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RESEARCH PAPER

Corticosteroid insensitivity is reversed by formoterol via phosphoinositide-3-kinase inhibition

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BACKGROUND AND PURPOSE

Patients with chronic obstructive pulmonary disease (COPD) show a poor response to corticosteroids, which has been linked to oxidative stress. Here we show that the long-acting β_2-agonist formoterol (FM) reversed corticosteroid insensitivity under oxidative stress via inhibition of phosphoinositide-3-kinase (PI3K) signalling.

EXPERIMENTAL APPROACH

Responsiveness to corticosteroids dexamethasone (Dex), budesonide (Bud) and fluticasone propionate (FP) was determined, as IC_{50} values on TNF-α-induced interleukin 8 release, in U937 monocytic cell line treated with hydrogen peroxide (H_2O_2) or peripheral blood mononuclear cells (PBMCs) from patients with COPD or severe asthma.

KEY RESULTS

PBMCs from severe asthma and COPD were less sensitive to Dex compared with those from healthy subjects. Both FM (10^{-9} M) and salmeterol (SM, 10^{-8} M) reversed Dex insensitivity in severe asthma, but only FM restored Dex sensitivity in COPD. Although H_2O_2 exposure decreased steroid sensitivity in U937 cells, FM restored responsiveness to Bud and FP while the effects of SM were weaker. Additionally, FM, but not SM, partially inhibited H_2O_2-induced PI3Kδ-dependent (PKB) phosphorylation. H_2O_2 decreased SM-induced cAMP production in U937 cells, but did not significantly affect the response to FM. The reduction of SM effects by H_2O_2 was reversed by pretreatment with LY294002, a PI3K inhibitor, or IC87114, a PI3Kδ inhibitor.

CONCLUSION AND IMPLICATIONS

FM reversed oxidative stress-induced corticosteroid insensitivity and decreased β_2 adrenoceptor-dependent cAMP production via inhibition of PI3Kδ signalling. FM will be more effective than SM, when combined with corticosteroids, for the treatment of respiratory diseases under conditions of high oxidative stress, such as in COPD.

Abbreviations

AS604850, 5-(2,2-difluoro-benzo[1,3]dioxol-5-ylmethylene)-thiazolidine-2,4-dione; COPD, chronic obstructive pulmonary disease; EPAC, exchange protein activated by cAMP; GR, glucocorticoid receptor; H-89, N-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrate dihydrochloride; H_2O_2, hydrogen peroxide; HDAC2, histone deacetylase-2; HSP27, heat shock protein 27; IC87114, 2-((6-amino-9H-purin-9-yl)methyl)-5-methyl-3-o-tolylquinazolin-4(3H)-one; IL-8, interleukin 8 (CXCL8); LABA, long-acting β_2-agonist; LY294002, 2-morpholin-4-yl-8-phenylchromen-4-one; LY303511, 2-(1-piperazinyl)-8-phenyl-4H-1-benzopyran-4-one; MSK2, mitogen- and stress-activated protein kinase; PBMC, peripheral blood mononuclear cell

Introduction

Although corticosteroids are the most potent anti-inflammatory agents for the treatment of chronic inflammatory diseases such as asthma, severe asthma patients, asthmatic patients who smoke and almost all patients with chronic obstructive pulmonary disease (COPD) show a poor response to corticosteroids (Thomson *et al.*, 2006; Drummond *et al.*, 2008; Ito and Mercado, 2009). These patients present considerable management problems and account for a huge part of the health care costs for treatment of airway diseases.

COPD is characterized by chronic airway inflammation, particularly neutrophilic inflammation, and several cytokines and chemokines were reported to be up-regulated in lungs from COPD (Barnes, 2009). Interleukin 8 (IL-8, CXCL8) is a well-known chemokine for neutrophils (Beeh *et al.*, 2003) and is abundant in bronchoalveolar lavage, sputum and blood from COPD patients (Nocker *et al.*, 1996; Brozyna *et al.*, 2009; Garcia-Rio *et al.*, 2010). The IL-8 production is less sensitive to corticosteroids (Ito *et al.*, 2006; Armstrong *et al.*, 2009), and we demonstrated that TNF-α-induced IL-8 production in peripheral blood mononuclear cells (PBMCs) is a good marker for evaluating corticosteroid sensitivity (To *et al.*, 2010).

COPD is also known to be caused by long-term inhalation of noxious gases and particles, such as cigarette smoke and woodsmoke (Stockley *et al.*, 2009). High levels of oxidative stress are seen in sputum, bronchoalveolar lavage fluid and exhaled breath obtained from smoking asthma patients and patients with COPD (Paredi *et al.*, 2002; Louhelainen *et al.*, 2008). Oxidative stress is reported to cause corticosteroid insensitivity via a reduction of the activity and expression of the critical nuclear enzyme histone deacetylase-2 (HDAC2), reduced nuclear translocation of glucocorticoid receptors (GRs) after ligand binding or degradation of GR by ubiquitination (Barnes and Adcock, 2009).

PI3K generates lipid second messengers that control an array of intracellular signalling pathways which have important roles in inflammation (Ito *et al.*, 2007). PI3Ks are subdivided into four classes (IA, IB, II and III) that possess lipid kinase activity, and the kinases largely responsible for PIP_3 are class I-PI3Ks. Class I- PI3K contains four isoforms of the catalytic subunit, known as p110α, p110β, p110γ and p110δ. The isoforms of p110γ and p110δ are predominantly expressed in leucocytes and lymphocytes, and are thought to be involved in inflammation to a greater extent than the other two isoforms (Ito *et al.*, 2007). We reported that the p110δ isoform is significantly up-regulated in peripheral lung tissue from COPD patients, and also IC87114, a selective PI3K-δ inhibitor, inhibited neutrophil accumulation when these patients were treated with dexamethasone (Dex) (To *et al.*, 2010). Furthermore, results by PI3K-δ knock-down by RNA interference in monocytic cell line and PI3K-δ knock-out mice supported the findings (Marwick *et al.*, 2009; To *et al.*, 2010). Thus, PI3Kδ is involved in corticosteroid insensitivity seen in COPD.

Recently, the combination of a long-acting β_2-agonist (LABA) with a low dose of inhaled corticosteroid has been reported to achieve better asthma control than either drug alone, or a higher dose of inhaled corticosteroid (Gibson *et al.*, 2007; O'Byrne *et al.*, 2008; Chung *et al.*, 2009). The efficacy of combination therapy in COPD is less clear although several studies showed beneficial effects (Nannini *et al.*, 2007). For example, the effect of the formoterol/budesonide combination significantly improved lung function and health-related quality of life with a reduction in exacerbations and symptoms (Calverley *et al.*, 2003; Szafranski *et al.*, 2003; Tashkin *et al.*, 2008). The large TORCH study also showed beneficial effects of a combination of salmeterol and fluticasone propionate (FP) in reducing exacerbations, although there was no effect on the decline of lung function over 3 years (Calverley *et al.*, 2007). The beneficial effects of add-on treatment of LABAs on corticosteroids have also been confirmed in several *in vitro* systems (Usmani *et al.*, 2005; Kaur *et al.*, 2008; Skevaki *et al.*, 2009), but the molecular mechanism responsible for this effect has not been fully elucidated.

