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RESEARCH PAPER

Rosiglitazone reverses salbutamol-induced β_2-adrenoceptor tolerance in airway smooth muscle

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BACKGROUND AND PURPOSE

β_2-Adrenoceptor agonists are important therapeutic agents in the treatment of asthma and chronic obstructive pulmonary disease. The regular use of these drugs has been associated with proasthmatic-like changes that limit their efficacy and increase the risk of severe adverse reactions. We investigated whether the peroxisome-proliferator-activated receptor (PPAR)γ agonist rosiglitazone modulated salbutamol-induced β_2-adrenoceptor desensitization *in vivo* and *in vitro*.

EXPERIMENTAL APPROACH

An *in vivo* model of homologous β_2-adrenoceptor desensitization, established in guinea-pigs by administering salbutamol continuously, was used to study the ability of rosiglitazone to prevent β_2-adrenoceptor tolerance. *In vitro* experiments on human bronchial smooth muscle cells were performed to increase the clinical relevance of the study.

KEY RESULTS

In tracheal smooth muscle tissues from desensitized animals, we observed a decrease in the protective effect of salbutamol on carbachol-induced contraction, a hyperresponsiveness to cholinergic stimuli, a modest underexpression of β_2-adrenoceptor gene and a marked decrease in β-adrenoceptor number, relative to control values. Treatment with rosiglitazone preserved salbutamol relaxant activity, mitigated carbachol hyperresponsiveness and partially restored β_2-adrenoceptor binding sites in tracheal tissues from homologously desensitized animals. The highly selective PPARγ agonist, GW1929, reproduced the effect of rosiglitazone, *in vivo*. *In vitro* β_2-adrenoceptor desensitization decreased salbutamol-mediated cAMP production, without affecting forskolin responses and β_2-adrenoceptor expression. Rosiglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 restored salbutamol sensitivity in homologously desensitized cells.

CONCLUSIONS AND IMPLICATIONS

These data suggest a potential pharmacodynamic interaction between PPARγ agonists and salbutamol on airway smooth muscle responsiveness, supporting the therapeutic potential of this combination in chronic airway disease.

Abbreviations

BSMC, human bronchial smooth muscle cells; EMSA, electrophoretic mobility shift assay; PPAR, peroxisome-proliferator-activated receptor

Introduction

β_2-Adrenoceptor agonists represent one of the most important drug classes in the treatment of asthma. Unfortunately, both inhaled and oral treatments with these drugs are known to induce decreased efficacy, due to receptor desensitization which, in turn, is thought to play a role in the onset of hyperresponsiveness (Broadley, 2006). Homologous and heterologous desensitization of β_2-adrenoceptor induced by chronic exposure to β_2-adrenoceptor agonists and pro-inflammatory cytokines, respectively, has been characterized in human airway smooth muscle cells (Shore and Moore, 2003). Molecular mechanisms underlying desensitization of β_2-adrenoceptors include uncoupling and internalization, which occur almost immediately, and down-regulation of expression of new receptors, which represents a long-term event (Shore and Moore, 2003).

The combination therapy with corticosteroids and β_2-adrenoceptor agonists is frequently used to treat patients with persistent asthma. While the precise mechanisms underlying the success of this combination therapy have not yet been fully elucidated, some lines of evidence suggest that corticosteroids and β_2-adrenoceptor agonists may positively interact at the molecular level to prevent signalling pathways involved in the inflammatory cascade (Barnes, 2009). However, a reduced responsiveness to corticosteroids observed in the clinical setting (Adcock and Lane, 2003) and the limited use of corticosteroid-based treatments in infants and preschool children (Allen, 2002) are characteristic features of human asthma, which may limit the therapeutic potential of these drugs.

Peroxisome-proliferator-activated receptors (PPARs) are a family of ligand-dependent transcription factors that play a pivotal role in controlling the expression of genes involved in metabolic and inflammatory processes by binding to sequence-specific PPAR response elements in the promoter region of target genes (Glass and Ogawa, 2006). Three PPAR isoforms, designated PPARα, PPARβ/δ and PPARγ, have been cloned and are differentially expressed in several tissues including liver, kidney, heart and muscle (Huang *et al.*, 2005). In terms of the distribution of PPARs in lung, the cellular expression profile of PPARγ has not been well characterized, but some studies have uncovered abundant expression of PPARγ in airway epithelium, bronchial submucosa, human alveolar macrophages, T lymphocytes, and bronchial epithelial and airway smooth muscle cells (Denning and Stoll, 2006). A growing body of evidence underlines the anti-inflammatory/immunomodulatory and anti-proliferative properties of PPAR ligands, particularly PPARα and PPARγ, in asthma and chronic obstructive pulmonary disease (Lee *et al.*, 2006; Roth and Black, 2006; Spears *et al.*, 2006), thus suggesting that PPAR targeting may represent a novel therapeutic strategy to selectively disrupt the signalling network critically involved in the pathophysiology of chronic airway inflammation.

Considering these premises, analysis of the potential synergism between PPAR agonists and β_2-adrenoceptor agonists on airway smooth muscle responsiveness may represent a logical approach to provide useful information to select novel PPAR-based combination regimens, worthy of being evaluated in prospective clinical trials. In this study, we have demonstrated that the PPARγ agonist, rosiglitazone, was able to reverse salbutamol-induced β_2-adrenoceptor tolerance in both tracheal muscular tissues derived from homologously desensitized guinea-pigs and human bronchial smooth muscle cells (BSMC).

Methods

Animals

All animal care and experimental procedures were carried out in accordance with the legislation of Italian authorities (D.L. 27/01/1992, n° 116), which complies with European Community guidelines (CEE Directive 86/609) for the care and handling of experimental animals, and with the approval of the Animal Care Committee of the University of Pisa. Experiments were carried out on male Dunkin-Hartley guinea-pigs (300–400 g), housed two per cage at 22°C under a 12:12 h light : dark cycle, and given free access to a normal diet and tap water. This species was selected because it is one of the most commonly used in pharmacological studies of the respiratory system (Canning and Chou, 2008).

In vivo *β_2-adrenoceptor desensitization and drug administration*

A chronic *in vivo* model of tracheal β_2-adrenoceptor desensitization was established in guinea-pigs based on the work of Finney and co-workers (Finney *et al.*, 2000). Briefly, a 1.5 cm incision on the back of the animals slightly posterior to the scapulae was made under light general anaesthesia with pentobarbital at 30–35 $mg \cdot kg^{-1}$ i.p. (absence of corneal reflex and motor response to nociceptive stimuli were confirmed before the surgical procedure). A small pocket was then formed by spreading apart the subcutaneous connective tissue with a haemostat; a minipump (Alzet, Palo Alto, CA, USA) was inserted and the skin closed with sutures. The contents of the

pump were delivered into the local subcutaneous space and absorption of the compound by local capillaries resulted in systemic drug administration. The rate of infusion of fluid from the minipump was 1 $\mu L \cdot h^{-1}$ delivering 40 $\mu g \cdot kg^{-1} \cdot h^{-1}$ of salbutamol, or its vehicle (sterile phosphate buffered saline) for 7 days. After model validation, rosiglitazone at 10 $mg \cdot kg^{-1} \cdot day^{-1}$, p.o. for six consecutive days was administered to control or desensitized animals, starting from 24 h after minipump implantation. A similar treatment schedule has been proven to modulate protein expression in obese-diabetic mice and healthy subjects (Combs *et al.*, 2002). GW1929 was used as alternative agonist to assess the specificity of rosiglitazone effect through PPARγ. For this, guinea-pigs were gavaged with vehicle (0.5% EtOH in saline solution) or GW1929 (1 $mg \cdot kg^{-1}$) once daily for 6 days. Dexamethasone was given at 2 $mg \cdot kg^{-1} \cdot day^{-1}$, i.p. for 6 days because preliminary experiments carried out at 0.2 $mg \cdot kg^{-1} \cdot day^{-1}$ s.c., a dose level previously reported to increase β-adrenoceptor density in rat lung (Mak *et al.*, 1995), failed to significantly modify the altered airway smooth muscle responsiveness in desensitized guinea-pigs (see *Results*).

