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Synergistic effects of the anti-cholinergic R,R-glycopyrrolate with anti-inflammatory drugs

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ARTICLE INFO

Article history:
Received 13 May 2006
Accepted 26 July 2006

Keywords:
Asthma
COPD
Inflammation
Anti-cholinergic
R,R-Glycopyrrolate

ABSTRACT

Currently, much effort is geared towards developing therapies that impact on the inflammation in respiratory diseases such as asthma and COPD, assuming that this will improve disease pathology. R,R-Glycopyrrolate, a quaternary ammonium compound, is a muscarinic receptor antagonist with the potential to be used as a long-acting bronchodilator in patients with asthma and COPD. In this study we evaluated whether the combination of R,R-glycopyrrolate with known anti-inflammatory drugs results in synergistic effects. Human primary monocytes were used as an *in vitro* model system. M3, M4, M1 and M2 receptors were expressed in these cells in descending order. The combinatory effects of the drugs on the release of TNF-α after lipopolysaccharide stimulation were analyzed. R,R-Glycopyrrolate alone did not affect LPS induced TNF-α release. The PDE4 inhibitor rolipram dose dependently inhibited the TNF-α release. Maximum inhibition was around 70%. The IC_{35} for rolipram was 68.9 ± 15.2 nM. The simultaneous administration of 10 μM R,R-glycopyrrolate reduced the IC_{35} to 1.70 ± 1.18 nM. The anti-histamine azelastine inhibited TNF-α release dose dependently. The simultaneous administration of R,R-glycopyrrolate did not influence the action of azelastine. The corticosteroid budesonide inhibited the TNF-α release dose dependently with an IC_{50} of 0.55 ± 0.13 nM. The simultaneous administration of 10 μM R,R-glycopyrrolate reduced the IC_{50} to 0.13 ± 0.03 nM. Finally, R,R-glycopyrrolate was most effective in the triple combination with budesonide and rolipram in the reduction of TNF-α release. In conclusion, R,R-glycopyrrolate acts synergistically with the PDE4 inhibitor rolipram and the steroid budesonide in inhibiting inflammatory mediators.

1. Introduction

Asthma bronchiale, affecting more than 150 million people worldwide, has emerged as a major public health problem over the past 20 years. Current data indicate that modern asthma therapy leads only to a limited decrease in death rates [1]. Chronic obstructive pulmonary disease (COPD) is a respiratory condition characterized by chronic obstruction of expiratory flow affecting peripheral airways, associated with chronic inflammation, mucus hypersecretion, goblet cell and submucosal gland hyperplasia, and, in the final stage, pulmonary remodeling resulting in emphysema together with fibrosis [2]. Although COPD is a major cause of chronic morbidity and mortality throughout the world, its recognition as a public health problem is low. The most important cause, by far, is cigarette smoking. However, 10–20% of COPD patients

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doi:10.1016/j.bcp.2006.07.025

are lifelong non-smokers. Additionally, COPD severity and incidence increase with age. As we face an ageing population, we will have more patients with COPD [3].

Today's asthma therapy consists of bronchodilators (reliever) and anti-inflammatory drugs (controller) [4]. Currently, inhaled glucocorticoids are the mainstay of long-term control therapy for persistent asthma. However, patients show a variable response to inhaled glucocorticoids and some exhibit resistance to this drug class [5]. Although long-acting bronchodilators have been an important advance for the management of chronic obstructive pulmonary disease, these drugs do not interfere with the underlying inflammatory process. No currently available treatments reduce the progression of COPD or suppress the inflammation in small airways and lung parenchyma [6]. Inhaled anticholinergic drugs are effective bronchodilators in the treatment of COPD, and tiotropium bromide has recently been introduced as a once-daily bronchodilator for use as a maintenance treatment.

