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# Protective effect of high-dose montelukast on salbutamol-induced homologous desensitisation in airway smooth muscle

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ABSTRACT

Montelukast (MK) is a potent cysteinyl-leukotriene receptor antagonist that causes dose-related improvements in chronic asthma. We sought to determine whether MK was able to prevent salbutamol-induced tolerance in airway smooth muscle. Homologous $\beta_2$-adrenoceptor desensitisation models were established in guinea-pigs and in human bronchial smooth muscle cells (BSMC) by chronic salbutamol administration. Characterisation tools included measurement of the response of tracheal smooth muscle tissues to salbutamol, analysis of gene expression and receptor trafficking, evaluation of intracellular cAMP levels and phosphodiesterase (PDE) activity in human bronchial smooth muscle cells. Salbutamol-induced $\beta_2$-adrenoceptor desensitisation was characterised by $\beta_2$-agonist hyporesponsiveness (−30%, $p < 0.001$) in desensitised tracheal smooth muscle, as compared to controls. MK, given intraperitoneally at 5 mg/kg/day for 6 consecutive days, completely restored tissue responsiveness to salbutamol. Prolonged salbutamol treatment significantly decreased cAMP synthesis, induced a complete removal of the $\beta_2$-adrenoceptor from plasma membrane with a parallel increase in the cytosol and increased PDE4D5 gene transcription and PDE activity in human bronchial smooth muscle cells. In homologously desensitised BSMC, MK 30 μM for 24 h was able to prevent salbutamol subsensitivity and such an effect was associated with inhibition of salbutamol-induced PDE4 activity and restoration of membrane $\beta_2$-adrenoceptor expression and function. These findings suggest the presence of a favourable interaction between MK and $\beta_2$-adrenoceptor agonists that might improve the therapeutic index of bronchodilators in patients with chronic respiratory diseases.



## 1. Introduction

It has been reported that patients with severe persistent asthma, chronic obstructive respiratory disease (COPD) and those asthmatics who smoke have poor response to corticosteroids [1]. Since the dose-response effect of corticosteroids is relatively flat, increasing the dose is expected to produce little improvement in lung function, whereas adding some other class of controller agent represents a more effective pharmacological strategy [2].

$\beta_2$-Adrenoceptor agonists are mainstays in the treatment of asthma and COPD. Unfortunately, prolonged administration of these drugs significantly increases the risk for asthma hospitalization and deaths, even when used in combination with inhaled corticosteroids [3,4]. While the precise mechanism underlying such an effect has not yet been fully elucidated, late $\beta_2$-adrenoceptor hyporesponsiveness caused by homologous $\beta_2$-adrenoceptor desensitisation in airway smooth muscle may have a role [5,6]. At variance with agonist-specific (homologous) $\beta_2$-adrenoceptor desensitisation, agonist-nonspecific (also termed heterologous) desensitisation is caused by chronic exposure to pro-inflammatory cytokines [1]. Noteworthy, despite the ability of corticosteroids to mitigate heterologous $\beta_2$-adrenoceptor desensitisation [1], several lines of evidence indicate that tolerance to β2-agonists cannot be restored by steroid therapy [6–8].

Montelukast (MK) is a potent and specific cysteinyl leukotriene (cysLT) receptor antagonist that causes benefit to chronic asthmatic patients. The recommended dose of MK for patients has been selected on the basis of its ability to block the direct effect of cysLTs on bronchial smooth muscles during the early asthmatic response [9]. However, there is much evidence obtained in animal models of allergic asthma sustaining that MK can exert a remarkable anti-inflammatory action at doses greater than needed to inhibit bronchial smooth muscle constriction [10–13]. This notion makes

*Abbreviations:* COPD, chronic obstructive pulmonary diseases; BSMC, bronchial smooth muscle cells.

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clinically attractive the possible use of high-dose MK treatment as adjunct to or a replacement for systemic corticosteroids in patients that do not respond to standard treatments.

Rovati and co-workers [14] demonstrated that MK was able to prevent heterologous $\beta_2$-adrenoceptor desensitisation in human airways smooth muscle cells treated with leukotriene D4 (LTD4) and hyporesponsiveness to $\beta_2$-adrenoceptor agonists in human bronchial rings passively sensitized and challenged with allergen. However, it is currently unknown whether MK can also protect from homologous $\beta_2$-adrenoceptor desensitisation in airway smooth muscle tissues.

Considering these premises and taking into account the clinical relevance of combination schedules including MK and $\beta_2$-adrenoceptor agonists, we sought to determine whether MK could reverse salbutamol-induced tolerance in vivo, on isolated guinea-pig trachea, and in vitro, on human bronchial smooth muscle cells.

## 2. Materials and methods

### 2.1. Animals

Male Dunkin-Hartley guinea-pigs of 300–400 g body weight were housed in a temperature controlled environment with food and water available ad libitum. The 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 (EU Directive 2010/63/EU) for the care and handling of experimental animals. The protocol was approved by the Animal Care Committee of the University of Pisa.