Here we show that formoterol, but not salmeterol, is able to restore corticosteroid sensitivity impaired under oxidative stress by an inhibitory effect on PI3K signalling.

Methods

Reagents

Formoterol and budesonide were kindly provided by Astra Zeneca (Lund, Sweden) and salmeterol by GlaxoSmithKline (Stevenage, UK). Dex, FP, AS604850, LY294002 hydrochloride, LY303511 and H-89 dihydrochloride were purchased from Sigma-Aldrich (Poole, UK); IC87114 from Echeron Bioscience, Inc. (Salt Lake City, UT, USA); KT5720, RP-cAMPs, 8-Bromo-cAMP (8Br) and 8-CPT cAMP from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and recombinant TNF-α from R&D Systems (Minneapolis, MN, USA).

Subjects

Seven age-matched healthy subjects, six mild asthma, and seven severe asthmatics defined by GINA (http://www.ginasthma.com) (Global Intiative for Asthma, 2008) (Table 1) as well as nine age-matched healthy subjects, seven smokers without COPD, and 13 moderate to severe COPD patients as defined by GOLD (http://www.goldcopd.com) (The Global Initiative for Chronic Obstructive Lung Disease (GOLD), 2008, http://www.goldcopd.com) (Table 2) were recruited. The PBMCs were separated by Accuspin (Sigma-Aldrich). This study was approved by the Ethics Committee of the Royal Brompton & Harefield Hospitals National Health Service Trust, and all subjects gave written informed consent.

Cytokine detection

PBMCs or U937 cells (a human monocytic cell line, the American Culture of Tissue Collection) were stimulated with TNF-α (1 or 10 $ng \cdot mL^{-1}$) in the presence or absence of corticosteroids, and incubated at 37°C for 16 h. IL-8 (CXCL8) in supernatant was determined by ELISA (R&D Systems). The IC_{50} values for corticosteroid response were calculated from sigmoid concentration–response curves with variable slope using GraphPad Prism® version 4 software (GraphPad Software, San Diego, CA, USA).

Table 1

Patient demographic data for healthy subjects, patients with mild asthma and severe asthma

	(Mean ± SD) Healthy	Mild asthma	Severe asthma
n	7	6	7
Age (years)	37.1±8.9	37.0±7.5	32.9 ± 10.7
Male/Female	3/4	2/4	1/6
Smoking history (pack year)	0	0	0
Pre-FEV_1 (% predicted)	93.7±11.2	88.6±12.7	59.6 ± 14.2$^{\#\#}$
Pre-FEV_1/FVC	87.9±4.8	95.3±8.6	68.3 ± 17.6$^{\#\#}$
ICS (ug) (*n*)	0	0	1429 ± 787 (6/7)
OC (mg) (*n*)	0	0	12.1 ± 13.5 (4/7)
Other medication	None	Albuterol on demand	Albuterol on demand

$^{\#\#}P < 0.01$ versus healthy, Kruskal–Wallis, Dunn's multiple comparison.
FEV_1, forced expiratory volume in one second; FVC, forced volume vital capacity; ICS, inhaled corticosteroid; OCS, oral corticosteroid.

Table 2

Patient demographic data for patients with COPD, smokers without COPD and age-matched non-smoker

	(Mean ± SD) Non-smoker	Smoker (w/o COPD)	COPD
n	9	7	13
Age (years)	56.6±5.5	56.1±9.5	64.5 ± 7.7
Male/Female	3/6	4/3	5/8
Pack year	0	43.7±18.1	46.2 ± 18.8
Pre-FEV_1 % predicted	87.4±15.7	96.3±9.0	41.7 ± 12.2$^{\#\#}$
Pre-FEV_1/FVC	78.0±6.6	73.8±4.3	39.6 ± 11.0$^{\#\#}$
ICS (ug) (*n*)	0	0	1154 ± 1068 (10/13)
OCS (mg) (*n*)	0	0	25.0 ± 13.2 (3/3)
Other medication	None	None	Anti-muscarinic (6/13)
			Theophylline (1/13)
			Albuterol on demand

$^{\#\#}P < 0.01$ versus healthy, Kruskal–Wallis, Dunn's multiple comparison.
FEV_1, forced expiratory volume in one second; FVC, forced volume vital capacity; ICS, inhaled corticosteroid; OCS, oral corticosteroid.

Western blot analysis for PKB detection

Total cell extracts prepared using RIPA buffer were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane (Amersham Biosciences, Amersham, UK) as previously reported (To *et al.*, 2010). The membranes were incubated with anti-phospho-PKB1 antibody or anti-total PKB1 antibody (Upstate, Southampton, UK) followed by a horseradish peroxidase-conjugated secondary antibody (Dako UK Ltd., Ely, UK).

Cell-based analysis of phosphorylated and total PKB

Phosphorylated PKB and total PKB were detected in U937 cells using PKB phosphorylation cell-based ELISA (FACE kit, Active Motif, Rixensart, Belgium). Briefly, cells (0.2 million per well) were added in poly-L-lysine (10 $\mu g \cdot mL^{-1}$) coated 96-well plates and stimulated with H_2O_2 (100 µM). At 7.5 min after stimulation, cells were fixed with 4% formaldehyde PBS solution and permeabilized with 0.1% TritonX100 in the wells. The levels of phosphorylated PKB and total PKB were determined using a double immunoenzymatic labelling.

Membrane protein extraction and β_2 adrenoceptor detection

Membrane protein extraction was performed using the ProteoExtract® Transmembrane protein extraction kit (Novagen, Madison, WI, USA) according to manufacturer's instructions.

The β_2 adrenoceptors were determined by SDS-PAGE/Western blotting using anti-β_2 adrenoceptor antibody (Abcam, Cambridge, UK).

Human phospho-MAPK array

U937 cells were treated with LABAs before stimulation with H_2O_2, or left untreated. Human phospho-MAPK array analysis was performed using Proteome Profiler™ antibody arrays (R&D Systems) according to the manufacturer's instructions.

cAMP assay

cAMP in cell extracts was determined by a cAMP assay kit (Cayman Chemicals, Tallinn, Estonia), according to the manufacturer's instructions.

Statistical analysis

Results are expressed as means ± SD (for clinical samples) or SEM (for *in vitro* studies), or median with inter-quartile range. Multiple comparisons were performed by ANOVA following Dunnett's multiple comparison test or Bonferroni's multiple comparison test. When the data were not normally distributed, determination of variance was done by Kruskal–Wallis analysis, followed by Dunn's multiple comparison tests. The comparisons between two groups were performed by Welch's *t*-test or Mann–Whitney *U*-test. A *P*-value < 0.05 was considered statistically significant. These analyses were performed using GraphPad Prism 4 software.