Despite considerable efforts by the pharmaceutical industry, it has been difficult to develop novel therapeutic agents for asthma and COPD. Anticholinergic medications have been accepted as an important treatment modality in COPD and chronic asthma. *R,R*-Glycopyrrolate, a quaternary ammonium compound, is a muscarinic receptor antagonist with the potential to be used typically as a long-acting bronchodilator in patients with asthma and COPD [7,8]. Since the combination therapy of long-acting β_2-agonists and inhaled steroids led to greater improvement in lung function and symptoms as compared to the single drugs [9], we hypothesized that the combination of *R,R*-glycopyrrolate with known anti-inflammatory drugs may result in synergistic effects. To this end, the effect of *R,R*-glycopyrrolate combined with the PDE4 inhibitor rolipram, the anti-histamine azelastine and corticosteroid budesonide on the release of TNF-α in lipopolysaccharide stimulated monocytes was analyzed.

2. Materials and methods

2.1. Materials

LPS from *E. coli* (Serotype 026:B6), dexamethasone, rolipram, and Histopaque 1077, were from Sigma, Deisenhofen, Germany. Roswell Park Memorial Institute (RPMI)-1640 medium was from Invitrogen, Karlsruhe, Germany. HANKS' balanced salt solution was supplied by a local pharmacy hospital (Universitätsklinikum, Erlangen, Germany). Fetal calf serum (FCS) was purchased from PAN Biotech, Aidenbach, Germany. RLT lysis buffer and RNeasy preparation kits were from QIAGEN, Hilden, Germany. Cytokine measurements in culture supernatants were performed with ELISA kits from BD Pharmingen (Heidelberg, Germany). Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Co.

2.2. Isolation of monocytes

About 50 ml blood was collected in 10 ml sodium-EDTA tubes by venapuncture from three different healthy donors for each experiment. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Histopaque 1077. Then cells were washed twice with HANKS solution, resuspended in RPMI-1640 medium and seeded in 24-well plates at a density of 0.5×10^6 cells per 0.5 ml of medium. Cells were allowed to attach overnight at 37 °C in a humidified growth chamber supplemented with 5% CO_2; afterwards the nonadherent cells were washed away and new medium was supplemented. Monocytes were then treated respectively. Culture supernatants were collected and stored at −20 °C.

2.3. Detection of muscarinic receptor subtypes

Total RNA was prepared from monocytes using RNeasy (QIAGEN, Hilden, Germany). One-tube RT-PCR was performed using Quantitect SYBR Green RT-PCR Kit from QIAGEN (Hilden, Germany). The amplification of different members of the human muscarinic receptor gene family was achieved by using subtype-specific primer pairs as described previously [10]. Primer pairs were synthesized by biomers (Ulm, Germany). The PCR products were analyzed by standard electrophoresis on 1.0% agarose gels, stained with ethidium bromide and photographed under UV illumination.

2.4. Cytokine production and inhibition with steroids

For cytokine measurement, monocytes were stimulated with LPS (100 ng/ml) for the indicated time periods. For cytokine inhibition, monocytes were pre-incubated with the respective substances (dissolved in DMSO) for 0.5 h before stimulation with LPS (100 ng/ml) for the indicated time periods.

2.5. Enzyme-linked immunosorbent assay

Cytokine measurements in culture supernatants were done by sandwich ELISA using matched antibody pairs (BD Pharmingen). ELISA plates (Maxisorb, Nunc) were coated overnight with anti-cytokine mAb in 0.1 M carbonate buffer, pH 9.5. After being washed, plates were blocked with assay diluent (Pharmingen) for 1 h and washed again. Appropriately diluted supernatant samples and standards were distributed in duplicates and the plates were incubated for 2 h at room temperature. Plates were washed, incubated for 1 h with working detector (biotinylated anti-cytokine Ab and avidin-horseradish peroxidase conjugate). After washing, substrate (tetramethylbenzidine and hydrogen peroxide) was added. The reaction was stopped by addition of 1 M H_3PO_4. Plates were read at 450 nm (reference 570 nm) in a microplate reader (Dynatech). The results were expressed as a percentage of the control level of cytokines production by cells stimulated in the presence of the vehicle of the corresponding compound. The detection limit of the ELISA for all cytokines was 15.6 pg/ml.