Ex vivo *assessment of tracheal β_2-adrenoceptor desensitization*

Experiments were performed on tracheal preparations isolated from guinea-pigs (Breschi *et al.*, 2007) under control conditions or following homologous desensitization to salbutamol (β_2-adrenoceptor agonist) in the presence or in the absence of drug treatment. Carbachol at 0.3 $\mu mol \cdot L^{-1}$ was selected to induce tonus in preparations because such a concentration had been found to elicit submaximal responses in preliminary experiments. When the carbachol-induced contraction reached a steady level (approximately after 5 min), salbutamol was applied in a cumulative manner in concentrations ranging from 0.001 to 100 $\mu mol \cdot L^{-1}$ in control and in desensitized tissues. A period of 3–5 min was allowed between subsequent increments of concentration in order to enable a full development of the effect of the agonist.

As selective airway smooth muscle hyperresponsiveness to cholinergic stimulation has been reported after chronic exposure to salbutamol (Loss *et al.*, 2001), tracheal contractility to 0.3 $\mu mol \cdot L^{-1}$ carbachol was measured in the different treatment groups.

Assessment of β_2-adrenoceptor mRNA levels in tracheal tissues by real-time PCR

The dorsal muscle portion of tracheae, immediately after their excision from animals, were dissected and stored at –20°C until their use. Total RNA was extracted by the RNeasy Fibrous Tissue kit (Qiagen, Valencia, CA, USA) and residual DNA was removed by on-column DNase digestion with the RNase-Free DNase Set (Qiagen, Valencia, CA, USA). The concentration and purity of total RNA were measured by 260 nm UV absorption and by 260/280 ratios, respectively, using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA); all RNAs displayed a 260/280 optical density ratio >1.9. The RNA integrity was verified by electrophoresis through 1.2% agarose-formaldehyde gel.

One microgram of total RNA from each sample was reverse-transcribed with oligo-dT and random primers by the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). For the experiments in pool, RNA samples from each group of treatment were mixed together and 1 µg of RNA from each pool was used for reverse transcription. PCR primers were designed by Beacon Designer 4.0 software (Premier Biosoft. International, Palo Alto, CA, USA) and synthesized by Sigma Genosys (Cambridge, UK). Primer sequences were: 5′-ACAAGGACGC CATCAACTG-3′ (F) and 5′-AAAGACCATAACCAC CAAGGG-3′ (R) for β_2-adrenoceptor; 5′-GCTGCC CCAGAACATCATCC-3′ (F) and 5′-GCCTGCTTCAC CACCTTC-3′ (R) for GAPDH; 5′-TGCGTGACATCA AGGAGAAG-3′ (F) and 5′-AAGGAGGGCTGGA AGAGAG-3′ (R) for β-actin; 5′-CGGCTACCACAT CCAAGGAA-3′ (F) and 5′-GCTGGAATTACC GCGGCT-3′ (R) for 18S. Primers for GAPDH and β-actin transcripts were designed using the corresponding guinea-pig mRNA sequences (GAPDH mRNA sequence: AB060340; β-actin mRNA sequence: AF508792). As no guinea-pig β_2-adrenoceptor mRNA sequence was retrievable from GenBank, we aligned *Rattus norvegicus* (NM_012492), *Homo sapiens* (M15169) and *Mus musculus* (NM_007420) β_2-adrenoceptor mRNA sequences by ClustalW (http://www.clustal.org/), and designed the primers in the region with the highest homology. For 18S rRNA transcript, we used the primers indicated in Chitano's paper (Chitano *et al.*, 2004).

Real-time PCRs were performed using Platinum SYBR GreenER qPCR Supermix UDG kit (Invitrogen, Carlesbad, CA, USA) and the iCycler iQ instrument (Bio-Rad, Hercules, CA, USA). The thermal cycling program consisted of 2 min incubation at 50°C with uracil-DNA glycosylase (UDG), 8.5 min at 95°C (DNA polymerase activation), 40 cycles at 95°C for 15 s (denaturation step) and 62°C for 1 min (annealing-extension step). Afterwards, a gradual increase in temperature from 55°C to 95°C at rate of 0.5°C·10 s^{-1} was utilized to build a melting curve.

SYBR Green fluorescence was detected during the annealing-extension step. For each primer pair, we tested the amplification efficiency by using five serial dilutions of cDNA carried out in duplicate. All primer pairs displayed efficiency between 90% and 100%. Each sample was run in triplicate and for each gene the standard deviation for the three experimental replicates was less than 0.4 arbitrary units.

Assessment of β-adrenoceptor density in tracheal tissues by radioligand binding assay

Frozen tissues (–80°C) derived from the dorsal muscle portion of tracheae from control and desensitized guinea-pigs, as well as those obtained from desensitized animals treated with rosiglitazone were thawed in ice-cold lysis buffer (20 mmol·L^{-1} $NaHCO_3$, pH 8.0), added with Triton 0.01% over approximately 1 h, and finely minced with scissors. The slurry was homogenized with an Ultraturrax (Janke & Kunkel, IKA Labortechnick, Germany) and sonicated (SONICS, Vibra Cell). The homogenate were centrifuged at 500× *g* for 10 min at 4°C. The pellets were discarded, the supernatants were filtered through four layers of cheese-cloth and then centrifuged at 40 000× *g* for 30 min at 4°C. The resulting pellets were washed once in lysis buffer by Potter-Elvehjem homogenizer and centrifuged at the same speed. The final pellets were gently resuspended in assay buffer A (50 mmol·L^{-1} Tris-HCl, ascorbic acid 0.01%, pH 7.4). Membrane protein concentrations were determined according to the method of Bradford (Biorad Protein Assay), using γ-globulin as standard.

The density of receptors in membrane fractions was determined by the radioligand binding assay using [^{3}H]-dihydroalprenolol ([^{3}H]-DHA, specific activity: 117.8 Ci·mmol). Membrane preparations (0.2 mg of proteins) were incubated with four different increasing concentrations of radioligand (0.5–11 nmol·L^{-1}) in a final volume of 0.5 mL. Incubation was performed at 4°C for 120 min in assay buffer A. Non-specific binding was determined in the presence of 10 µmol·L^{-1} propanolol. Specific binding was calculated by subtracting non-specific binding from total binding. Because [^{3}H]-DHA is an antagonist for β_1- and β_2-adrenoceptors, the specific binding was relative to both receptor subtypes. Therefore, saturation binding experiments were also executed in the presence of β_1-adrenoceptor antagonist 200 nmol·L^{-1} CGP20712A and the specific binding represented the bound to β_2-adrenoceptors. Incubation was stopped by addition of 5 mL of ice-cold buffer B (50 mmol·L^{-1} Tris-HCl, 0.1% bovine albumin serum, pH 7.4) and rapid vacuum filtration through Whatman GF/C glass fibre filters by means of a harvester (Brandel). The filters were washed twice with 5 mL of buffer B and placed in vials containing 4 mL of Packard Ultima Gold MV scintillation fluid. Residual radioactivity was determined using a liquid scintillation counter (TRI-CARB 2800 TR, PerkinElmer Life Science).