Results

Formoterol reversed corticosteroid insensitivity in PBMCs of COPD patients

PBMCs obtained from healthy subjects, current smokers without COPD, patients with moderate to severe COPD, and patients with severe asthma or mild asthma were stimulated with TNF-α for 16 h in the absence or presence of serial dilutions of Dex, and IL-8 was measured in the supernatant. The IC_{50} value of Dex (Dex-IC_{50}) was calculated and used as a measurement of corticosteroid sensitivity. The Dex-IC_{50} of severe asthma patients was 178.8 ± 83.0 nM (mean ± SD) and significantly higher than those of healthy control and mild asthmatics (26.6 ± 24.2 and 20.0 ± 7.9 nM, respectively) (Figure 1A, Supporting Information Table S1). The Dex-IC_{50} of COPD patients was 218.6 ± 225.1 nM, higher than those of non-smoker controls (37.1 ± 30.4 nM, $P < 0.05$) and smoker controls (47.5 ± 51.8 nM) (Figure 1A, Supporting Information Table S1). Thus, PBMCs from severe asthma and COPD patients were less sensitive to Dex compared with cells from healthy subjects.

As seen in Figure 1B, salmeterol showed slow onset of cAMP production in PBMCs from healthy subjects compared with formoterol. In addition, formoterol (1 nM) and salmeterol (100 nM) induced similar amounts of cAMP formation at 20 min after treatment. Therefore, 20 min incubation time was used for subsequent experiments. As summarized in Supporting Information Table S1, add-on treatment with formoterol (1 nM) or salmeterol (100 nM) had no effect on Dex sensitivity in PBMCs from healthy subjects ($n = 7$). In contrast, the Dex-IC_{50} was significantly reduced by treatment with formoterol (1 nM) and salmeterol (100 nM) in PBMCs from severe asthma patients (Supporting Information Table S1). In PBMCs from COPD patients, formoterol, but not salmeterol, shifted the concentration–response curve of Dex leftwards (Figure 1C,D), and therefore, formoterol significantly reduced the Dex-IC_{50} value [60.3 ± 57.8 nM (mean ± SD) vs. 218.6 ± 225.1 nM with vehicle, $P < 0.05$] while salmeterol did not significantly affect Dex sensitivity (Dex-IC_{50}: 126.2 ± 137.9 nM with salmeterol 100 nM and 157.7 ± 194.8 nM with salmeterol 10 nM) (Figure 1E,F, Supporting Information Table S1).

As LABA is reported to enhance GR nuclear translocation (Usmani *et al.*, 2005), we determined the level of GR nuclear translocation in PBMCs as fluorescein isothiocyanate (FITC) fluorescence level in nuclei after treatment with FITC-dexamethasone (FITC-Dex) (Mercado *et al.*, 2011). GR nuclear translocation was significantly impaired in COPD cells (FITC-Dex in nuclei in COPD, 3.3 nM, 1.9–7.4, median, inter-quartile range, $n = 13$) compared with non-smokers (7.8 nM, 6.5–8.8, median, inter-quartile range, $n = 8$, $P < 0.05$ with Bonferroni's correction) and smokers without COPD (6.6 nM, 2.6–8.2, median, inter-quartile range, $n = 7$, $P < 0.05$ with Bonferroni's correction) (Supporting Information Figure S1A). Add-on treatment with formoterol (1 or 10 nM) significantly enhanced GR nuclear translocation in COPD cells. Although salmeterol (100 nM) slightly increased it, the effect was not statistically significant.

Formoterol reversed corticosteroid insensitivity induced by oxidative stress

U937 cells were stimulated with TNF-α (1 ng·mL^{-1}) in the absence or presence of 30 min pre-incubation with serial dilutions of budesonide (0.01–100 nM) and IL-8 was measured in the supernatant collected at 16 h after TNF-α stimulation. Budesonide inhibited TNF-α-induced IL-8 production in a concentration-dependent manner with IC_{50} of 0.38 ± 0.081 nM (mean ± SEM) and maximal inhibition (Imax) of 87.1 ± 2.8% (Table 3, Figure 1G). TNF-α-induced IL-8 was significantly increased when the cells were pre-incubated with H_2O_2 (100 μM) for 10 min before TNF-α stimulation (mean ± SEM: 1977 ± 101 ng·mL^{-1} with TNF-α vs. 3000 ± 439 ng·mL^{-1} with H_2O_2 and TNF-α, $n = 3$, $P < 0.05$), and the budesonide IC_{50} was increased and the maximum inhibition was reduced by H_2O_2 pretreatment (IC_{50}: 1.5 ± 0.14 nM and Imax 60.0 ± 1.8% with H_2O_2; Table 3, Figure 1G).

When the cells were pretreated with formoterol (1 nM) for 20 min before budesonide treatment, the budesonide concentration–dependent curve shifted to the left. This suggests increased budesonide sensitivity (IC_{50}: 0.23 ± 0.031 nM and Imax 76.5 ± 7.3% with H_2O_2 + formoterol) (Figure 1G, Table 3). In contrast, salmeterol (100 nM) did not significantly restore budesonide sensitivity reduced in the presence of H_2O_2 (Table 3).

The sensitivity to FP and its Imax were also reduced by H_2O_2 pretreatment (IC_{50}: 0.074 ± 0.016 nM and Imax 84.7 ± 0.68% without H_2O_2, IC_{50}: 0.68 ± 0.11 nM and Imax 68.5 ± 3.4% with H_2O_2). Formoterol (1 nM) restored FP sensitivity but salmeterol (100 nM) did not (Table 3).