2.6. Data analysis

Analysis was performed using GraphPad Prism Version 4.02 (Graphpad Inc., San Diego, CA). Data were expressed as means ± S.E.M. Differences were further analyzed statistically by the Student's paired *t*-test, since cells from the same three donors were used for all concentrations and conditions in one experiment. Dependent on the efficacy of the inhibitors in the

various test systems, corresponding IC values for half-maximum inhibition were calculated from concentration–inhibition curves by nonlinear regression analysis using the program GraphPad Prism (Version 4.02) from GraphPad Software Inc.

3. Results

3.1. Determination of the muscarinic receptor subtype mRNA expression in human monocytes

RT-PCR experiments were performed to assess the muscarinic receptor subtype mRNA expression of unstimulated and LPS stimulated monocytes. Genomic DNA was used as positive control since the genomic DNA sequences of all mAChR subtypes are intronless, and thus cannot be distinguished from cDNA by differences in size. As shown in Fig. 1 the M3 subtype was most strongly expressed. The M4 and M1 subtype were moderately expressed, whereas only a faint band was detectable for the M2 subtype and no band for the M5 subtype. Stimulation with LPS increased the expression of the M1 and decreased the expression of the M3 receptor. Since these results have been obtained by qualitative PCR, a quantitative comparison of muscarinic receptor expression levels cannot be made.

3.2. Effect of R,R-glycopyrrolate on LPS induced TNF-α release from human monocytes

First we investigated whether R,R-glycopyrrolate *per se* inhibits TNF-α production. Human primary monocytes were isolated from whole blood and incubated with various concentrations of R,R-glycopyrrolate. LPS strongly induced the secretion of TNF-α in the supernatant after 24 h (Fig. 2). R,R-Glycopyrrolate in concentrations up to 10 μM did not affect the LPS stimulated TNF-α release (Fig. 2).

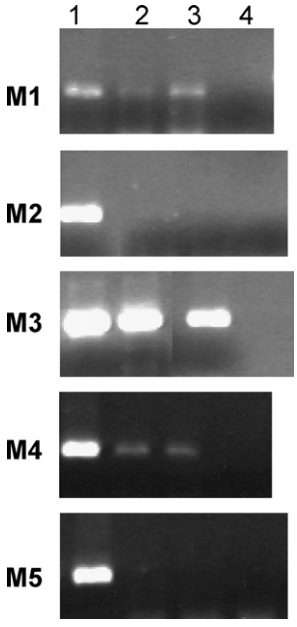


Fig. 1 – RT-PCR of muscarinic receptor subtypes M1–M5. Total RNA was isolated from resting (lane 2) and LPS stimulated (lane 3) human monocytes and subjected to RT-PCR. As a positive control genomic DNA was included (lane 1). The no template control is shown in lane 4. The PCR products were analyzed by standard electrophoresis on 1.0% agarose gels, stained with ethidium bromide and photographed under UV illumination.

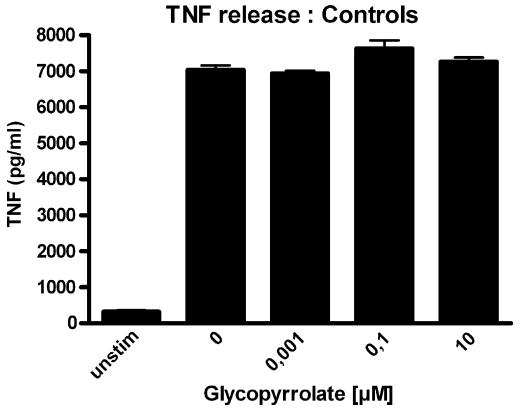


Fig. 2 – R,R-Glycopyrrolate does not affect LPS-stimulated TNF-α release. Human primary monocytes were isolated from whole blood. Stimulation was performed with LPS (100 ng/ml), unstimulated cells were exposed to vehicle only. Vehicle or different concentrations of R,R-glycopyrrolate were added 30 min before stimulation. TNF-α protein levels were determined by ELISA after 24 h. Each data point represents mean ± S.E.M. of three independent ELISA measurements.