Cell culture conditions

Human bronchial smooth muscle cells were purchased from Lonza (Walkersville, MD, USA). Cells were maintained exactly as recommended by the manufacturer in an optimized medium containing 5% fetal bovine serum, 5 ng·mL^{-1} insulin, 2 ng·mL^{-1} basic fibroblast growth factor and 0.5 ng·mL^{-1} epidermal growth factor (SmGM-2 Bullet Kit, Lonza).

cAMP assay

Homologous β_2-adrenoceptor desensitization was performed by exposing BSMC to salbutamol at 1 µmol·L^{-1} for 24 h. Intracellular cAMP levels were measured by the cAMP-Glo™ Assay (Promega, Madison, WI, USA) in control and desensitized cells after stimulation with 10 µmol·L^{-1} salbutamol, in the presence or absence of rosiglitazone, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 or dexamethasone at 10 µmol·L^{-1} for 24 h.

RT-PCR analysis

RNA from cells was extracted by using the RNeasy Mini kit and reverse-transcribed by the QuantiTect Reverse Transcription kit. PCR was performed by the Hot StartTaq Master Mix kit. Primers used were: 5′-ACCAGGAAGCCATCAACTG-3′ (F) and 5′-GAAG ACCATGATCACCAGGGG-3′ (R) for β_2-adrenoc eptor; 5′-TTCAGAAATGCCTTGCAGTG-3′ (F) and 5′-CACCTCTTTGCTCTGCTCCT-3′ (R) for PPARγ; 5′-GTGAAGGTCGGAGTCAACG-3′ (F) and 5′-GGTG AAGACGGCCAGTGGACTC-3′ (R) for GAPDH and the expected amplification products were 119, 332 and 300 bp long respectively. Relative densitometry of bands was measured using NIH ImageJ gel analysis.

Electrophoretic mobility shift assay for PPARγ

Five micrograms of nuclear protein extract was preincubated for 20 min on ice with 2 µg poly-(dI-dC) (Sigma, USA) and then incubated for 30 min on ice with ^{32}P-labelled PPAR oligonucleotide (Perkin Elmer Life Sciences, Boston, USA) in binding buffer (50% glycerol; 10 mmol·L^{-1} Tris-HCl, pH 7.6; 500 mmol·L^{-1} KCl, 10 mmol·L^{-1} EDTA, 1 mmol·L^{-1} dithiothreitol) in a final volume of 30 µL. Consensus oligonucleotide for PPAR was 5′-CAAAACTA GGTCAAAGGTCA-3′. The interference assay was performed by using a selective PPARγ antibody (Santa Cruz Biotechnology, Santa Cruz, USA). DNA/

protein complex was size fractionated on a non-denaturing 5% polyacrylamide gel in TBE buffer (100 mmol·L^{-1} Tris-HCl, 100 mmol·L^{-1} boric acid, 2 mmol·L^{-1} EDTA) and detected by autoradiography. Densitometric analysis was performed by using the 'Quantity One' software (Bio-Rad Laboratories).

Data analysis and statistical procedures

Data were expressed as mean ± standard error of the mean (SEM). In functional studies, the pD_2 value, an index of agonist potency, was calculated as $-\log EC_{50}$ (molar concentration exerting half maximal effect). Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparison. $P < 0.05$ was taken as level of significance. Results were plotted by Prism software (Graphpad Software, San Diego, CA, USA).

In real-time PCR analyses, the relative expression levels of the three housekeeping genes (18S rRNA, β-actin and GAPDH) and their stability were evaluated by geNorm software (Vandesompele *et al.*, 2002). The relative expression levels of β_2-adrenoceptor gene were calculated with the Pfaffl method (Pfaffl, 2001) by the Gene Expression MacroTM 1.1 application (Bio-Rad, Hercules, CA, USA) and reported as fold increase or decrease. Three Student's *t*-tests were applied to detect significant group-wise differences in the relative expression levels. A constant level $P = 0.05$ was used for rejection of the null hypothesis. Descriptive statistics and *t*-tests for independent samples were performed by R V2.7.0 http://www.R-project.org (R Development Core Team, 2008).

In binding studies, the saturation data were subjected to curve-fitting procedures, using non-linear regression analysis of Graphpad Prism 3 and Scatchard plot to obtain the maximal number of [^{3}H]-DHA binding sites (B_{max}, fmol·mg protein) and the equilibrium dissociation constant (K_d, nmol·L^{-1}).

Materials

The following drugs were used: salbutamol hemisulphate, forskolin, carbachol hydrochloride, dexamethasone, propanolol, fenofibrate and CGP20712A from Sigma Aldrich (USA), sodium pentobarbital from Sessa (Milan, Italy), rosiglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 from Cayman Chemical Company (Ann Arbor, Michigan) and GW1929 from Tocris Bioscience (Ellisville, USA). [3H]-DHA was purchased from PerkinElmer Life Science. Sodium pentobarbital was dissolved in saline solution to obtain a final concentration of 50 mg·mL^{-1}. Salbutamol was dissolved in sterile phosphate buffered saline and this solution was employed to fill minipumps. For functional studies salbutamol and carbachol were dissolved in Krebs-Henseleit solution. Rosiglitazone was mixed with powdered pellet and administered orally. Dexamethasone was dissolved in phosphate buffered saline immediately before i.p. injection. Drug and receptor nomenclature follows Alexander *et al.* (2009).

Results

In vivo *model of homologous β_2-adrenoceptor desensitization*

Our data showed that acute response to salbutamol was reduced by approximately 25% in tracheal preparations isolated from homologously desensitized guinea-pigs, as compared with controls (Figure 1A), while no significant difference was observed between the two groups with forskolin (Figure 1B). Noteworthy, a hyperresponsiveness to carbachol was also demonstrated in salbutamol-desensitized animals with respect to controls (Figure 1C). Finally, the PPAR-DNA binding activity was measured by electrophoretic mobility shift assay and the presence of PPARγ protein was confirmed by selective inhibition (decrease in the shifted band) of PPAR-DNA binding by anti-PPARγ antibody, both in control and desensitized samples (Figure 1D). Prolonged *in vivo* exposure to the β_2-adrenoceptor agonist slightly induced nuclear translocation of total PPAR protein, as compared with controls (Figure 1D).

Tracheal smooth muscle responsiveness to salbutamol and carbachol after drug treatments

Chronic administration of the PPARγ agonist rosiglitazone at 10 mg·kg^{-1}·day^{-1} p.o. for 6 days was able to partially restore tracheal smooth muscle responsiveness to salbutamol in desensitized animals. Indeed, the concentration–response curve obtained in desensitized animals given rosiglitazone tended to that of control animals (Figure 2A) with similar pD_2 and E_{max} values (Table 1). Rosiglitazone administration did not change tissue responsiveness to salbutamol in control animals (Figure 2A; Table 1).

The highly selective and orally active PPARγ agonist, GW1929, administered chronically at 1 mg·kg^{-1}·day^{-1} for 6 days, was able to reproduce the effect of rosiglitazone. In particular, GW1929 completely reversed salbutamol-induced β_2-adrenoceptor tolerance in tracheal smooth muscle tissues derived from desensitized guinea-pigs, without changing salbutamol responsiveness in control animals (Figure 2B; Table 2).

