor necrosis factor α; pAkt, phosphorylated Akt; ELISA, enzyme-linked immunosorbent assay; Nt, nortriptyline; SHT-2, 5-hydroxytryptamine type 2; LY294002, 2-(4-morpholino)-8-phenyl-4H-1-benzopyran-4-one; IC87114, 2-(6-aminoquinolin-9-ylmethyl)-3-(2-chlorophenyl)-6,7-dimethoxy-3H-quinoxalin-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 addition 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 (TNFα) 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 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 × 10⁶ 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-µm filter to sterilize and remove particulate matter and was used immediately. The optical density was measured at 320 nm and the IC₅₀ value was calculated to be 1.67 µM (Fig. 1B). LY294002 was added at the desired final concentrations to a mixture of phosphatidylinositol bisphosphate substrate and recombinant PI3Kα, δ, or γ enzymes (Millipore), and the mixture was incubated for 2 h at room temperature. After this incubation period, ATP (20 µM) was added to the enzyme/compound/phosphatidylinositol bisphosphate substate 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 ± 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 (BISOPFT, 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 µM; 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 µM (Fig. 1B). LY294002 also produced a concentration-dependent inhibition of CSE-induced pAkt with an IC₅₀ at 0.25 µM (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 PI3Kα, γ, and δ
activity, nortriptyline concentration-dependently inhibited PI3Kα activity (IC\textsubscript{50} 0.82 μM), and the efficacy was similar to that of LY294002 (IC\textsubscript{50} 0.98 μM), whereas nortriptyline had no effect on PI3Kα or PI3Kγ (Table 1).

**Nortriptyline Prevents CSE or H\textsubscript{2}O\textsubscript{2} Reduction of HDAC Activity.** H\textsubscript{2}O\textsubscript{2} 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 EC\textsubscript{50} value of 9.3 \( \times \) 10\textsuperscript{-10} M and \( E_{\text{max}} \) of 56%. Pretreatment with CSE (33%) resulted in a decrease in budesonide sensitivity (EC\textsubscript{50} 1.2 \( \times \) 10\textsuperscript{-9} M; \( p < 0.05 \) versus Nt) and reduced \( E_{\text{max}} \) (24%; \( p < 0.05 \) versus Nt) (Table 2). This was inhibited by pretreatment with NAC (10 mM) (EC\textsubscript{50} 6.1 \( \times \) 10\textsuperscript{-10} M with NAC), suggesting the reduction of budesonide sensitivity was mediated by oxidative stress (Fig. 3A).

**TABLE 1**

Inhibitory effects of nortriptyline and LY294002 on PI3K enzyme activity

<table>
<thead>
<tr>
<th>PI3Kα</th>
<th>PI3Kγ</th>
<th>PI3Kδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC\textsubscript{50} μM</td>
<td>No effect at 10 μM</td>
<td>No effect at 3.3 μM</td>
</tr>
</tbody>
</table>

Nortriptyline 0.82 | No effect at 10 μM | No effect at 3.3 μM |
LY294002 0.98 | 12.6 | 0.63 |

**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 H\textsubscript{2}O\textsubscript{2} (200 μM) for 15 min. Levels of phosphorylase 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 phosphorylase 473 Akt and total Akt1 were measured by Western blot in whole-cell extracts, and IC\textsubscript{50} values were calculated. *, \( p < 0.05 \); **, \( p < 0.01 \) compared with controls.

**Fig. 2.** Nortriptyline prevents oxidative stress reduction of HDAC activity. A, U937 cells were pretreated with Nt (1 μM) before stimulation with H\textsubscript{2}O\textsubscript{2} (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.
Inhibitory effects of nortriptyline and LY294002 on EC_{50} and E_{max} of budesonide

<table>
<thead>
<tr>
<th>Effects of Nortriptyline</th>
<th>Control</th>
<th>CSE</th>
<th>CSE + Nt (3.3 μM)</th>
<th>CSE + Nt (1 μM)</th>
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<tr>
<td>EC_{50} (nM)</td>
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<td>5.9</td>
<td>1.00</td>
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<td>E_{max} (%)</td>
<td>56 ± 5</td>
<td>24 ± 2</td>
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<table>
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<tr>
<th>Effects of LY294002</th>
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<th>CSE + LY294002 (1 μM)</th>
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</thead>
<tbody>
<tr>
<td>EC_{50} (nM)</td>
<td>1.28</td>
<td>2.67</td>
<td>1.07</td>
</tr>
<tr>
<td>E_{max} (%)</td>
<td>49 ± 6</td>
<td>12 ± 3</td>
<td>18 ± 4</td>
</tr>
</tbody>
</table>

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^{-3} M) 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^{-7} M) 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 EC_{50}.