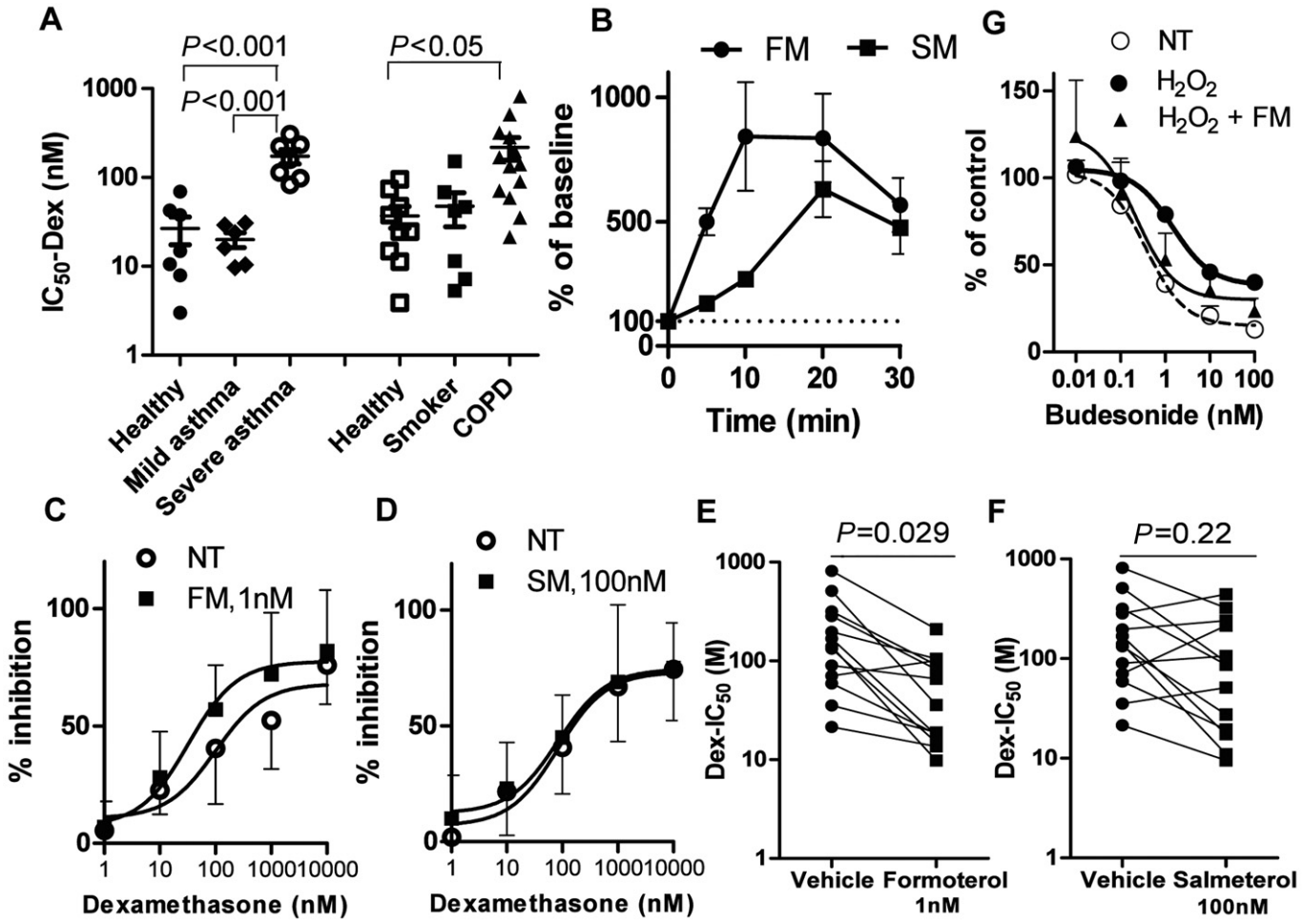

Figure 1

Effect of formoterol and salmeterol on corticosteroid insensitivity in PBMCs from COPD patients and in U937 cells exposed to oxidative stress. PBMCs were collected from healthy subjects, smokers without COPD, moderate to severe COPD, mild asthma and severe asthma patients. (A) IC_{50} values of dexamethasone (Dex) on TNF-α-induced IL-8 release were calculated and used as the marker of corticosteroid sensitivity (individual values with mean ± SEM). (B) PBMCs from healthy subjects were treated with formoterol (FM: 1 nM) or salmeterol (SM: 100 nM), and cAMP level was determined at each time point. (C–F) Cells from COPD patients were pretreated with FM (1 nM), SM (100 nM) or vehicle (DMSO, 0.5%) for 20 min before Dex treatment, and Dex-IC_{50} values were calculated. Dex concentration-dependence with/without FM (C) or SM (D). Dex-IC_{50} values; individual plot with/without FM (E) or SM (F). (G) TNF-α-induced IL-8 release from U937 cells pretreated first with formoterol (1 nM) for 20 min and then budesonide for 30 min. Cells were exposed to H_2O_2 (100 μM) 10 min before LABA treatment or left untreated (NT).

Table 3

Efficacy of budesonide and fluticasone propionate on TNF-α-induced IL-8 in the presence or absence (NT) of oxidative stress (H_2O_2 100 μM) in U937 cells

	Budesonide IC_{50} (nM)	Imax (%)	Fluticasone propionate IC_{50} (nM)	Imax (%)
NT	0.38 ± 0.081	87 ± 2.8	0.074 ± 0.016	85 ± 0.7
H_2O_2	1.5 ± 0.14$^{\#\#}$	60 ± 1.8	0.68 ± 0.11$^{\#}$	68 ± 3.4
H_2O_2 + formoterol (1 nM)	0.23 ± 0.031**	77 ± 7.3	0.15 ± 0.035*	73 ± 8.2
H_2O_2 + salmeterol (100 nM)	1.0 ± 0.21	66 ± 1.6	0.48 ± 0.11	69 ± 2.5

$^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus NT (Welch's test).
$^{**}P < 0.01$, $^{*}P < 0.05$ versus H_2O_2 (Dunnett's multiple comparison).
Imax, maximal inhibition. Data are expressed as the mean ± SEM.

Formoterol inhibited oxidative stress-induced PKB phosphorylation

Kinase phosphorylation arrays showed that H_2O_2 strongly induced phosphorylation of p38MAPKα, mitogen- and stress-activated protein kinase (MSK2), heat shock protein 27 (HSP27) as well as PKB kinases in U937 cells. Neither salmeterol (100 nM) nor formoterol (10 nM) inhibited phosphorylation of p38MAPKα, MSK2 and HSP27 (Figure 2A,B). Western blot analysis confirmed H_2O_2-induced PKB phosphorylation (Figure 2C), which was inhibited by LY294002 (3.3 μM), a PI3K inhibitor, but not by LY303511, a LY294002 inactive analogue (Supporting Information Figure S1B). As shown in Figure 2C,D, Western blot analysis demonstrated that formoterol (0.1–10 nM) concentration-dependently inhibited H_2O_2-induced PKB phosphorylation with maximum inhibition of 54%. In contrast, salmeterol did not significantly inhibit it even at 1000 nM (33% inhibition). For further optimization, pPKB levels were determined by pPKB cell-based ELISA. Formoterol significantly inhibited H_2O_2-induced phosphorylation of PKB when pretreated for 5 min before H_2O_2 stimulation and the inhibitory effect of formoterol was still seen when pretreated for 20 min before the incubation with H_2O_2. However, salmeterol (100 nM) did not show any effects on PKB phosphorylation when treated for up to 30 min before H_2O_2 stimulation (Figure 2E). As shown in Figure 2F, this H_2O_2-induced PKB phosphorylation was PI3Kδ-dependent as only IC87114 (IC), but not AS604850 (AS), inhibited it.