In this study, preliminary experiments performed in desensitized animals had demonstrated

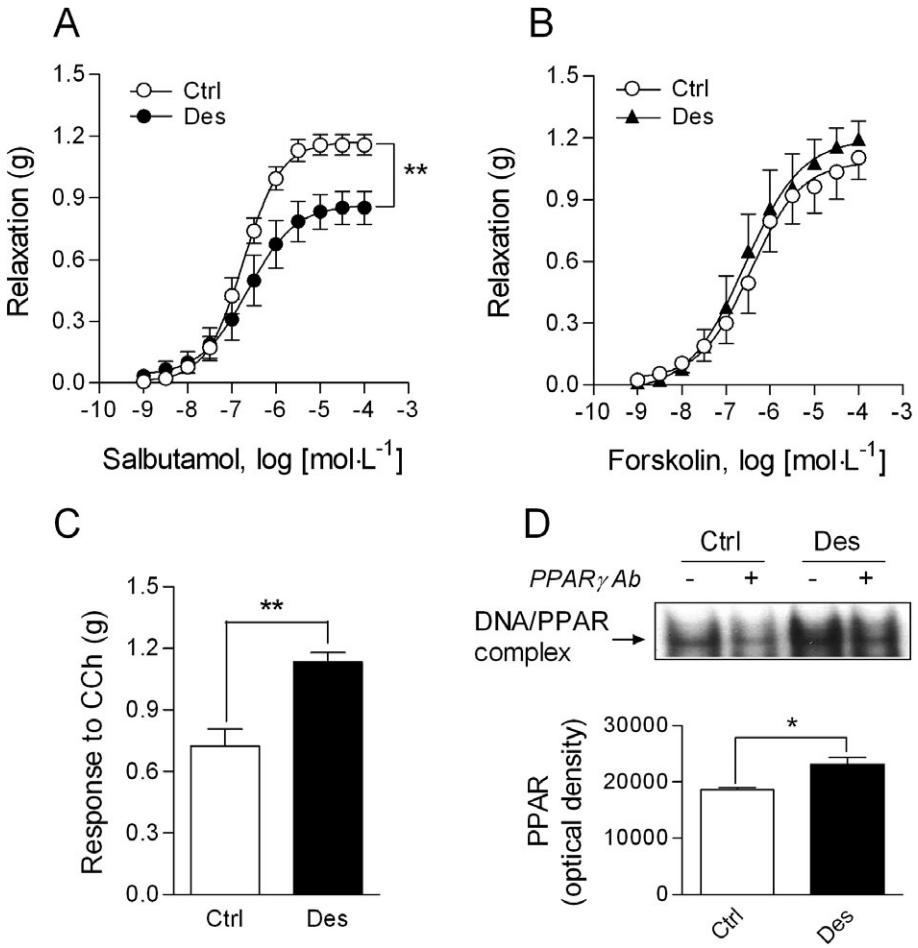


Figure 1

In vivo model of homologous β_2-adrenoceptor desensitization. Cumulative relaxation–response curves for (A) salbutamol and (B) forskolin, and (C) contractile response to carbachol (CCh), in control and desensitized animals. (D) A representative electrophoretic mobility shift assay gel showing the binding specificity of peroxisome-proliferator-activated receptor (PPAR)γ to the DNA probe and a bar graph illustrating the densitometric analyses of PPAR-binding activity. Ctrl: control; Des: desensitized. Results are average of five separate experiments. Data are reported as mean ± standard error of the mean (SEM). *$P < 0.05$; **$P < 0.01$ (compared with the control group).

that dexamethasone at 0.2 mg·kg^{-1}·day^{-1} s.c., was not able to ameliorate salbutamol responsiveness (E_{max} of 0.75 ± 0.04 g and 0.87 ± 0.06 g in dexamethasone treated and not-treated animals respectively) and did not prevent the carbachol-induced hypercontractility (1.06 ± 0.12 g and 1.08 ± 0.04 g, in dexamethasone treated and not-treated animals respectively). Afterwards, we used a 10-fold higher dose of dexamethasone (2 mg·kg^{-1}·day^{-1} for 6 days) given intraperitoneally to homologously desensitized animals. However, dexamethasone, also at this dose, failed to significantly modify the altered tissue responsiveness induced by β_2-adrenoceptor desensitization (Figure 2C; Table 3).

Rosiglitazone and GW1929 also reduced tissue hyperresponsiveness to carbachol in desensitized guinea-pigs ($P < 0.05$; Figure 2D,E respectively), which was not significantly reversed by treatment with dexamethasone (Figure 2F).

Quantitation of β_2-adrenoceptor mRNA levels

GAPDH and β-actin were used as reference genes because their expression levels did not significantly change in all of our experimental conditions, while the expression of 18S rRNA was not stable enough. The statistical analysis highlighted a modest but significant underexpression of β_2-adrenoceptor gene in desensitized versus control animals. Treatment with rosiglitazone or dexamethasone did not significantly modify the β_2-adrenoceptor expression observed in desensitized animals (Figure 3).

In order to weight the influence of biological variability on the fold change measurements, three experiments were carried out by pooling together