Discussion

Oxidative stress, such as H_{2}O_{2} and CSE, may play an important role in the development of corticosteroid insensitivity in COPD and severe asthma (Amed and Barnes, 2008). As shown in Fig. 3, CSE reduced the effects of budesonide with nortriptyline showed greater inhibitory effects than either compound alone, and isobologram analysis demonstrated that this combination showed synergy (Fig. 4).

![Graph](https://example.com/graph.png)

<table>
<thead>
<tr>
<th>Nortriptyline (μM)</th>
<th>100</th>
<th>33</th>
<th>10</th>
<th>3.3</th>
<th>1</th>
<th>0.33</th>
<th>0.1</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Budesonide (nM)</td>
<td>75.4</td>
<td>75.0</td>
<td>76.9</td>
<td>83.4</td>
<td>88.2</td>
<td>87.2</td>
<td>85.7</td>
<td>85.5</td>
</tr>
<tr>
<td>(% inhibition)</td>
<td>0</td>
<td>1</td>
<td>3.3</td>
<td>10</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Synergistic interaction between nortriptyline and budesonide. U937 cells were treated with H_{2}O_{2} (200 μM) for 60 min and then stimulated with budesonide (10^{-10} to 10^{-3} M) 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.
increased activation of the PI3Kδ isoform (To et al., 2010). Another study found that PI3Kδ(−/−) 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-quinoxalino-4-one (IC87114), a selective PI3Kδ 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 PI3Kδ by RNA interference did not develop corticosteroid insensitivity in response to H2O2 (To et al., 2010). Thus, PI3Kδ 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 TNFα 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 TNFα-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 PI3Kδ enzyme activity (Table 1). At lower concentrations nortriptyline also restored the levels of HDAC activity after both H2O2 and CSE exposure. Thus, nortriptyline was able to restore corticosteroid insensitivity by inhibiting PI3Kδ 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 Emax, but not E50 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), 5-HT2 receptors (Sánchez and Hyttel, 1999), and histamine H1 receptor antagonists and antagonists of 5-HT2 receptors (Sañchez and Hyttel, 1999). The use of an antagonist for these receptors did not have any impact on Emax but not EC50, whereas nortriptyline seems to act at a different site in the enzyme, suggesting that it may act at a different site than theophylline.

Another widely used drug, theophylline, is also able to restore corticosteroid responsiveness under conditions of oxidative stress by increasing HDAC2 through selective inhibition of PI3Kδ (Ito et al., 2002; Cosio et al., 2004; To et al., 2010). The inhibitory effect of theophylline on PI3Kδ 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 PI3Kδ 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.

References


Lehár J, Krueger AS, Avery W, Heilbut AM, Johansen LM, Price ER, Rickles RJ,


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**Supplement Table 1.** Effects of nortriptyline, prazosin, ketanserin and mepyramine on EC$_{50}$ and E-max of budesonide.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CSE</th>
<th>CSE +P (1 μM)</th>
<th>CSE +Nt (1 μM)</th>
<th>CSE +K (1 μM)</th>
<th>CSE +M (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$ (nM)</td>
<td>0.143</td>
<td>0.640 *</td>
<td>0.726</td>
<td>0.080 #</td>
<td>0.366</td>
<td>0.795</td>
</tr>
<tr>
<td>Emax (%)</td>
<td>63± 5</td>
<td>34± 7</td>
<td>28± 12</td>
<td>38± 5</td>
<td>30± 2</td>
<td>35± 2</td>
</tr>
</tbody>
</table>

CSE: cigarette smoke extract, Nt: Nortriptyline, P: Prazosin, K: Ketanserin, M: Mepyramine, Emax: Maximal % inhibition of TNFα induced IL-8, EC$_{50}$: Value of budesonide that inhibits 50% TNFα induced IL-8, LY: LY294002. * p<0.01 vs Control.
# p<0.01 vs CSE.
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JPET

**Supplement Figure 1A.** U937 cells were pre-incubated with LY294002 (LY: 0.033-1 μM) and treated with cigarette smoke extract (CSE) for 5 min. Levels of phospho-serine 473 Akt and total Akt1 were measured by Western blot in whole-cell extracts and IC\textsubscript{50} values were calculated. * p<0.05, ** p<0.01 compared to controls.

**Supplement Figure 1B.** U937 cells were treated with cigarette smoke extract (CSE: 100%) for 2 h and HDAC2 and lamin A/C protein expression measured from nuclear extracts using SDS-PAGE/Western blotting.
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