3.3. Synergistic inhibition of TNF-α release by R,R-glycopyrrolate and the PDE4-inhibitor rolipram

We analyzed the effect of rolipram alone or in combination with 10 μM R,R-glycopyrrolate on LPS induced TNF-α release. The PDE4 inhibitor rolipram dose dependently inhibited the TNF-α release (Fig. 3). Even the highest concentration of 10 μM rolipram did not completely inhibit the TNF-α release, maximum inhibition observed was around 70%. Therefore, IC_{35} values were calculated. The IC_{35} for rolipram was 68.9 ± 15.2 nM. The simultaneous addition of 10 μM R,R-glycopyrrolate reduced the IC_{35} to 1.70 ± 1.18 nM ($p = 0.0151$).

3.4. Combination of R,R-glycopyrrolate with the antihistamine azelastine

The anti-histamine azelastine dose dependently inhibited the TNF-α release. The simultaneous addition of 10 μM R,R-glycopyrrolate did not interact synergistically, additively or even antagonistically on the azelastine effect on the TNF-α release (Fig. 4).

3.5. Synergistic inhibition of TNF-α release by R,R-glycopyrrolate and the glucocorticoid budesonide

The effect of the glucocorticoid budesonide alone or in combination with 10 μM R,R-glycopyrrolate on the LPS induced TNF-α release from human primary monocytes was analyzed. Budesonide dose dependently inhibited the TNF-α

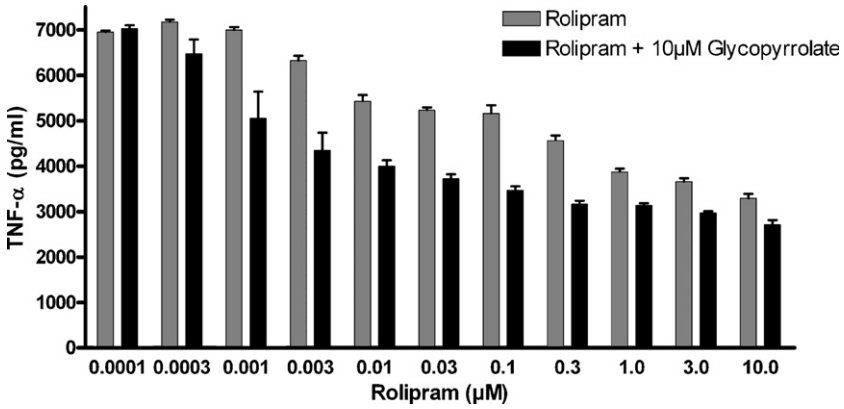


Fig. 3 – Synergistic inhibition of TNF-α release by rolipram and R,R-glycopyrrolate. Human primary monocytes were isolated from whole blood. Stimulation was performed with LPS (100 ng/ml). Different concentrations of rolipram alone or in combination with 10 μM R,R-glycopyrrolate were added 30 min before stimulation. TNF-α protein levels were determined by ELISA after 24 h. TNF protein levels of vehicle treated cells were 7093 pg/ml. Each data point represents mean ± S.E.M. of three independent ELISA measurements.

release (Fig. 5). The IC_{50} for budesonide was 0.55 ± 0.13 nM. The simultaneous addition of 10 μM R,R-glycopyrrolate acted synergistically on the inhibition of the TNF-α release. The addition of R,R-glycopyrrolate reduced the IC_{50} to 0.13 ± 0.03 nM ($p = 0.0251$).

3.6. *Synergistic effect of the triple combination R,R-glycopyrrolate, budesonide and rolipram*

Finally, the simultaneous addition of R,R-glycopyrrolate, budesonide and rolipram was analyzed. Concentrations below the IC_{35} for rolipram and IC_{50} for budesonide were chosen in order to detect possible synergistic effects. The concentration chosen for rolipram was 10 nM and for budesonide 0.1 nM. These concentrations were combined with 10 μM R,R-glycopyrrolate in all possible combinations. Each drug alone hardly affected the LPS induced TNF-α secretion (Fig. 6). Each double combination caused a significant inhibition of TNF-α secretion. The triple combination of rolipram, budesonide and R,R-glycopyrrolate was most effective in the reduction of the TNF-α secretion (Fig. 6).