### 2.2. In vivo $\beta_2$-adrenoceptor desensitisation and drug administration

A chronic in vivo model of tracheal $\beta_2$-adrenoceptor desensitisation was established in guinea-pigs as previously reported [6]. Briefly, animals were sedated with pentobarbital at 30–35 mg/kg i.p. and implanted subcutaneously with osmotic minipumps (Alzet, Palo Alto, California, USA) delivering salbutamol (40 μg/kg/h) or vehicle (sterile phosphate buffered saline) for 7 days. The content of the pump was delivered into the local subcutaneous space and absorption of the compound by local capillaries resulted in systemic drug administration. MK was given i.p. at 1 and 5 mg/kg/day for 6 consecutive days to control or desensitised animals, starting from 24 h after minipump implantation. Drug doses was selected in accordance with previous studies showing that MK administered from 1 to 100 mg/kg has a dose-dependent anti-inflammatory response on allergen-induced lung inflammation in an animal model of persistent asthma [10,11,15].

Experiments were performed on tracheal preparations isolated from guinea-pigs [6] under control conditions or following homologous desensitisation to salbutamol in the presence or in the absence of MK treatment. Carbachol at 0.3 μM 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 μM in control and desensitised 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.

### 2.3. In vitro $\beta_2$-adrenoceptor desensitisation

Human bronchial smooth muscle cells (BSMC; Lonza, Walkersville, MD, USA) were maintained in an optimized medium containing 5% foetal bovine serum, 5 ng/ml insulin, 2 ng/ml basic fibroblast growth factor and 0.5 ng/ml epidermal growth factor (SmGM-2 Bullet Kit, Lonza). Prolonged homologous $\beta_2$-adrenoceptor desensitisation was performed by exposing human BMSC to salbutamol at 1 μM for 24 h. This concentration level was selected on the basis of the EC50 (concentration of salbutamol that gave half-maximal effect, i.e., 0.46 ± 0.01 μM) previously reported [6]. Intracellular cAMP levels were measured by the cAMP-Glo™ Assay (Promega, Madison, WI, USA) in control and desensitised cells after stimulation with 10 μM salbutamol, in the presence or absence of MK from 1 to 50 μM for 24 h. BSMC were used between passages 4 and 8.

### 2.4. Assessment of $\beta_2$-adrenoceptor and PDE4D5 mRNA levels in BSMC

Total RNA was obtained from the dorsal muscle portion of tracheae and from human BSMC by using the RNeasy Fibrous Tissue kit and the RNeasy Mini kit (Qiagen, Valencia, CA, USA), respectively, after the same salbutamol treatments as above in terms of times and doses. The concentration and purity of total RNA were measured by 260 nm UV absorption and by 260/280 ratios, respectively, using Gene Quant pro Spectrophotometer; good quality RNA 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). PCR was performed by using the Hot StartTaq Master Mix kit. Primers sequences were as follows: 5′-ACC AGG AAG CCA TCA ACTG-3′ (F) and 5′-GAA GAC CAT GAT CAC CAG GGG-3′ (R) for $\beta_2$-adrenoceptor; 5′- TGC CAG CTG TAC AAA GTT GACC-3′ (F) and 5′-TTC TCG GAG AGA TCA CTG GAGA-3′ (R) for PDE4D5; 5′-GTG AAG GTC GGA GTCA ACG-3′ (F) and 5′-GGT GAA GAC GGC CAG TGG ACTC-3′ (R) for GAPDH and the expected amplification products were 119, 212 and 300 bp long, respectively. Relative densitometry of bands was measured using NIH ImageJ gel analysis.

### 2.5. Detection of $\beta_2$-adrenoceptor protein levels by Western Blot analysis

BSMC were treated with medium alone (control), salbutamol (1 μM), and/or MK (30 μM) for 24 h. For total cell lysates, samples were then lysed for 60 min at 4 °C by adding RIPA buffer (9.1 mM $NaH_2PO_4$, 1.7 mM $Na_2HPO_4$, 150 mM NaCl, pH 7.4, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS and a protease inhibitor cocktail). Equal amounts of cell extracts (40 μg) were diluted in Laemmli solution, resolved by SDS-PAGE (8.5%), transferred to PVDF membranes and probed overnight at 4 °C with a primary anti-$\beta_2$-adrenoceptor (1:100, H-73, Santa-Cruz Biotechnology) antibody. The primary antibody was detected using anti-rabbit IgG light chains conjugated to peroxidase (diluted 1:10,000). The peroxidase was detected using a chemiluminescent substrate (ECL, Perkin Elmer). In order to evaluate receptor trafficking from plasma membrane to cytosol, Western Blot analysis was also performed on membrane and cytosolic fraction. Briefly, BSMC, treated as described above, were washed thoroughly in PBS buffer and rapidly lysed in a hypotonic solution containing 10 mM Tris, pH 7.4, 5 mM EDTA, 2 μg/ml pepstatin, 5 μg/ml benzamidine and 5 μg/ml soybean trypsin inhibitor. Membrane and cytosolic fraction were prepared by centrifugation at 40,000× g for 20 min. Equal amount (30 μg) of cytosolic and membrane fraction proteins were resolved by SDS-PAGE electrophoresis and $\beta_2$-