The inhibitory effect of formoterol on H_2O_2-induced PKB phosphorylation was reduced by H89 dihydrochloride, a PKA inhibitor in Western blot analysis (Figure 3A,B). This effect of PKA inhibition was also confirmed with another PKA inhibitor, KT5720 (Figure 3C). Exchange protein activated by cAMP (EPAC) specific agonists, 8-pCPT-2′-O-Me-cAMP (8CPT) or 8Br, did not inhibit PKB phosphorylation (Figure 3C). In addition, 8CPT did not reverse the inhibition of pPKB formation by formoterol (Figure 3C). Thus, the effect of formoterol is PKA- dependent, and unlikely to be mediated via EPAC signalling.

Oxidative stress affected LABA-induced cAMP production

Both formoterol and salmeterol increased cAMP production in intact U937 cells concentration-dependently, and the concentrations to show 50% induction over baseline (50% enhancement concentration: EnC_{50}) were 0.37 ± 0.22 and 9.9 ± 1.0 nM, respectively. Pretreatment of the cells with H_2O_2 for 20 min significantly reduced the salmeterol effect (EnC_{50}, 223 ± 96 nM with H_2O_2 vs. 9.9 ± 1.0 nM without H_2O_2, $P < 0.05$), but H_2O_2 treatment did not affect the formoterol-induced cAMP production (EnC_{50}, 1.1 ± 0.65 nM with H_2O_2 vs. 0.37 ± 0.22 nM without H_2O_2, $P = 0.39$) (Figure 4A,B). The H_2O_2-induced reduction in the ability of salmeterol to induce cAMP formation was reversed by a pan PI3K inhibitor, LY-294002, which was pretreated for 20 min before H_2O_2 exposure (EnC_{50}, 11.1 ± 4.6 nM with LY294002 vs. 223 ± 9 nM H_2O_2 control, $P < 0.05$). LY294002 (3.3 μM) was confirmed to inhibit PKB phosphorylation in U937 cells by more than 80% (Supporting Information Figure S1B).

As LY294002 is a non-selective PI3K inhibitor, isoform selective inhibitors, such as IC87114, a PI3Kδ inhibitor, and AS604850, a PI3Kγ inhibitor, were used. As shown in Figure 4C, although formoterol's effect was not affected by H_2O_2, salmeterol-induced cAMP production was reduced in H_2O_2-treated cells. This reduction was reversed by pretreatment of the cells with IC87114, but not AS604850. Thus, PI3Kδ is a key kinase in the inhibition of salmeterol effects induced by H_2O_2 treatment.

Both formoterol (1 nM) and salmeterol (100 nM) significantly increased cAMP in PBMCs obtained from healthy subjects (formoterol: 391 ± 73% increase; salmeterol: 348 ± 58% increase, $n = 6$) (Figure 4D). In contrast, in PBMCs from COPD patients, although formoterol significantly increased cAMP (240%, $n = 6$, $P < 0.05$), the increase in cAMP formation induced by salmeterol (170%) was not statistically significant (Figure 4F), and a similar trend was observed in the control group of smokers (Figure 4E).

Oxidative stress modified β_2-adrenoceptor internalization

Neither formoterol (1 nM, 10 nM) nor salmeterol (100 nM) altered β_2 adrenoceptor expression in the membrane fraction of U937 cells after 5–30 min incubation, although salbutamol (10 μM) reduced it after 5 and 15 min incubation (data not shown). Thus, LABAs (salmeterol and formoterol) did not induce continuous β_2 adrenoceptor internalization under normal conditions. H_2O_2 slightly reduced β_2 adrenoceptor expression in membrane after 240 min exposure. In cells that had been pre-exposed to H_2O_2 for 30, 60 or 240 min, a 30 min incubation with formoterol (10 nM) did not alter the levels of β_2 adrenoceptors in the cell membrane (Figure 4G,H). In contrast, a 30 min incubation with salmeterol (100 nM) in cells that had been pre-exposed to H_2O_2 for 240 min induced a reduction in membrane β_2 adrenoceptors (Figure 4G,H), indicating that salmeterol induces β_2 adrenoceptor internalization in the cells exposed to H_2O_2.

Discussion and conclusions

Both severe asthma and COPD are recognized to be diseases with impaired corticosteroid sensitivity (Holgate and Polosa, 2006; Stockley *et al.*, 2009). We showed that the ability of a corticosteroid (Dex) to inhibit TNF-α-induced IL-8 release *in vitro* was reduced in PBMCs obtained from patients with severe asthma or COPD by five- to sevenfold (increased IC_{50}) compared with cells from healthy subjects (including smokers and non-smokers). Both salmeterol and formoterol enhanced the effect of Dex on TNF-α-induced IL-8 release in PBMCs obtained from severe asthma patients. However, only formoterol, but not salmeterol, significantly increased the sensitivity of PBMCs from COPD patients to Dex. Before the main study, we evaluated the time course and concentration-dependence of the cAMP formation induced by formoterol and salmeterol; from these results we chose a 20 min pretreatment as the time point at which formoterol (1 nM) and salmeterol (100 nM) showed similar cAMP levels in U937 cells. This similar induction of cAMP by formoterol (1 nM) and salmeterol (100 nM) was also confirmed in PBMCs from

