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Nortriptyline reverses corticosteroid insensitivity by inhibition of PI3K-δ

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Abbreviations:

CSE, cigarette smoke extract

COPD, chronic obstructive pulmonary disease

FCS, foetal calf serum

GR, glucocorticoid receptor

HDAC2, histone deacetylase-2

H_2O_2, hydrogen peroxide

IL-8; interleukin-8

NAC, *N*-acetyl cysteine

PBMC, peripheral blood mononuclear cells

PI3K, phosphoinositide-3-kinase

ROS, reactive oxygen species

TNFα, tumour necrosis factor alpha

Recommended section

Inflammation and pharmacology

Abstract

Corticosteroid insensitivity represents a major barrier to the treatment of chronic obstructive pulmonary disease (COPD) and severe asthma. This is caused by oxidative stress, leading to reduced histone deacetylase-2 (HDAC2) function through activation of phophoinositide-3-kinase-δ (PI3Kδ). The tricyclic anti-depressant, 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 (pAkt), 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 TNFα-induced IL-8 production by budesonide. Hydrogen peroxide (H_2O_2) or cigarette smoke-conditioned extract (CSE) increased the level of pAkt and reduced HDAC activity. Pre-treatment with nortriptyline inhibited pAkt induced by CSE and H_2O_2 as well as restoring HDAC activity that had been decreased by H_2O_2 and CSE. In addition, nortriptyline inhibited PI3Kδ activity, with no effect on the PI3Kα and γ 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 in 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 (GR) and subsequent nuclear translocation. Activated GR inhibits pro-inflammatory gene transcription via inhibition of nuclear factor-κB (NF-κB)-associated histone acetylation by both direct inhibitions of CREB-binding protein (CBP)-associated histone acetyltransferases (HAT) activity and recruitment of histone deacetylase 2 (HDAC2) to the promoter of actively transcribed inflammatory genes (Ito et al., 2007b). HDAC2 expression and activity are reduced in bronchial biopsies, bronchoalveolar lavage (BAL) 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 smoking asthmatics (Ito and Mercado, 2009). Moreover, knock-down of HDAC2 expression in BAL macrophages induces corticosteroid insensitivity, whereas HDAC2 over-expression restores corticosteroid function (Ito et al., 2006). Reactive oxygen species (ROS) 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 (H_2O_2), a peroxynitrate generator SIN-1 (Osoata et al., 2009) or cigarette smoke (Adenuga et al.,

2009). In these studies, pre-treatment with anti-oxidants, such as *N*-acetylcysteine (NAC) and glutathione, prevent the post-translational modification and down-regulation of HDAC2 (Adenuga et al., 2009; Osoata et al., 2009). Recently, we have shown that oxidative stress induced phosphorylation and inactivation of HDAC2 through activation of the phosphoinositol-3-kinase (PI3K)/Akt pathway (To et al., 2010).

Nortriptyline is a second-generation tricyclic anti-depressant which has also been used to treat nicotine addition and smoking cessation in COPD patients but has also shown a marked improvement in certain respiratory symptoms (Borson et al., 1992). Recently high throughput screening has been used to identify drugs showing synergistic effects with corticosteroids on TNFα production in peripheral blood mononuclear cells (PBMC) (Lehar 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 ROS 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, 5% CO_2 in RPMI-1640 medium containing 10% foetal calf serum (FCS) and 15mM glutamine. For stimulation with hydrogen peroxide (H_2O_2) (Sigma, Poole, UK) or cigarette smoke extract (CSE), U937 cells were seeded (0.5x 10^6 cells/ml) using starvation media RPMI-1640 (phenol red free), with 1% FCS and 15 mM L-glutamine at 37°C, 5% CO_2.