4. Discussion

Glycopyrrolate is a quaternary ammonium derivative with minimal mucosal absorption and neglegible bioavailability when inhaled. In normal subjects, i.v. glycopyrrolate has been shown to cause bronchodilation [11] while nebulized administration caused a long lasting bronchodilation [7] without the systemic anticholinergic effects of inhaled atropine [12]. We pursued the hypothesis, that the combination of R,R-glycopyrrolate with anti-inflammatory drugs may yield synergistic effects.

The work presented here investigated this hypothesis in the *in vitro* model of LPS induced TNF-α release from human

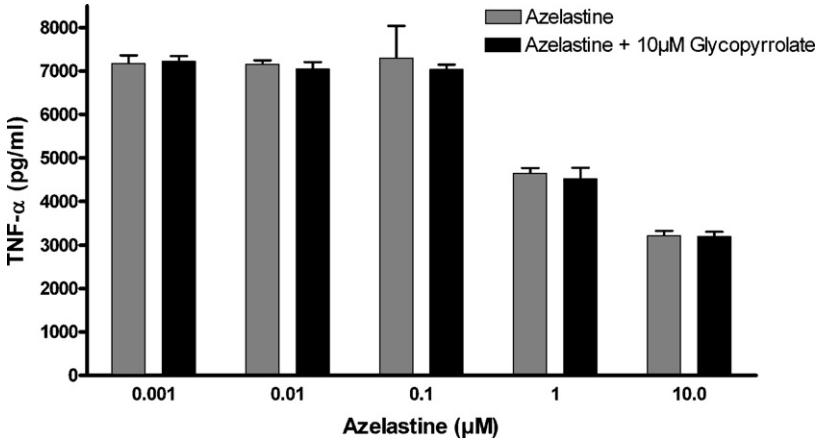


Fig. 4 – Azelastine does not act synergistically with R,R-glycopyrrolate. Human primary monocytes were isolated from whole blood. Stimulation was performed with LPS (100 ng/ml). Different concentrations of azelastine alone or in combination with 10 μM R,R-glycopyrrolate were added 30 min before stimulation. TNF-α protein levels were determined by ELISA after 24 h. TNF protein levels of vehicle treated cells were 7093 pg/ml. Each data point represents mean ± S.E.M. of three independent ELISA measurements.

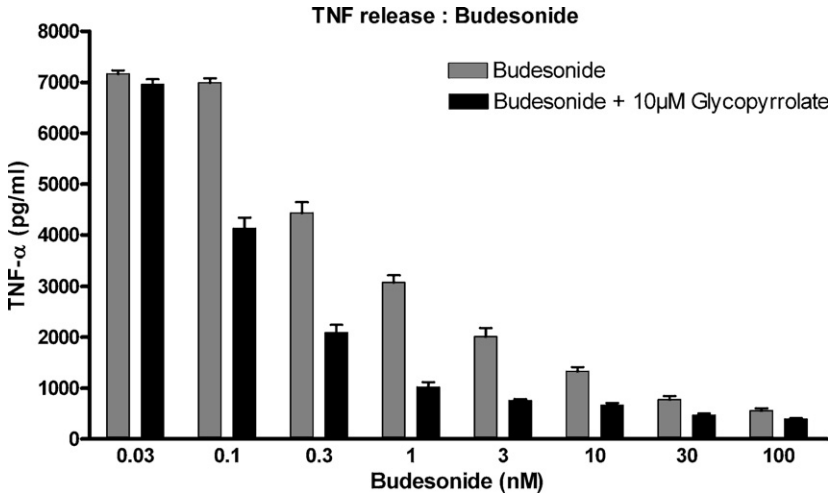
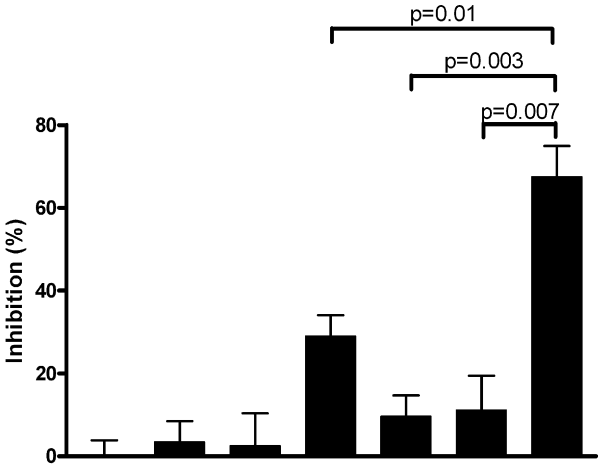