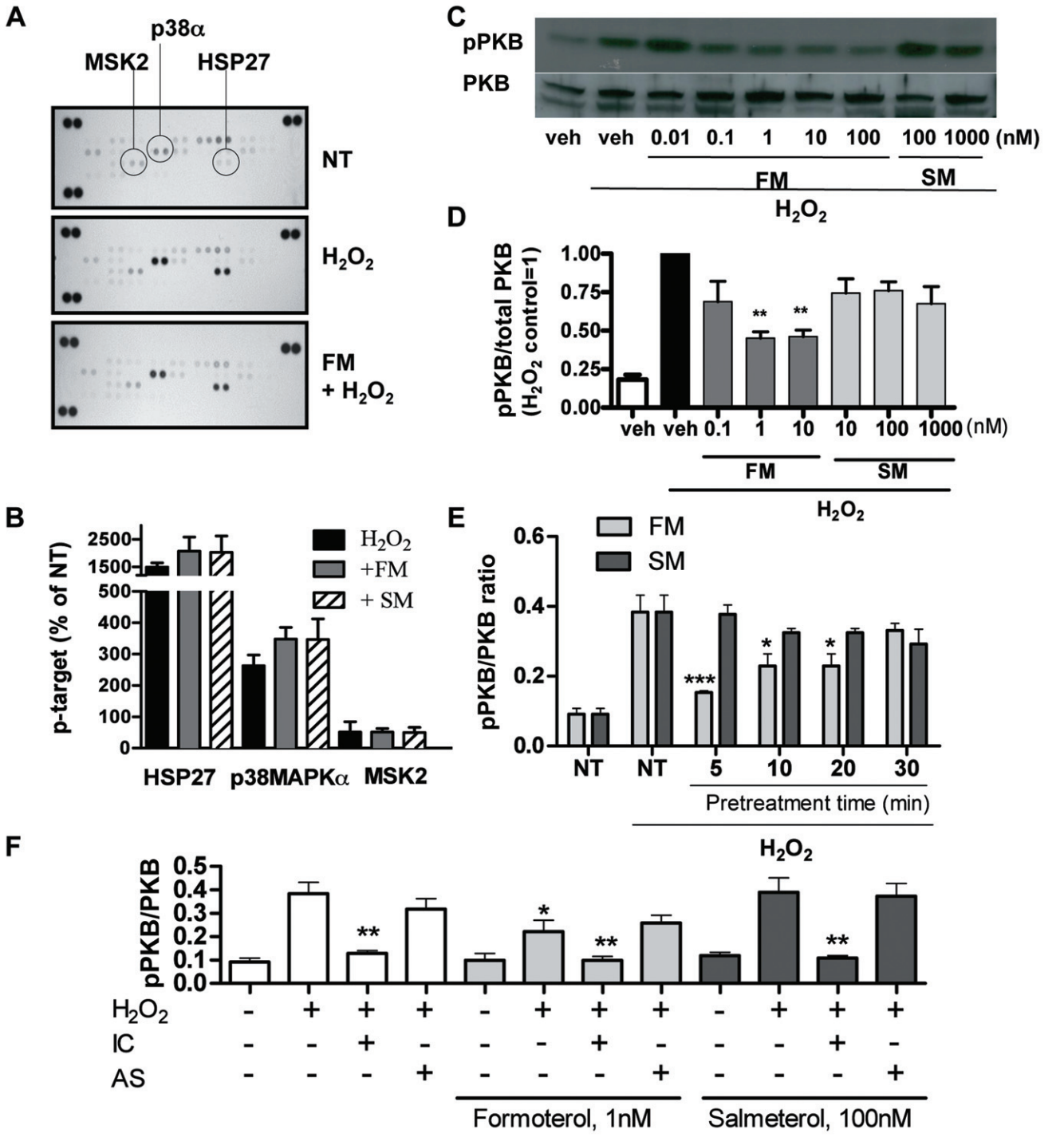

Figure 2

Formoterol effect on PI3K signalling and β_2-adrenoceptor down-regulation by oxidative stress in U937 cells. (A) U937 cells were stimulated with H_2O_2 (100 μM) for 15 min or not treated (NT), and the cell extracts were analysed in a MAP kinase phosphorylation array. The cells were treated with formoterol (FM: 10 nM) or salmeterol (SM: 100 nM) for 20 min before H_2O_2 exposure. The representative images of FM effects are shown in (A). Band densities of p38MAPKα, MSK2 and HSP27 (activated by H_2O_2) were measured by densitometry (B). (C, D) U937 cells were stimulated with H_2O_2 (100 μM) for 7.5 min or vehicle (veh; 0.5% DMSO), and PKB phosphorylation was visualized by Western blotting. Cells were treated with FM or SM for 20 min or vehicle (veh; 0.5% DMSO) before H_2O_2 exposure. Representative image (C) and densitometric analysis (D). $^{**}P < 0.01$ versus vehicle + H_2O_2, Dunn's test. (E) Effects of different pretreatment time of FM and SM before H_2O_2 exposure. The ratio of pPKB and total PKB was determined by PKB cell-based ELISA. (F) U937 cells were pretreated with IC87114 (IC: 1 μM) or AS604850 (AS: 1 μM) for 20 min before treatment with FM or SM for 20 min. Cells were then stimulated with H_2O_2 for 7.5 min, and phospho-PKB and total PKB levels were determined by cell-based ELISA.

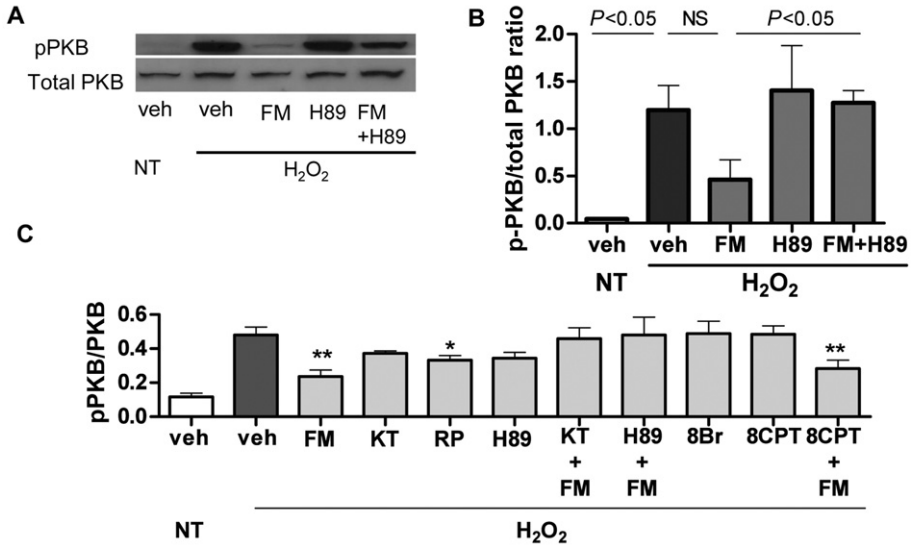

Figure 3

Effects of PKA and EPAC signalling on formoterol-dependent pPKB inhibition in H_2O_2-treated U937 cells. (A,B) U937 cells were treated with vehicle (veh, 0.5% DMSO), 10 nM formoterol (FM), 1 μM H89, a PKA inhibitor or combination of H89 and FM. For combination, H89 was pretreated for 15 min before FM. H_2O_2 at 200 μM was then applied for 7.5 min and cells were harvested for whole cell extraction and following Western blotting. NT: non-treatment. Representative image (A) and densitometric analysis (B, n = 3). In addition, U937 cells were pretreated with formoterol (FM: 10 nM), KT5720 (KT, 200 nM) with/without FM, Rp-cAMP (RP, 10 μM), H89 (10 μM) with/without FM, 8-Br-cAMP (8Br, 10 μM) or 8-pCPT-2′-O-Me-cAMP (8CPT, 10 μM) with/without FM for 20 min. Cells were then stimulated with H_2O_2 for 7.5 min. phospho-PKB and total PKB levels were determined by cell-based ELISA. Mean ± SEM. $^{**}P < 0.01$ versus H_2O_2 control.

healthy subjects. Therefore, the conditions we used were not unfair to salmeterol. In addition, as the patients in the study had not been treated with either salmeterol of formoterol previously (ie were salmeterol- and formoterol-naïve), tachphylaxis to these agonists can be ruled out in this study.