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 1640 medium, as previously described (Walters et al., 2005). CSE was then passed through a 0.25μm filter to sterilize and remove particulate matter and was used immediately. The optical density was measured at 320λ 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 (0.5x10^6 cells/ml) in starvation media and left overnight at 37°C, 5% CO_2. Cells were incubated with nortriptyline (Sigma, 1, 3.3, 10 and 33 μM) or a non-selective PI3K inhibitor LY294002 (Sigma, 0.033, 0.1, 0.33, and 1 μM) for 30 minutes and then stimulated with H_2O_2 (200 μM) for 15 minutes or CSE for 5 minutes. Phosphatase Inhibitor (Active Motif, 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) and 40 μg of protein samples were separated using 10% SDS-PAGE/Western blot (Invitrogen, Paisley, UK). Serine 473 phosphorylation of Akt and total Akt1 were detected with mouse monoclonal anti-S473 Akt (Cell Signalling, Hitchin, UK) and rabbit polyclonal anti-Akt1 (Cell Signalling) respectively. Band density of S473Akt was normalized to total Akt1.

HDAC activity

Cells seeded at $0.5x10^6$ cells/ml in starvation media were treated with nortriptyline (1, 0, 3.3 and 10 μM) for 30 minutes and then stimulated with H_2O_2 (200 μM) for 15 minutes or CSE (100% and 33%) for 2 hours. Nuclear extracts were prepared as previously described (Ito et al., 2002) and HDAC activity (Biomol, Exeter, UK) was measured in 10 μl from nuclear extracts and normalized to protein concentrations.

HDAC2 expression

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

Corticosteroid sensitivity assay

U937 cells (seeded at $0.5x10^6$ cells/ml) were treated with nortriptyline (Klein et al., 1991) (1 and 3.3 μM) (Sigma), N-acetylcysteine (NAC) (10 mM), LY294002 (Gharbi et al., 2007) (1 μM) (Sigma), prazosin (Faridbod et al., 2010) (0.1 μM) (Sigma), mepyramine (Kiss et al., 2004) (1 μM) (Sigma) and ketanserin (Herndon et al., 1992) (0.1 μM) (Sigma) for 30 minutes prior stimulation of CSE (33%) for 2 hours. Cells were collected by centrifugation and pellets re-suspended in starvation media. Cells were seeded in 96-

well plates in the presence of budesonide (AstraZeneca, Lund, Sweden, 10^{-12} to 10^{-7}M) for 45 minutes prior to overnight stimulation with TNFα (10 ng/ml, R&D Systems, Abingdon, UK). Supernatants were collected and IL-8 expression was measured by ELISA (R&D Systems). The % inhibition of IL-8 by budesonide was calculated and corticosteroid sensitivity measured as $EC_{50.}$

PI3K activity assay

PI3K enzyme inhibitory activity was determined by the time-resolved fluorescence resonance energy transfer (TR-FRET) (HTRF®PI3K enzyme assay, Millipore, Watford, UK). Nortriptyline or LY294002 was added, at the desired final concentrations, to a mixture of PIP2 substrate and recombinant PI3 kinase α, δ or γ enzymes (Millipore), and the mixture incubated for 2h at room temperature. Following this incubation period, ATP (20 μM) was added to the enzyme/compound/PIP_2 substrate mixture and the resulting mixture was incubated for 30 min at room temperature. The percentage inhibition of each reaction was calculated relative to vehicle-treated control, and the 50% inhibitory concentration (IC_{50} value) then calculated from the concentration-response curve.

Statistical Analysis

Data are expressed as means ± SEM. Results were analyzed using paired t-test or one-way Anova for repeated measures with Dunnett post-test for multiple comparisons. EC_{50} of budesonide was determined from concentration-inhibitory response curve of IL-8 production, and the differences of EC_{50} values of budesonide were assessed using Bonferroni's multiple comparison test. The Graph Pad Prism Software (Prism, 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

analysed by isobologram by the method of Chou-Talalay (Chou and Talalay, 1977) using CalcuSyn software (BISOFT®, Cambridge, UK)

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Results

Nortriptyline prevents CSE and H_2O_2 induced phosphorylation of Akt

Phosphorylation of Akt at serine 473 was used to measure PI3K activation. S473Akt phosphorylation was increased by H_2O_2 (3.4±0.2 fold increase *vs.* control, $p<0.01$) and CSE (3.3±0.2 fold increase *vs*. control, $p<0.01$) (Figures 1A and 1B). Pre-incubation with nortriptyline (1-10 μM significantly ($p<0.01$)) prevented Akt phosphorylation by H_2O_2 (Figure 1A) and by CSE ($p<0.01$) and the IC_{50} was calculated to be 1.67 μM (Figure 1B). LY294002 also produced a concentration-dependent inhibition of CSE-induced pAkt with an IC_{50} at 0.25 μM (Supplement 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 PI3Kδ activity (IC_{50}: 0.82 μM) and the efficacy was similar to that of LY294002 (IC_{50}: 0.98 μM), whereas nortriptyline had no effect on PI3Kα or PI3Kγ (Table 1).