Fig. 5 – Synergistic inhibition of TNF-α release by budesonide and *R,R*-glycopyrrolate. Human primary monocytes were isolated from whole blood. Stimulation was performed with LPS (100 ng/ml). Different concentrations of budesonide alone or in combination with 10 μM *R,R*-glycopyrrolate were added 30 min before stimulation. TNF-α protein levels were determined by ELISA after 24 h. TNF protein levels of vehicle treated cells were 7093 pg/ml. Each data point represents mean ± S.E.M. of three independent ELISA measurements.

monocytes. The release of TNF-α after stimulation with LPS is a valid model system to test compounds for potential anti-inflammatory effects [13]. Furthermore, we could detect the expression of M3, M4, M1 and M2 receptors with and without LPS stimulation.

It has been shown, that *R,R*-glycopyrrolate displays no selectivity in its binding to the M1–M4 receptors [14]. In kinetic studies glycopyrrolate demonstrated compared to ipratropium a similar fast onset of action at the receptors, but a more slow dissociation from them. *R,R*-Glycopyrrolate did not cause any effect in our model system *per se*. However, in a model of induced sputum from COPD patients it was recently shown that muscarinic receptors may be involved in airway inflammation through another mechanism. Acetylcholine induced LTB_4 release in human blood monocytes through an ERK1/2-dependent (extra cellular signal-regulated kinases) pathway [15]. By this mechanism muscarinic antagonism may contribute to reduce neutrophil infiltration and activation in



10 μM R,R-glycopyrrolate	+			+		+	+
10 nM rolipram		+		+	+		+
0.1 nM budesonide			+		+	+	+

Fig. 6 – Synergistic inhibition of TNF-α release by budesonide, rolipram and *R,R*-glycopyrrolate. Human primary monocytes were isolated from whole blood. Stimulation was performed with LPS (100 ng/ml). The table indicates drug and concentration used. TNF-α protein levels were determined by ELISA after 24 h. The TNF level of vehicle treated and LPS stimulated cells were set to 100%. Only the triple combination versus each double combination was statistically analyzed. Each data point represents mean ± S.E.M. of three independent ELISA measurements.

COPD. Furthermore anticholinergics may reduce the mucus formation which is also a major problem in COPD [16].

As the first class of anti-inflammatory compounds, which may act synergistically on the TNF-α release, we chose a PDE4 inhibitor. PDE4 inhibitors are novel anti-inflammatory agents for the treatment of respiratory diseases [17]. The PDE4 inhibitor rolipram alone inhibited LPS induced TNF-α release with an IC_{35} of 68.9 ± 15.2 nM, which is similar to published reports [18]. The incomplete inhibition of TNF-α release is due to the remaining activity of other phosphodiesterases such as PDE3 [18]. The addition of *R*,*R*-glycopyrrolate reduced the IC_{35} about 40-fold to 1.70 ± 1.18 nM. This effect was statistically significant. This is the first description of a synergistic effect of a PDE4 inhibitor with a bronchodilatory agent such as an anticholinergic compound.

The antihistamine azelastine inhibited the TNF-α release only in high concentrations of one micromolar or above. This observation correlates with reports in mast cells [19]. In these cells azelastine inhibits IgE mediated TNF-α production with an IC_{50} of 6 μM [20]. However, *R*,*R*-glycopyrrolate did not cause any synergistic or additive effect in combination with this drug.