The corticosteroid insensitivity of COPD and severe asthma might be induced by several different factors. IL-2 and IL-4 have both been shown to mimic the corticosteroid insensitivity seen in severe asthma reported (Irusen *et al.*, 2002), whereas oxidative stress is thought to be the predominant cause of corticosteroid insensitivity in COPD because oxidative stress has been found to impair corticosteroid actions (Ito *et al.*, 2004). In fact, we showed here that H_2O_2 decreased the inhibitory effects of budesonide and FP on TNF-α-induced IL-8 release in U937 cells (Table 3). In this system, formoterol increased the sensitivity of budesonide and FP, while salmeterol had no effect despite the fact that we used 10–100-fold higher concentrations of salmeterol than formoterol to adjust for its lower potency for cAMP production in intact cells, and that we used optimal incubation time for salmeterol (Figure 1B). In addition, H_2O_2 caused a rightward shift of the concentration–response curve for the effect of salmeterol on cAMP production (Figure 4B), but did not affect the concentration–response curve of formoterol on cAMP (Figure 4A). Thus, in contrast to formoterol, the effects of salmeterol appear to be sensitive to oxidative stress.

As shown in Figure 2A, H_2O_2 caused phosphorylation of several kinases, including MAPKs and downstream kinases. Neither formoterol nor salmeterol inhibited the phosphorylation of p38MAPKα and its downstream molecules, HSP27 and MSK2. PKB phosphorylation is an indicator of PI3K activation by H_2O_2, which was confirmed by Western blot analysis and pPKB cell-based ELISA (Figure 2C–F). Activation of the PI3K-PKB pathway by oxidative stress, such as cigarette smoke, has previously been reported in macrophage-like U937 cells (Marwick *et al.*, 2009). PI3Ks are subdivided into four classes (IA, IB, II and III) that possess lipid kinase activity, and the kinases largely responsible for PIP_3 production are class I-PI3Ks (Ito *et al.*, 2007). Class I-PI3K contains four isoforms of the catalytic subunit, known as p110α, p110β, p110γ and p110δ. Recently, oxidative stress-induced PI3Kδ activation has been reported to be involved in corticosteroid insensitivity (Marwick *et al.*, 2009). We also found that the activation and expression of the PI3Kδ isoform is increased in COPD peripheral lung tissue (To *et al.*, 2010). In this study, we confirmed that H_2O_2-induced pPKB was PI3Kδ-, but not PI3Kγ- dependent in U937 cells (Figure 3F). Although PI3K is reported to cause corticosteroid insensitivity by inactivation of HDAC2, a cofactor of GR (To *et al.*, 2010), PI3K is also reported to cause desensitization of β_2 adrenoceptors (Naga Prasad *et al.*, 2002). Thus, oxidative stress affects the actions of both corticosteroids and β_2 adrenoceptor agonists through PI3K signalling activation, possibly via PI3Kδ (Figure 5).

As shown in Figure 2C,D, using Western blotting analysis it was demonstrated that formoterol (0.1–10 nM) concentration-dependently and significantly decreased H_2O_2-induced PKB phosphorylation with a maximum inhibition of 54%. This effect of formoterol was also confirmed by a pPKB cell-based ELISA (Figure 2E,F). In contrast, the effects of salmeterol were not statistically significant with Western blot analysis revealing a maximum inhibition of 32% even at 1000 nM. Thus, formoterol was more effective than

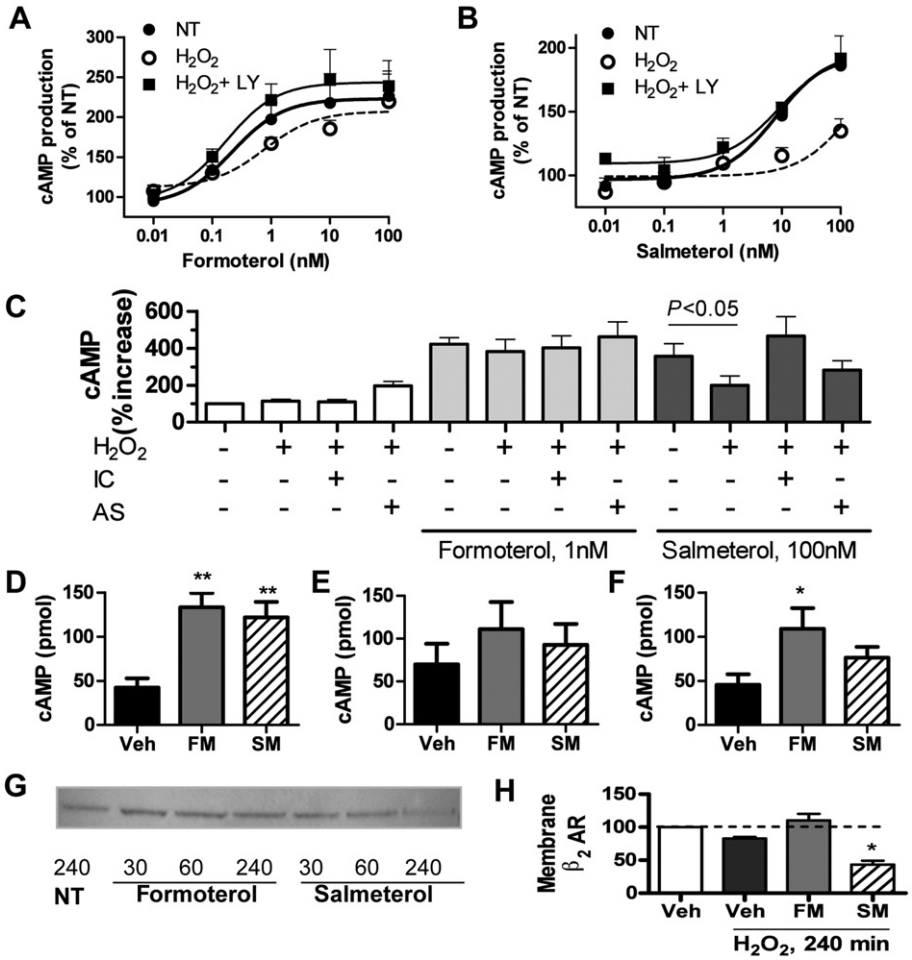

Figure 4

The effects of formoterol and salmeterol on cAMP production in U937 cells exposed to oxidative stress and in PBMCs from COPD patients. U937 cells were pretreated with H_2O_2 (100 μM) or not (NT) for 20 min, and then treated with formoterol (FM) or salmeterol (SM) for 20 min Cells were also treated with LY-294002 (LY, 3.3 μM) 20 min before H_2O_2 exposure (A and B). (C) U937 cells were pretreated with IC87114 (IC: 1 μM) or AS604850 (AS: 1 μM) for 20 min before the application of H_2O_2 for 20 min. The cells were then treated with FM or SM for 20 min, and collected for cAMP assay. (D, E, F) The concentration of cAMP induced by 20 min treatment with FM (1 nM) or SM (100 nM) was determined in PBMCs (4 million cells each) from non-smoker controls (D), smoker controls (E) and COPD patients (F). *$P < 0.05$, **$P < 0.01$ versus vehicle (veh; 0.5% DMSO) control by Dunnett's test. Results are expressed as mean ± SEM. (G, H) U937 cells were exposed to H_2O_2 for indicated time interval (30, 60 or 240 min), and then treated with FM (10 nM), SM (100 nM) or vehicle (veh, 0.5% DMSO). At 30 min after LABA treatment, membrane protein extracts were prepared and β_2 adrenoceptor (β_2AR) expression was visualized. Representative image (G) and densitometric analysis (H). *$P < 0.05$ in Dunnett's test versus vehicle. Results are expressed as mean ± SEM.