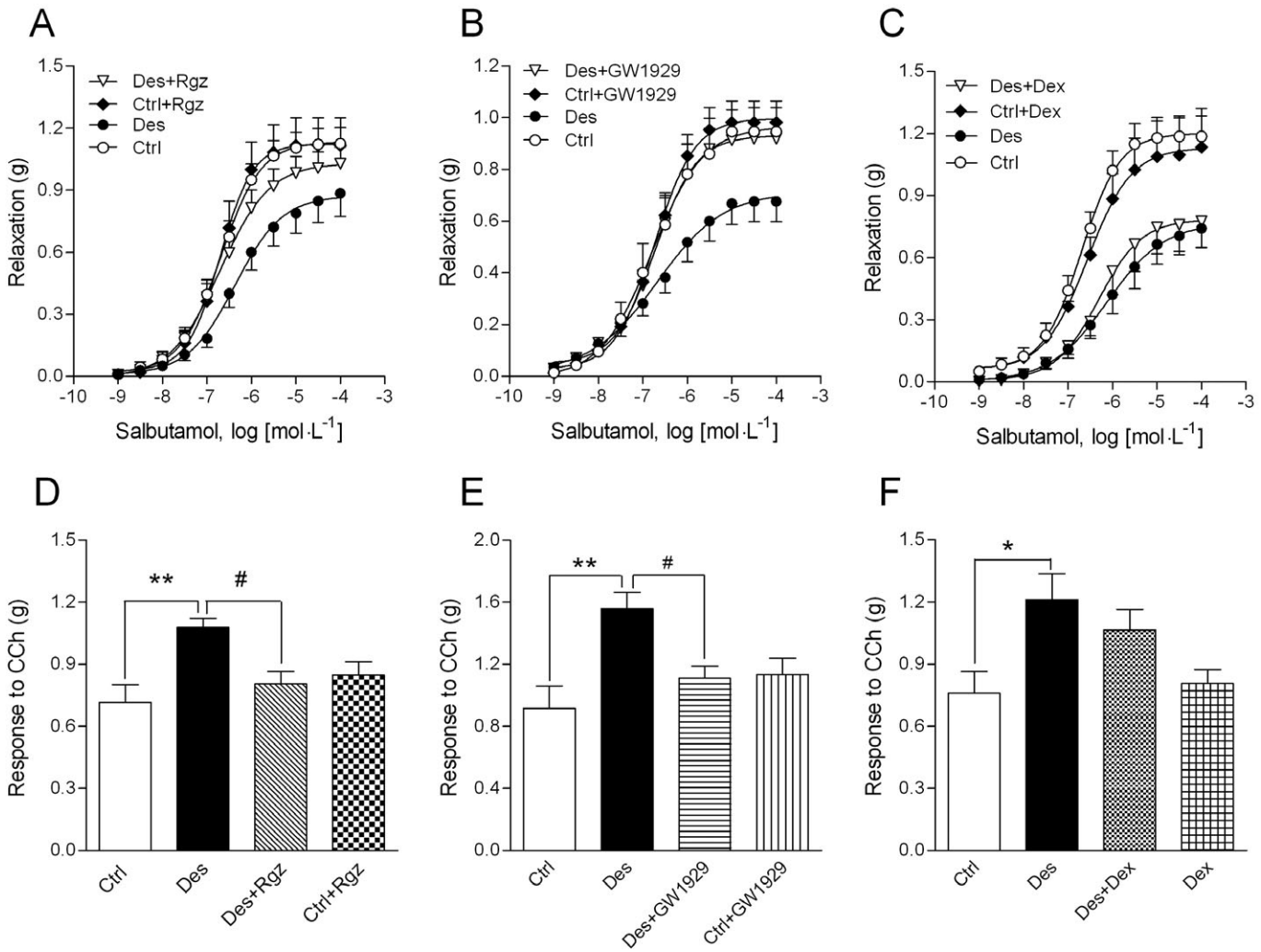


Figure 2

Effect of peroxisome-proliferator-activated receptor (PPAR)γ agonists and dexamethasone on tracheal smooth muscle responsiveness in control and homologously desensitized guinea-pigs. (A–C) Cumulative relaxation–response curves for salbutamol and (D–F) contractile response to carbachol (CCh) in isolated tracheae from control and desensitized guinea-pigs, in the absence or presence of rosiglitazone 10 mg·kg^{-1}·day^{-1}, GW1929 1 mg·kg^{-1}·day^{-1} or dexamethasone 2 mg·kg^{-1}·day^{-1} administered for six consecutive days. Ctrl: control; Des: desensitized; Rgz: rosiglitazone; Dex: dexamethasone. Results are average of five to eight separate experiments for each treatment group. Data are reported as mean ± standard error of the mean (SEM). *P < 0.05; **P < 0.01 (compared with the control group); #P < 0.05 (compared with the desensitized group).

the RNAs from each class of samples. The pool experiments showed mean fold changes equal to those observed by analysing samples individually (data available on request).

Radioligand binding studies

Preliminary experiments confirmed the high prevalence of the β_2-adrenoceptor subtype in the guinea-pig airways observed previously (Carswell and Nahorski, 1983). Binding experiments performed on the dorsal muscle portion of trachea (i.e. the specific tissue also used for functional and real-time PCR characterization of β_2-adrenoceptors) derived from control animals showed a K_d of 2.5 nmol·L^{-1} and a B_{max} of 123 fmol·mg of protein. Analyses of tissues from salbutamol-desensitized animals showed a decrease in β_2-adrenoceptor numbers to below the detection limit of our assay, making impossible to estimate B_{max} and K_d values in these samples. However, in desensitized animals, rosiglitazone treatment was able to partially restore receptor binding sites with a B_{max} of 55.5 fmol·mg protein and a K_d value of 2.48 nmol·L^{-1} (Figure 4).

In vitro *model of homologous β_2-adrenoceptor desensitization*

After salbutamol treatment at 0.001–100 μmol·L^{-1}, a concentration-dependent increase in the

Table 1

Potency (pD_2) and intrinsic activity (E_{max}) for salbutamol-induced smooth muscle relaxation after treatment with rosiglitazone

	pD_2 (–log [mol·L^{-1}])	E_{max} (g)
Ctrl	6.69 ± 0.09	1.13 ± 0.04
Des	6.37 ± 0.15	0.87 ± 0.06**
Des + Rgz	6.70 ± 0.12	1.03 ± 0.05$^{\#}$
Rgz	6.71 ± 0.14	1.12 ± 0.06

Values (means ± standard error of the mean, SEM) were determined from the concentration–response data in Figure 2A. Results are average of five to eight separate experiments for each treatment group.
**$P < 0.01$ (compared with the control group); $^{\#}P < 0.05$ (compared with the desensitized group).
Ctrl, control (vehicle alone); Des, desensitized; Rgz, rosiglitazone; pD_2, molar concentration exerting half maximal effect; E_{max}, maximal effect.

Table 2

Potency (pD_2) and intrinsic activity (E_{max}) for salbutamol-induced tracheal smooth muscle relaxation after treatment with GW1929

	pD_2 (–log [mol·L^{-1}])	E_{max} (g)
Ctrl	6.79 ± 0.20	0.96 ± 0.07
Des	6.68 ± 0.25	0.71 ± 0.06*
Des + GW1929	6.72 ± 0.16	0.93 ± 0.05$^{\#}$
GW1929	6.74 ± 0.10	0.99 ± 0.04

Values (means ± standard error of the mean, SEM) were determined from the concentration–response data in Figure 2B. Results are average of five separate experiments for each treatment group.
*$P < 0.05$ (compared with the control group); $^{\#}P < 0.05$ (compared with the desensitized group).
Ctrl, control (vehicle alone); Des, desensitized; pD_2, molar concentration exerting half maximal effect; E_{max}, maximal effect.

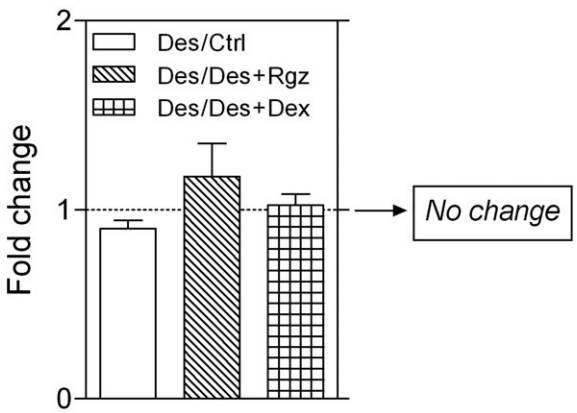
intracellular cAMP levels was observed in control cells with an EC_{50} of 0.46 ± 0.11 µmol·L^{-1} (Figure 5A). *In vitro*, chronic exposure to salbutamol (1 µmol·L^{-1} for 24 h) induced β_2-adrenoceptor desensitization, resulting in a significant reduction of salbutamol responsiveness (~70%; $P < 0.05$) as compared with control samples (Figure 5A,B). On the contrary, homologous desensitization did not affect cAMP production by forskolin 1 µmol·L^{-1} (i.e. a concentration that approximates the EC_{50} value for forskolin concentration–response curve) (Figure 5B), suggesting that adenylyl cyclase was not directly involved in this process. Noteworthy, the β_2-adrenoceptor/GAPDH expression ratio was not altered significantly in desensitized versus control cells (0.78 ± 0.14 and 0.71 ± 0.12, respectively;

Table 3

Potency (pD_2) and intrinsic activity (E_{max}) for salbutamol-induced smooth muscle relaxation after treatment with dexamethasone

	pD_2 (–log [mol·L^{-1}])	E_{max} (g)
Ctrl	6.70 ± 0.10	1.20 ± 0.04
Des	6.13 ± 0.21	0.77 ± 0.08**
Des + Dex	6.31 ± 0.19	0.78 ± 0.07
Dex	6.56 ± 0.23	1.13 ± 0.08

Values (means ± standard error of the mean, SEM) were determined from the concentration–response data in Figure 2C. Results are average of five separate experiments for each treatment group.
**$P < 0.01$ (compared with the control group).
Ctrl, control (vehicle alone); Des, desensitized; Rgz, rosiglitazone; Dex, dexamethasone; pD_2, molar concentration exerting half maximal effect; E_{max}, maximal effect.