The IC_{50} for the inhibition of TNF-α release by the glucocorticoid budesonide was 0.55 ± 0.13 nM. This steroid has been well characterized *in vitro*. The IC_{50} values for budesonide in various *in vitro* models are all in the range of 0.2–2 nM [21]. The management of asthma has evolved over the last 20 years from the treatment of bronchoconstriction with short-acting β_2-agonists, *via* the management of airway inflammation with inhaled corticosteroids, to the current trend for combination therapy with an inhaled corticosteroid and a long-acting β_2-agonist [22]. Superior control of both asthma and chronic obstructive pulmonary disease by combination inhaled corticosteroid/long-acting β_2-agonist therapy has been shown in many clinical studies over the last decade [9]. Therefore, it was tempting to investigate the combination of the bronchodilatory agent *R*,*R*-glycopyrrolate and the inhaled corticosteroid budesonide. Indeed a synergistic effect could be detected. The addition of *R*,*R*-glycopyrrolate reduced the IC_{50} value of budesonide to 0.13 ± 0.03 nM.

Finally the triple combination was analyzed. Rolipram, budesonide and *R*,*R*-glycopyrrolate were added simultaneously to the cells. Since the concentrations chosen were well below the IC_{50} each drug alone hardly affected the LPS induced TNF-α secretion. The triple combination of rolipram, budesonide and *R*,*R*-glycopyrrolate most effectively inhibited TNF-α secretion. This effect was statistically significant when compared to each double combination. To our knowledge this is the first time that such a combination has been analyzed. The observed synergistic effect indicates that such a combination may lead to dramatic dose reduction of the single drugs used.

Whereas the function of acetylcholine as a neurotransmitter is well described increasing evidence has been accumulated showing the presence of acteylcholine in non-neuronal cells as well. It has been shown that choline acetyltransferase, the CHT1 transporter for its substrate choline and its product acetylcholine are present in many non-neuronal cells in the lung and airways as well as in various cells of the immune system (reviewed in ref. [23]). For example, in humans, the synthesizing enzyme, choline acetyltransferase, has been demonstrated in alveolar macrophages [24]. Accordingly, human monocytes used in our experimental setup are probably the source of acetylcholine, whose autocrine or paracrine action can be blocked by *R*,*R*-glycopyrrolate. However, we could not observe an effect of glycopyrrolate by its own. Since muscarinic receptors couple only partially to signalling cascades involved in the induction of proinflammatory mediators [25], antagonizing acetylcholine action is presumably not sufficient to cause an anti-inflammatory effect by its own.

Several modes of inhibition of steroids on acetylcholine action have been described. Corticosterone is known to inhibit all three isoforms of the organic cation transporter, which are responsible for the export of acetylcholine from non-neuronal cells [26]. Treatment of rats with dexamethasone for one week resulted in decreased acetylcholine concentration in the surface epithelium of trachea and intestine, which was accompanied by a reduction in choline acetyltransferase activity [27]. Furthermore treatment with glucocorticoids, as examined in dogs, led to a decrease of both M2 and M3 muscarinic receptor density [28]. These are possible ways by which steroids in addition to their own mode of action may synergise with an anti-cholinergic drug such as glycopyrrolate.

Nothing is known about any effect of PDE4 inhibitors on the acetylcholine pathway. However, the M2 and M4 couple to G-proteins belonging to the Gi/o family [25]. Further on, Gi/o proteins couple to adenylyl cyclase in an inhibitory manner. This makes it possible, that anti-cholinergics similarly to PDE4 inhibitors elevate intracellular cAMP levels leading to a synergistic action of anti-cholinergics and PDE4 inhibitors.

Asthma treatment guidelines advocate the use of long-acting β_2-agonists in addition to inhaled corticosteroids in patients whose asthma is uncontrolled by steroids alone, thereby addressing two processes fundamental to asthma: bronchoconstriction and inflammation. The approach to treat both processes by a combination of drugs is pursued in COPD therapy as well. The results obtained in this study indicate, that the combination of the anticholinergic *R*,*R*-glycopyrrolate as a bronchodilatory agent with an anti-inflammatory drug such as a PDE4 inhibitor or a steroid may work in a similar fashion.

Acknowledgement

The authors thank Brigitte Neupert for her expert technical assistance.

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