Nortriptyline prevents CSE or H_2O_2 reduction of HDAC activity

H_2O_2 significantly reduced HDAC activity (39±12% vs. control, $p<0.05$) and this was completely prevented by nortriptyline at 1 μM ($p<0.05$) (Figure 2A). Incubation with CSE also resulted in a significant decrease of HDAC activity (9±3% at 33% CSE and 19±3% at 100% CSE, $p<0.05$) (Figure 2B). Pre-incubation with nortriptyline (1 μM) also completely restored HDAC activity ($p<0.05$ vs CSE) reduced by CSE (Figure 2B). As HDAC2 protein expression was not reduced after CSE (Supplement 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_{50} value of $9.3x10^{-10}$ M and Emax of 56%. Pre-treatment with CSE (33%) resulted in a decrease in budesonide sensitivity (EC_{50}: $1.2x10^{-9}$ M, $p<0.05$ vs. NT) and reduced E-max (24%, $p<0.05$ vs. NT) (Table 2). This was inhibited by pre-treatment with NAC (10 mM) (EC_{50}: $6.1x10^{-10}$ M with NAC), suggesting the reduction of budesonide sensitivity was mediated by oxidative stress (Figure 3A). Nortriptyline also prevented CSE-induced corticosteroid insensitivity. CSE (EC_{50}: 5.9 nM, Emax: 24±2%) induced budesonide insensitivity compared to control (EC_{50}: 0.85 nM, $p<0.01$ vs. NT and Emax: 56±5%, $p<0.05$ vs. NT) (Figure 3B). Nortriptyline reversed budesonide insensitivity with CSE (EC_{50} at 1 μM: 0.54 nM, $p<0.001$ vs. CSE and EC_{50} at 3.3 μM: 1.00 nM, $p<0.001$ vs CSE). Emax was also reduced by nortriptyline however not significantly (Emax at 1 μM: 33±6% and at 3.3 μM: 32±8%). LY294002 (1 μM) also reversed CSE-induced budesonide insensitivity (EC_{50} of budesonide: 1.07 nM with LY294002 in the presence of CSE vs. 2.67 nM in CSE control, $p<0.05$), but did not have any impact on Emax (Figure 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 (Sanchez 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 EC_{50} or Emax (Supplement Table 1) whereas nortriptyline was shown to restore EC_{50} as previously reported.

Nortriptyline and budesonide showed synergistic effects

U937 cells were treated with H_2O_2 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 combination of budesonide with nortriptyline showed greater inhibitory effects than either compound alone and isobologram analysis demonstrated that this combination showed synergy (Figure 4).

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Discussion

Oxidative stress, such as H_2O_2 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 Figure 3, CSE reduced the effects of budesonide on TNFα-induced IL-8 release in U937 cells, as previously reported (Cosio et al., 2004; Ito et al., 2001). As NAC, an anti-oxidant 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. H_2O_2 at high concentrations are 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 a 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 1 alpha (HIF-1α) has recently 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. Nitrative stress causes nitration of tyrosine residues on HDAC2, resulting in decreased activity (Osoata et al., 2009). Recently, PI3K-dependent Akt pathway is reported to induce HDAC2 phosphorylation and decreased activity (Adenuga et al., 2009). In fact, both H_2O_2 and CSE induce phosphorylation of Akt at serine 473 (Lahair et al., 2006) (Figure 1) with concomitant reduction of HDAC activity (Figure 2) (To et al., 2010). LY294002, a non-selective PI3K inhibitor, concentration-dependently inhibited CSE-

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induced Akt phosphorylation (Supplement Figure 1). We previously reported that LY294002 inhibited Akt phosphorylation and reversed HDAC activity in U937 cells *in vitro* and in cigarette smoke exposed mice *in vivo* (To et al., 2010).