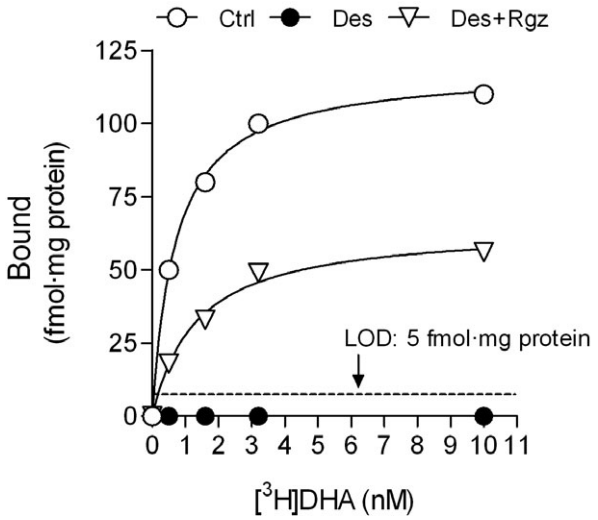


Group	n	Fold change[a]	P value
Des/Ctrl	8	0.90 ± 0.04	0.028
Des/Des + Rgz	8	1.17 ± 0.17	0.818
Des/Des + Dex	8	1.02 ± 0.05	0.543

Figure 3

Real-time PCR assessment of β_2-adrenoceptor gene expression in the guinea-pig tracheal smooth muscle. Ctrl: control; Des: desensitized; Rgz: rosiglitazone; Dex: dexamethasone. [a]Values were expressed as mean ± standard error of the mean (SEM).

$P = 0.37$) (Figure 5C). The PPARγ gene was highly expressed in BSMC and homologous β_2-adrenoceptor desensitization did not substantially change mRNA levels (Figure 5D). The nuclear PPAR protein was identified by electrophoretic mobility shift assay in cell nuclei and the specificity of the DNA binding assay was confirmed by selective inhibition of PPAR-DNA binding by anti-PPARγ



Group	n	Kd (-log M)	Bmax (fmol·mg protein)
Ctrl	5	2.51	123
Des	5	ND	ND
Des+Rgz	5	2.48	55.5

Figure 4

Radioligand binding assay of β-adrenoceptor in pooled preparations of isolated tracheal smooth muscle derived from different sample groups. Ctrl: control; Des: desensitized; Rgz: rosiglitazone; LOD, limit of detection; ND, not determined.

antibody (decrease in the PPAR signal and appearance of supershift bands) (Figure 5E). While protein expression of total PPAR was significantly increased in desensitized versus control cells, PPARγ was not substantially affected after homologous desensitization (supershifted bands) (Figure 5E).

Effect of PPARγ agonists and dexamethasone in homologously desensitized BSMC

The treatment with the PPARγ agonists, rosiglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, at 10 μmol·L^{-1} for 24 h significantly restored responsiveness to salbutamol in homologously desensitized cells, whereas no significant effect was observed in control cells after treatment with drugs (Figure 6A,B). Treatment with 10 μM fenofibrate (PPARα agonist) did not prevent salbutamol subsensitivity in desensitized BSMC, thus suggesting that the effect is specific to PPARγ agonists (Figure 6C). Dexamethasone at 10 μmol·L^{-1} for 24 h tended to improve salbutamol-induced cAMP accumulation in control cells, while it failed to reverse salbutamol subsensitivity in desensitized cells (Figure 6D).

The protective effect against homologous desensitization observed for rosiglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 did not seem to be related to transcriptional β_2-adrenoceptor changes, as treatment with PPARγ agonists did not significantly affect β_2-adrenoceptor expression (Figure 6E). Fenofibrate or dexamethasone at 10 μmol·L^{-1} for 24 h did not change β_2-adrenoceptor expression in control as well as in desensitized cells (Figure 6E).

Discussion

G-protein-coupled receptor-mediated regulation of airway smooth muscle tone is one of the major determinants of the acute and chronic features of asthma (Deshpande and Penn, 2006). In the present study, we used an *in vivo* model of tracheal β_2-adrenoceptor desensitization, established in guinea-pigs by administering salbutamol continuously to mimic the effect of regular therapy in asthmatic patients treated with this class of drugs. Specifically, we used a treatment schedule (i.e. 40 μg·kg^{-1}·h^{-1} for seven consecutive days) with proven efficacy in inducing pulmonary β_2-adrenoceptor desensitization in rats (Finney *et al.*, 2000). Noteworthy, our model was characterized by a substantial change in the airway smooth muscle responsiveness to relaxant and contractor stimuli. In particular, chronic exposure to salbutamol significantly decreased the protective effect of the β_2-adrenoceptor agonist on carbachol-induced bronchoconstriction by ~25%, without affecting activation of adenylyl cyclase; such an effect was accompanied by a hyperreactivity to cholinergic stimuli. Recently, convincing evidence has been provided that proasthmatic changes in airway smooth muscle function (i.e. late β_2-adrenoceptor hyporesponsiveness and hyperreactivity mediated by muscarinic receptors) are a common feature of homologous β_2-adrenoceptor desensitization (Nino *et al.*, 2009). Finally, the guinea-pig model of salbutamol-induced β_2-adrenoceptor tolerance described in the present study closely resembles the clinical setting as the regular use of β_2-adrenoceptor agonist bronchodilators has been associated with bronchial hyperresponsiveness and loss of bronchoprotection in asthma patients, a proasthmatic phenotype that increases the risk of asthma exacerbation during long-term use of these drugs (Spitzer *et al.*, 1992; Cates *et al.*, 2008).

With regard to the mechanisms underlying such an effect, we demonstrated that functional changes in tracheal responsiveness to adrenergic and cholinergic stimulants in desensitized guinea-pigs were accompanied by a marked decrease in tracheal