salmeterol at inhibiting the phosphorylation of PKB in these cells. In addition, we showed that the reduction in salmeterol-stimulated cAMP production induced by oxidative stress is reversed by a non-selective PI3K inhibitor (Figure 4B) and a PI3Kδ inhibitor (Figure 4C). Taken together, these results indicate that PI3Kδ inhibition seems to be at least in part involved in the preserved efficacy of formoterol under conditions of oxidative stress. As formoterol did not inhibit PI3K enzyme activity itself in the *in vitro* kinase assay (data not shown), the effect of formoterol seems to be through functional PI3K inhibition. Interestingly, the functional inhibition of PI3K by formoterol is PKA-dependent but not EPAC-dependent (Figure 3). Although H89 is known to be a PKA inhibitor, it has also been demonstrated to be a β_2 adrenoceptor antagonist (Penn *et al.*, 1999). However, as we obtained similar results using KT5720, another PKA inhibitor, we believe that formoterol-induced inhibition of PKB phosphorylation is PKA- dependent. The mechanisms by which formoterol inhibits PI3K signalling functionally and are responsible for the differences between formoterol and salmeterol are not clear. Formoterol is cationic and nearly a full agonist whereas salmeterol is more lipophilic and only a partial agonist. This difference might be important for inhibitory effects on PI3K signalling. We recently reported that formoterol also inhibits p38MAPKγ by dephosphorylation, possibly via activation of phosphatase (Mercado *et al.*, 2011). PP2A, one of the phosphatases, is known to be activated by cAMP inducers (Feschenko *et al.*, 2002), and also PP2A

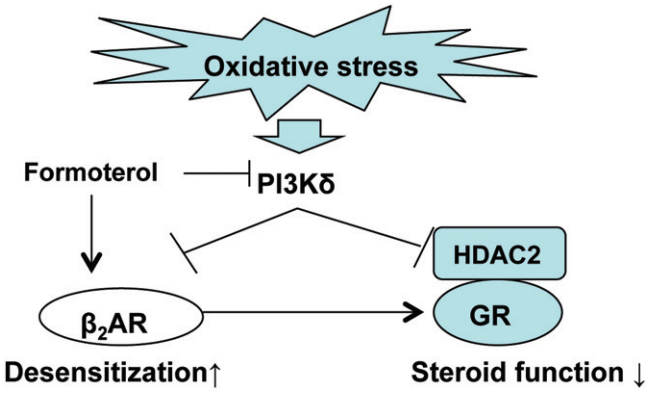

Figure 5

Schematic illustration on how formoterol works. Oxidative stress decreases corticosteroid sensitivity via inactivation of histone deacetylase 2 (HDAC2), a co-factor of the glucocorticoid receptor (GR). Although salmeterol enhances corticosteroid sensitivity in severe asthma, the efficacy of salmeterol is limited under oxidative stress as PI3K affects its β_2 adrenoceptor-dependent cAMP production. However, as formoterol has partial PI3K inhibitory activity, it is able to reverse both events during oxidative stress.

dephosphorylates PKB (Kim *et al.*, 2008). Thus, it is possible that formoterol inhibits PI3K-dependent PKB phosphorylation by increasing phosphatase activity and the efficacy of salmeterol at enhancing PP2A might be weaker than that of formoterol. Another possibility is that PI3K-PKB may phosphorylate β_2 adrenoceptors, and this post-translational modification affects the binding of salmeterol. Thus, additional experiments are required to clarify the molecular mechanism whereby formoterol inhibits PI3K signalling.

The clinical benefits of combination inhalers with LABAs and corticosteroids are less well documented in COPD than in asthma. A direct comparison of the add-on effects of formoterol versus salmeterol to corticosteroid has not been conducted. However, Cote and colleagues showed that formoterol has a faster onset of action than salmeterol on lung function in COPD patients (Cote *et al.*, 2009). Partridge and colleagues also showed that budesonide/formoterol were better than fluticasone/salmeterol at improving the total morning activity scores of patients with COPD (Partridge *et al.*, 2009). In *in vitro* studies, formoterol but not salmeterol was found to elevate cAMP levels in monocyte-derived macrophages (Donnelly *et al.*, 2010). In addition, Rabe and colleagues showed that formoterol, but not salmeterol, inhibited LTB_4-induced H_2O_2 generation in guinea pig peritoneal eosinophils (Rabe *et al.*, 1993). As shown in Supplementary Information Figure 1A, formoterol but not salmeterol induced significant GR nuclear translocation and this may provide a basis for the differences observed between the two agonists. Thus, the qualitative differences of efficacies between formoterol and salmeterol are well documented. Recently, Adner and colleagues showed that budesonide prevented the inhibitory effects of cytokines on formoterol- but not salmeterol-induced tracheal relaxation and cAMP signalling (Adner *et al.*, 2010). Thus a meta-analysis and/or further clinical studies are needed to elucidate the differences between the efficacies of salmeterol and formoterol, alone and in combination with inhaled corticosteroids, in the treatment of COPD.

In conclusion, the increase in cAMP production and enhancement of the anti-inflammatory actions of corticosteroids induced by formoterol were resistant to oxidative stress, whereas those induced by salmeterol were impaired by oxidative stress. This difference appears to be explained, at least in part, by the partial inhibition of PI3Kδ signalling by formoterol but not by salmeterol (Figure 5). Thus, formoterol may have greater clinical benefits compared with salmeterol (alone and in combination with inhaled corticosteroids) for the treatment of respiratory diseases where a high level of oxidative stress is seen, such as in COPD.

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Conflict of interest

Peter J. Barnes and Kazuhiro Ito received an academic project grant from Astra Zeneca and non-restricted funding by GlaxoSmithKline (GSK). Kazuhiro Ito is currently an employee of RespiVert Ltd.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effects of formoterol and salmeterol on glucocorticoid receptor (GR) nuclear translocation in PBMCs from COPD patients (A) and effects of LY294002 on PKB phosphorylation in PBMCs from COPD patients (B).
Table S1 The effects of formoterol and salmeterol on sensitivity to dexamethasone on TNF-α-induced IL-8 in PBMCs

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