Four different isoforms of in PI3K have been identified, namely α, β, γ and δ (Ito et al., 2007a). Recent studies have found that pAkt is increased in COPD lung tissue and cells and that this is due to 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, IC87114, a selective PI3Kδ inhibitor (Sadhu et al., 2003), increased the effects of a corticosteroid in smoking mice (To et al., 2010). In addition, cells with knocked-down PI3Kδ by RNA interference did not develop corticosteroid insensitivity in response to H_2O_2 (To et al., 2010). Thus PI3Kδ appears to be a crucial in mediating corticosteroid insensitivity after oxidative stress via decreased activity of HDAC2.

Nortriptyline is a tricyclic drug which is the major metabolite of amitriptyline and has been used for a long time in the treatment of depression and also 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 (Lehar 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 H_2O_2 for 20 min using isobologram analysis (Figure 4). In this study, nortriptyline also selectively inhibited both CSE and H_2O_2-induced Akt phosphorylation and selectively inhibited PI3Kδ enzyme activity (Table 1). At lower concentrations nortriptyline also restored the reduced levels of HDAC activity after both H_2O_2 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 Figure 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 Emax after CSE, suggesting that the reduction of Emax was not PI3K-dependent. We have previously reported that LY294002 modified EC_{50} but not Emax in PBMCs obtained from COPD (To et al., 2010). Nortriptyline is also known to inhibit Histamine (H1) (Taylor and Richelson, 1980), α1 adrenergic (Brown et al., 1980) and 5-HT2 receptors (Sanchez and Hyttel, 1999). The use of antagonist for these receptors did not have any impact on EC_{50} or Emax 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 PI3Kδ (Cosio et al., 2004; Ito et al., 2002; 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 appears to act directly in the enzyme, suggesting that it may act at a different site to 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 useful treatment of corticosteroid-insensitive diseases, such as severe asthma and COPD. Since 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 COPD patients are now indicated since the

inhibitory effect of this drug is within the range of drug concentrations currently used in the treatment of depression.

Authorship contribution:

Participated in research design: N. Mercado, P.J. Barnes and K. Ito

Conducted experiments: N. Mercado and Y. To.

Performed data analysis: N. Mercado and Y. To.

Wrote or contributed to the writing of the manuscript: N. Mercado, P.J. Barnes and K. Ito

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Footnotes

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Figure legends

Figure 1. Nortriptyline prevents oxidative stress activation of PI3K/Akt pathway. (A) U937 cells were pre-incubated with nortriptyline (Nt: 1-10 μM) for 30 min and treated with hydrogen peroxide (H_2O_2: 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 pre-incubated with nortriptyline (1-33 μM) and treated with cigarette smoke extract (CSE) for 5 min. Levels of phosphoserine 473 Akt and total Akt1 were measured by Western blot in whole-cell extracts and. IC_{50} values were calculated. * $p<0.05$, ** $p<0.01$ compared to controls.

Figure 2. Nortriptyline prevents oxidative stress reduction of HDAC activity. (A) U937 cells were pre-treated with nortriptyline (Nt: 1 μM) before stimulation with hydrogen peroxide (H_2O_2: 200 μM) for 15 min. HDAC activity was measured by fluorometric activity assay in nuclear extracts. (B) U937 cells were pre-incubated with nortriptyline (Nt: 1 μM) for 30 minutes before stimulation with cigarette smoke extract (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 to controls.

Figure 3. Nortriptyline prevents cigarette smoke-induced corticosteroid insensitivity. (A) U937 cells were pre-treated with N-acetylcysteine (NAC: 10 mM) for 30 min followed by cigarette smoke extract (CSE: 33%) for 2 h. Cells were seeded in 96-well plates and treated with budesonide (10^{-12} to 10^{-7}M) for 45 min prior to overnight stimulation with tumor necrosis factor-α (TNFα: 10 ng/ml). Supernatants were collected and interleukin (IL)-8 expression was measured by ELISA. (B) U937 cells were pre-treated with nortriptyline (Nt; 1 and 3.3 μM) or (C) LY294002 (LY: 1 μM) 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 prior overnight stimulation with TNFα (10 ng/ml). Supernatants were collected and IL-8 expression. The % inhibition of IL-8 by budesonide were calculated and corticosteroid sensitivity measured by EC_{50}.