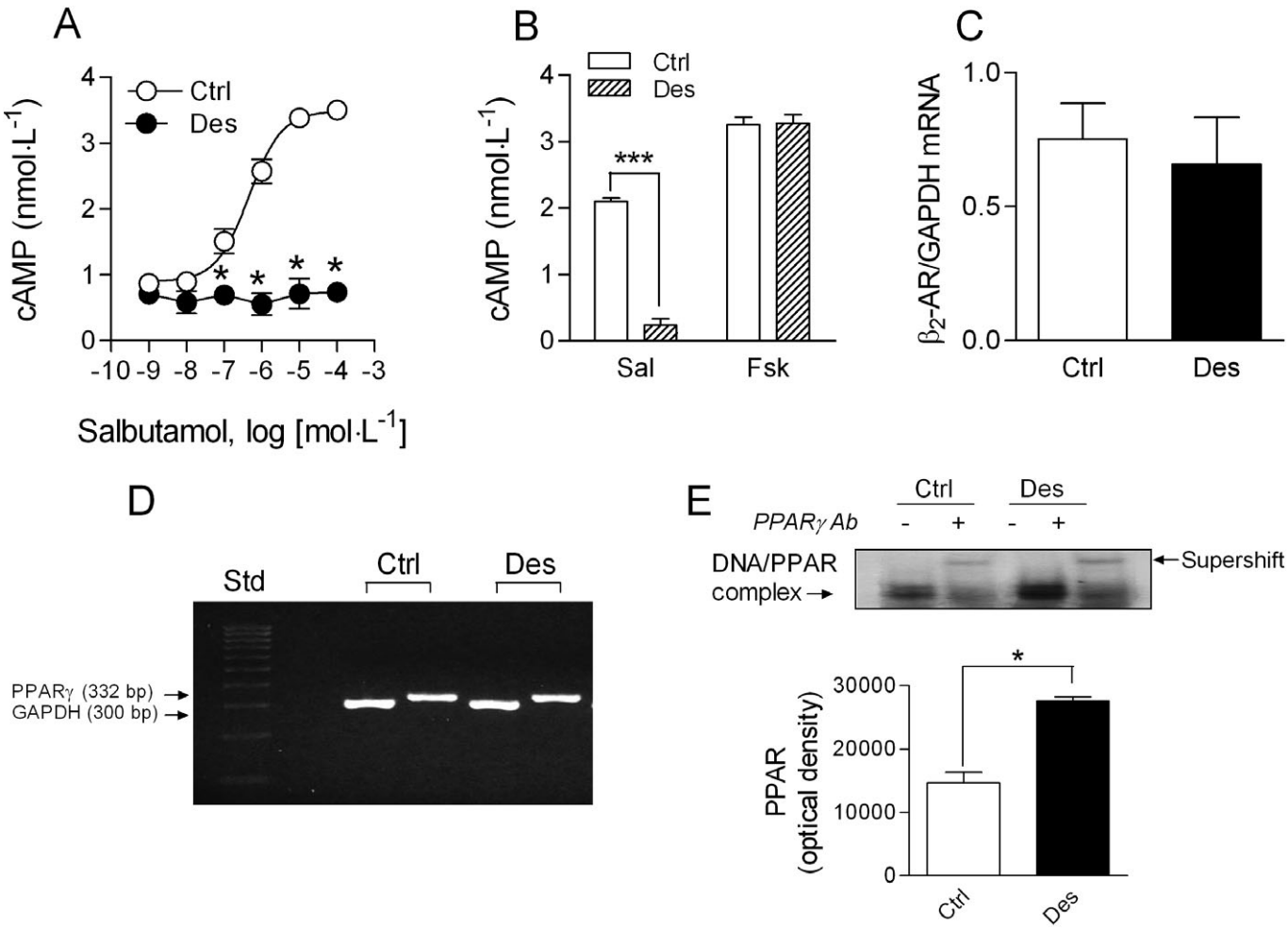


Figure 5

In vitro model of homologous β_2-adrenoceptor desensitization. (A) Concentration–response curves for salbutamol in human bronchial smooth muscle cells treated with vehicle (Ctrl) or homologously desensitized (Des) with salbutamol 1 μmol·L^{-1} for 24 h. (B) Effect of homologous β_2-adrenoceptor desensitization on the *in vitro* responsiveness to 10 μmol·L^{-1} salbutamol (Sal) or 1 μmol·L^{-1} forskolin (Fsk). (C) Densitometric analyses of β_2-adrenoceptor. (D) RT-PCR image of peroxisome-proliferator-activated receptor (PPAR)γ gene expression and (E) a representative electrophoretic mobility shift assay gel showing the binding specificity of PPARγ to the DNA probe and a bar graph illustrating the densitometric analyses of PPAR-binding activity. Results are average of five separate experiments. Data are reported as mean ± standard error of the mean (SEM). *$P < 0.05$; ***$P < 0.001$.

smooth muscle β_2-adrenoceptor cell-surface number. This evidence was in agreement with data that demonstrated a loss of binding activity to β_2-adrenoceptors in rat (Finney *et al.*, 2000) and guinea-pig (Nishikawa *et al.*, 1994) lung following chronic treatment with β-adrenoceptor agonists.

Our real-time PCR data showed a modest, although significant, β_2-adrenoceptor gene underexpression in guinea-pig tracheal smooth muscle after homologous desensitization. Although down-regulation of β_2-adrenoceptors has already been demonstrated in lungs of rats and guinea-pigs chronically treated with isoprenaline or noradrenaline (Nishikawa *et al.*, 1993; 1994; Mak *et al.*, 1995), to our knowledge this is the first report that quantitatively assessed this phenomenon in the tracheal smooth muscle of guinea-pigs exposed to a β_2-adrenoceptor selective drug used in clinical practice.

Collectively, these *in vivo* results support the notion that prolonged β_2-adrenoceptor activation may trigger a counteractive response (i.e. cholinergic signal amplification) in the airway smooth muscle cells, aimed to retain a given level of airway smooth muscle tone. In particular, uncoupling and internalization of cell-surface β_2-adrenoceptors appear to play a key role in the adaptive modulation of tracheal smooth muscle responsiveness to pharmacological stimuli *in vivo*, whereas transcriptional events seem to be less involved in this process. In

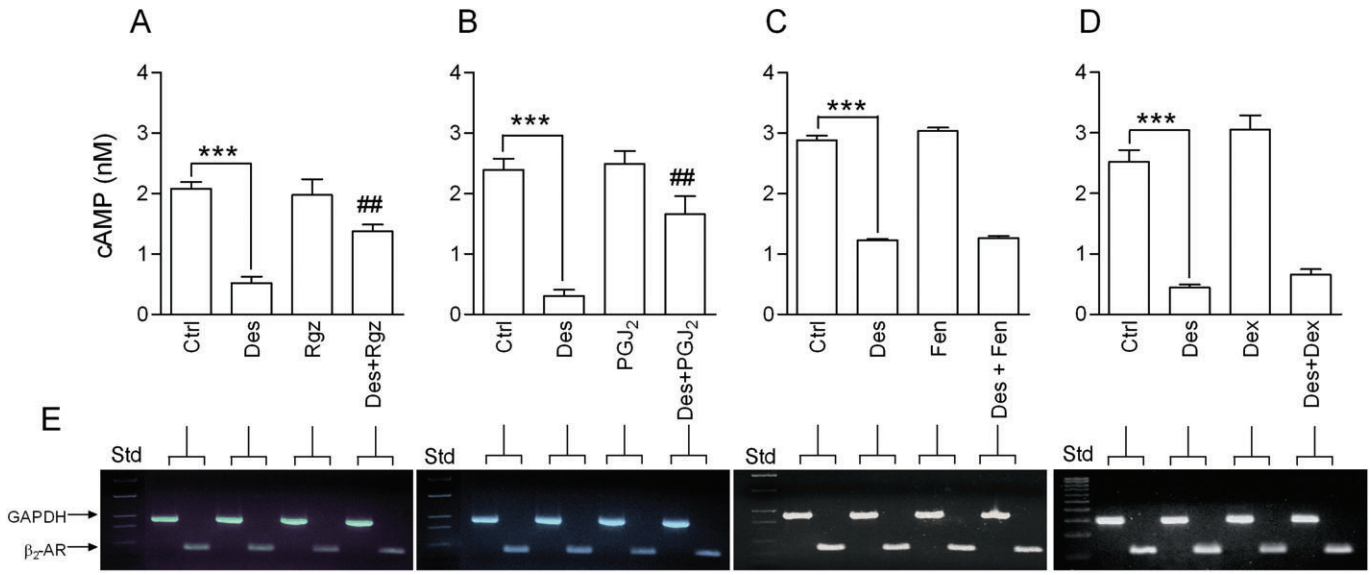


Figure 6

Effect of peroxisome-proliferator-activated receptor (PPAR)γ agonists and dexamethasone in homologously desensitized human bronchial smooth muscle cells. cAMP intracellular production after stimulation with 10 μmol·L^{-1} salbutamol in control (Ctrl) and desensitized (Des) cells, in the absence or presence of (A) rosiglitazone (Rgz) (B) 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2), or (C) fenofibrate (Fen) and (D) dexamethasone (Dex) given at 10 μmol·L^{-1} for 24 h. (E) β_2-adrenoceptor gene expression levels for each treatment group were also reported. Results are average of five separate experiments. Data are reported as mean ± standard error of the mean (SEM). ***$P < 0.001$ (compared with control group); ##$P < 0.01$ (compared with desensitized group).

line with this concept, it has been recently showed that isolated airway smooth muscle tissues from rabbits and cultured human airway smooth muscle cells exhibited constrictor hyperresponsiveness to acetylcholine and impaired β_2-adrenoceptor-mediated relaxation and cAMP accumulation following long-term exposure to cAMP-stimulating agents (Hu *et al.*, 2008; Nino *et al.*, 2009). Furthermore, subcutaneous administration with osmotic minipumps delivering the long-acting β_2-adrenoceptor agonist salmeterol was demonstrated to reduce pulmonary β_2-adrenoceptor number in rats by 70%, without affecting the levels of β_2-adrenoceptor mRNA transcripts (Finney *et al.*, 2001).

In vitro experiments on human BSMC were performed to enhance the clinical relevance of the study. In agreement with *in vivo* findings, we demonstrated that activation of adenylyl cyclase was unaffected by homologous β_2-adrenoceptor desensitization, while salbutamol-induced cAMP stimulation was substantially reduced in the absence of changes in β_2-adrenoceptor expression levels. These results are also in line with evidence obtained in human small airways (Cooper and Panettieri, 2008), thus suggesting that the mechanism of β_2-adrenoceptor desensitization occurs upstream of adenylyl cyclase in the absence of β_2-adrenoceptor down-regulation.

The PPAR agonists have been recently proposed as potential new therapeutic agents in the treatment of chronic airway diseases, due to their ability to minimize the contribution of inflammation to airway remodelling and dysfunction (Belvisi *et al.*, 2006). The most relevant finding of the present study was the ability of the PPARγ agonist, rosiglitazone, to simultaneously restore salbutamol relaxant activity and mitigate carbachol hyperresponsiveness in tracheal tissues from homologously desensitized animals. Such an effect seems to be mediated by PPARγ activation as *in vivo* treatment with GW1929, a selective PPARγ agonist chemically unrelated to thiazolidinediones, was able to reproduce the effect of rosiglitazone. We also demonstrated that therapeutic concentrations of rosiglitazone and the endogenous PPARγ agonist 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 were able to prevent β_2-adrenoceptor desensitization, *in vitro*; such an effect seemed to be PPARγ-specific, as treatment with the PPARα agonist, fenofibrate, did not restore responsiveness to salbutamol. Noteworthy, we showed the presence of PPARγ both in control and desensitized airway smooth muscle tissues, a condition that may offer a pharmacodynamic basis for the use of PPAR targeting strategies in combination with β_2-adrenoceptor agonists. Several lines of evidence suggest the existence of cross-talk between the sympathetic nervous system and

PPARγ-mediated signal transduction pathways in adipose tissue (Sell *et al.*, 2004; Hughes *et al.*, 2006; Bogacka *et al.*, 2007). However, to our knowledge, no data on the synergistic effects of PPARγ and β_2-adrenoceptor agonists on airway smooth muscle *in vivo* and *in vitro* have been reported before the present study.

The precise molecular mechanism by which rosiglitazone interacts with β_2-adrenoceptor signalling remains to be clarified and further investigation is in progress. In our experimental setting, *in vivo* functional β_2-adrenoceptor desensitization was fully prevented by co-exposure to rosiglitazone; however, β_2-adrenoceptor number increased by approximately 50% compared with desensitized values. This difference may be ascribed to the presence of 'spare receptors' in airway smooth muscle tissues or to additional effects of rosiglitazone on molecular elements involved in the cross-talk between β_2-adrenoceptor and muscarinic cholinoceptor systems. In this regard, it is worth mentioning that pre-treatments with either the phosphodiesterase-4 selective inhibitor rolipram, the protein kinase A (PKA) inhibitor H89 or the extracellular regulated kinase (ERK)1/2 inhibitor U0126 prevent the proasthmatic-like changes induced by salmeterol-induced β_2-adrenoceptor activation (Nino *et al.*, 2009), suggesting possible molecular targets of rosiglitazone action, which need to be investigated.

Overall, our *in vivo* and *in vitro* findings suggest that rosiglitazone protection against β_2-adren oceptor desensitization occurred through a mechanism in part dependent on up-regulation of surface receptors, without changes in β_2-adrenoceptor gene transcription.

Corticosteroids are the most potent anti-inflammatory agents used to treat chronic inflammatory diseases. The combination with β_2-adrenoceptor agonists is frequently used in the control of asthma and the important molecular interactions between these two classes of drugs are now recognized (Barnes, 2009). Chronically administered dexamethasone has been proven to increase β-adrenoceptor density in rat lungs without a significant increase in β-adrenoceptor mRNA levels (Mak *et al.*, 1995). We investigated whether dexamethasone, given by different schedules of administration, was able to preserve salbutamol response in homologously desensitized guinea-pig tracheal smooth muscle. In our model, dexamethasone failed to restore salbutamol relaxant activity and did not significantly modify tissue hyperresponsiveness to carbachol in desensitized animals, regardless of dose level and route of administration. Noteworthy, our *in vitro* findings demonstrate that dexamethasone did not reverse β_2-adrenoceptor desensitization induced in human BSMC by long-term exposure to salbutamol. The same result was obtained by Hall *et al.* (1993) in human airway smooth muscle cells chronically exposed to isoprenaline, and there is evidence indicating that tolerance to β_2-adrenoceptor agonists cannot be restored by systemic steroid therapy in asthma patients (Grootendorst *et al.*, 2001). On the other hand, 1 h incubation of human lung slices with dexamethasone prevented β_2-adrenoceptor desensitization (Cooper and Panettieri, 2008). Although these discrepancies may be apparent and related to the different model systems employed, further investigation are required due to the clinical relevance of steroid/β_2-agonist combination therapy. It should be noted that the scientific rationale for combination therapy including corticosteroids and β_2-adrenoceptor agonists is mainly the complementary actions of these drugs on the pathophysiology of asthma including their additive inhibitory effect on serum-induced bronchial smooth muscle cell proliferation (Roth *et al.*, 2002) and the ability of corticosteroids to prevent cytokine-induced β_2-adrenoceptor desensitization (Barnes, 2002). Finally, it has been demonstrated that glucocorticoids can favourably interact with PPARγ agonists by reducing IL-1β-induced COX-2 expression (Pang *et al.*, 2003) and by inhibiting TNFα-induced production of chemokines (Nie *et al.*, 2005) in human airway smooth muscle cells, thus supporting the potential combined benefit of PPARγ agonists and drugs currently used in the treatment of asthma.

In summary, our pharmacological model *in vivo* reproduces the proasthmatic-like changes observed in asthma patients during long-term use of β_2-adrenoceptor agonists, that is, enhanced reactivity to contraction mediated by M_3 muscarinic receptors and diminished relaxation mediated by β_2-adrenoceptors. In our experimental setting, regulation of β_2-adrenoceptor density appears to be essential to modulate tracheal smooth muscle response to contractor or relaxant stimuli. Finally, the PPARγ-mediated ability of rosiglitazone to reverse the proasthmatic-like changes induced by persistent β_2-adrenoceptor activation might improve the therapeutic index of β_2-adrenoceptor agonists not only increasing drug efficacy but also decreasing risk of serious adverse events associated with the regular use of this class of drugs.

Acknowledgements

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Conflict of interests

None.

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