Figure 4. Synergistic interaction between nortriptyline and budesonide. U937 cells were treated hydrogen peroxide (H_2O_2: 200 μM) for 60 min and then stimulated with budesonide (10^{-10} to 10^{-7}M) and/or nortriptyline (Nt: 1-33 μM) for 30 min prior stimulation with tumor necrosis factor-α (TNFα: 10 ng/ml). The supernatant was collected 24 h after treatment and interleukin (IL)-8 was determined by ELISA. Inhibitory percent on TNFα-induced IL-8 release is shown in the figure.

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Table 1. Inhibitory effects of nortriptyline and LY294002 on PI3K enzyme activity.

IC_{50} (μM)	**PI3Kδ**	**PI3Kγ**	**PI3Kα**
Nortriptyline	0.82	NE at 10μM	NE at 3.3μM
LY294002	0.98	12.6	0.63

NE: no effect; PI3K: phosphoionositol-3 kinase; IC_{50}: Value of nortriptyline required for 50 % inhibition of PI3K activity.

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Table 2. Inhibitory effects of nortriptyline and LY294002 on EC_{50} and E-max of budesonide.

	Control	**CSE**	**CSE +Nt (3.3 µM)**	**CSE +Nt (1 µM)**
EC_{50} (nM)	0.85	5.9	1.00	0.54
Emax (%)	56± 5	24± 2	32± 8	33± 6

	Control	**CSE**	**CSE +LY(1 µM)**
EC_{50} (nM)	1.28	2.67	1.07
Emax (%)	49± 6	12± 3	18± 4

CSE: cigarette smoke extract; Nt: Nortriptyline; Emax: Maximal % inhibition of TNFα induced IL-8; EC_{50}: Value of budesonide that inhibits 50% TNFα induced IL-8. LY: LY294002

Figure 1

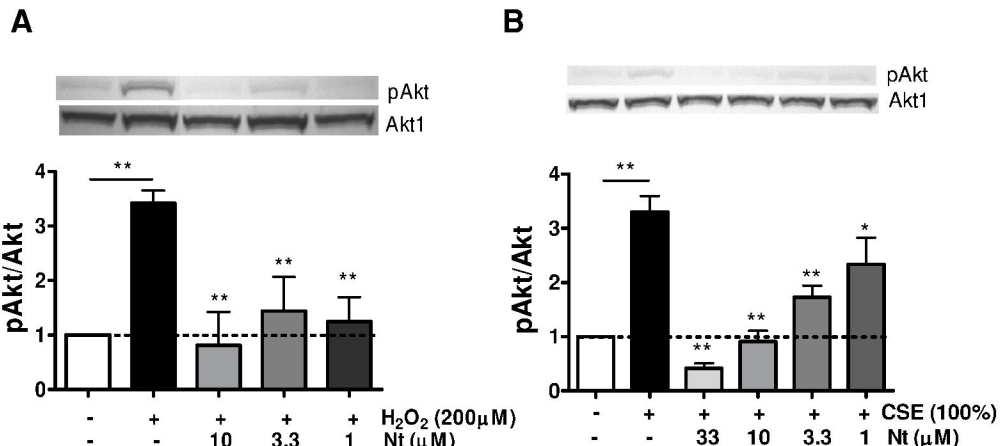
A
pAkt
Akt1
pAkt/Akt
0
1
2
3
4
**
**
**
**
- + + + + H2O2 (200µM)
- - 10 3.3 1 Nt (µM)
B
pAkt
Akt1
pAkt/Akt
0
1
2
3
4
**
**
**
**
*
- + + + + + CSE (100%)
- - 33 10 3.3 1 Nt (µM)

Figure 2

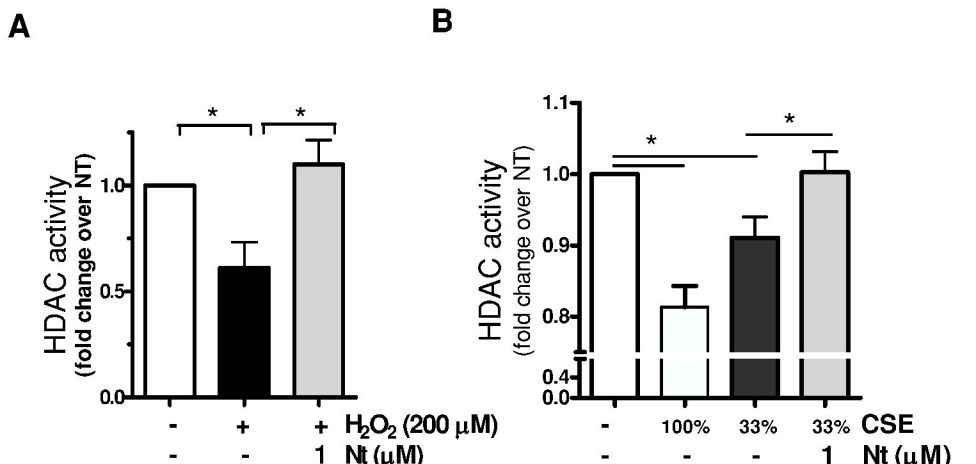
A
B
HDAC activity
(fold change over NT)
1.0
0.5
0.0
*
*
- + + H2O2 (200 μM)
- - 1 Nt (μM)
1.1
1.0
0.9
0.8
0.4
0.0
- 100% 33% 33% CSE
- - - 1 Nt (μM)

Figure 3

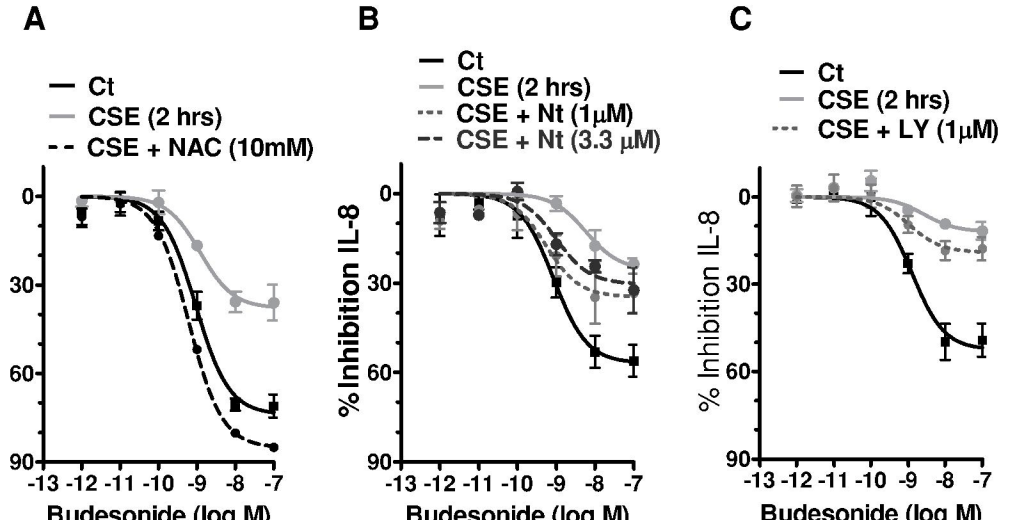
A
Ct
CSE (2 hrs)
CSE + NAC (10mM)
0
30
60
90
-13 -12 -11 -10 -9 -8 -7
Budesonide (log M)
B
Ct
CSE (2 hrs)
CSE + Nt (1μM)
CSE + Nt (3.3 μM)
% Inhibition IL-8
0
30
60
90
-13 -12 -11 -10 -9 -8 -7
Budesonide (log M)
C
Ct
CSE (2 hrs)
CSE + LY (1μM)
% Inhibition IL-8
0
30
60
90
-13 -12 -11 -10 -9 -8 -7
Budesonide (log M)

Figure 4

Budesonide (nM)	Nortriptyline (μM)				
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**