Current Research in Pharmacology and Drug Discovery 2 (2021) 100010

Contents lists available at ScienceDirect

Current Research in Pharmacology and Drug Discovery

journal homepage: www.journals.elsevier.com/current-research-in-pharmacology-and-drug-discovery

Beclomethasone dipropionate and sodium cromoglycate protect against airway hyperresponsiveness in a human ex vivo model of cow's milk aspiration

Beatrice Ludovica Ritondo [a], Paola Rogliani [a], Francesco Facciolo [b], Silvia Falco [a], Aurora Vocale [a], Luigino Calzetta [c,*]

[a] Unit of Respiratory Medicine, Department of Experimental Medicine, University of Rome "Tor Vergata", Via Montpellier 1, 00133, Rome, Italy
[b] Thoracic Surgery Unit, Regina Elena National Cancer Institute, Via Elio Chianesi 53, 00144, Rome, Italy
[c] Department of Medicine and Surgery, Respiratory Disease and Lung Function Unit, University of Parma, Via Rasori 10, 43126, Parma, Italy

ARTICLE INFO

Keywords:
Beclomethasone dipropionate
Sodium cromoglycate
Airway hyperresponsiveness
Cow milk aspiration
Children

ABSTRACT

Background: Recurrent cow's milk (CM) aspiration is often associated with gastroesophageal reflux in infants and toddlers and it seems to be implicated in the etiology of different inflammatory lung disorders. This study aimed to investigate ex vivo the impact of CM aspiration on human airways and whether treatment with beclomethasone dipropionate (BDP) or sodium cromoglycate (SCG) may prevent the potential CM-induced airway hyperresponsiveness (AHR).

Methods: Human isolated bronchi were contracted by electrical field stimulation (EFS_{10Hz}) to mimic the contractile tone induced by the parasympathetic activity and challenged with CM, fat/lactose-free CM, or human breast milk (HM). The effect of pre-treatment with beclomethasone dipropionate (BDP) and sodium cromoglycate (SCG) was also investigated on the AHR induced by CM.

Results: After a 60 min-challenge with CM 1:10 v/v and fat/lactose-free CM 1:10 v/v, ASM significantly ($P < 0.05$) increased compared to control ($+67.04 \pm 17.08\%$ and $+77.91 \pm 1.34\%$, respectively), a condition that remained stable for 150 min post-treatment, whereas HM did not alter ASM contractility. BDP 1 μM and 10 μM significantly ($P < 0.05$) reduced the AHR elicited by CM ($-52.49 \pm 10.97\%$ and $-66.98 \pm 7.90\%$, respectively vs. control). At the same manner, SCG 1 μM and 10 μM significantly ($P < 0.05$) inhibited the CM-induced AHR ($-59.03 \pm 9.24\%$ and $-73.52 \pm 7.41\%$, respectively vs. control).

Conclusion: CM induces AHR in human ASM by eliciting an increased parasympathetic contractile response. Preventive treatment with nebulized SCG may be indicated in infants or toddlers fed with CM, rather than with BDP due to a superior safety profile.

1. Introduction

Gastroesophageal reflux (GER) often represents a physiological condition in neonates and infants, characterized by the involuntary retrograde movement of gastric contents into the oesophagus (Rybak et al., 2017). However, the clinical presentation of GER is variable, from innocent regurgitation to severe extra-oesophageal complications such as aspiration pneumonia and further chronic pulmonary disorders including asthma and recurrent bronchopulmonary infections (Iwadate et al., 2001; Colombo and Hallberg, 2000). Unfortunately, although very rarely, GER may lead to sudden infant death (Iwadate et al., 2001). Cow's milk (CM) aspiration is often associated with GER in infants and toddlers and even though occasional aspiration is generally asymptomatic, large amount of aspirated milk seems to be associated with sudden death in childhood (Iwadate et al., 2001).

Evidence indicates that recurrent CM aspiration induces persistent inflammation and airway hyperresponsiveness (AHR) related with abnormalities in the parasympathetic control of airway smooth muscle (ASM), and it has been implicated in the etiology of different inflammatory lung disorders. In a murine animal model, CM aspiration has been

Abbreviations: AHR, airway hyperresponsiveness; ASM, airway smooth muscle; BDP, beclomethasone dipropionate; CM, cow's milk; EFS, electrical field stimulation; GER, gastroesophageal reflux; HM, human breast milk; SCG, sodium cromoglycate.

* Corresponding author.
E-mail address: luigino.calzetta@unipr.it (L. Calzetta).

https://doi.org/10.1016/j.crphar.2020.100010
Received 7 September 2020; Received in revised form 24 November 2020; Accepted 27 November 2020

Table 1
Main characteristics of donors and normal ranges in agreement with GINA (GINA and Global Initiative for Asthma (GINA), 2020) and GOLD recommendations (GOLD and Global Initiative for Chronic Obstructive Lung Disease, 2020).

Variables	Values	Normal range
Gender (male/female)	8/8	/
Age (years)	50.0 ± 3.0	/
Height (cm)	164.8 ± 2.0	/
Weight (Kg)	68.3 ± 3.0	/
Smoking status:		
Current	4	/
Former	5	/
Never	7	/
IgE	55.8 ± 5.7	<100
CM specific IgE	ND	ND
Pack years	24.4 ± 5.6	/
FEV_1 (L)	2.71 ± 0.07	/
FEV_1 (% predicted)	93.1 ± 2.4	>80
FEV_1 reversibility (%)	4.8 ± 1.3	<12%
FVC (L)	3.34 ± 0.09	/
FEV_1/FVC	0.81 ± 0.01	>0.7

CM: cow's milk; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; GINA: Global Initiative for Asthma; GOLD: Global Initiative for Chronic Obstructive Lung Disease; IgE: immunoglobulin E; IU: international units; ND: not detectable.

associated with airway dysfunction, lung eosinophilia, and goblet cell hyperplasia (Janahi et al., 2000). Interestingly, recurrent small-volume CM intratracheal instillation in rabbits caused persistent inflammation and it was associated with enhanced airway reactivity compared to sham controls (Colombo and Hallberg, 2000). Moreover, this inflammatory state was characterized by either neutrophilia or eosinophilia in bronchial lavage specimens (Colombo and Hallberg, 2000). These findings lent support to the hypothesis that CM microaspiration might significantly contribute to airway inflammation, thus predisposing patients to increased airway reactivity in patients with GER (Colombo and Hallberg, 2000).

Although recurrent aspiration of CM has been shown to alter the neural control of bronchi in young rabbits (Cazzola et al., 2011), to date limited data are available concerning the detrimental effect of such condition in human airways. Children often suffer from both GER and asthma, however it is difficult to establish a causal relationship between CM aspiration, GER, and pulmonary sequelae (Orenstein and Orenstein, 1988). Considering the weakness of the current scientific background, the aim of this study was to investigate ex vivo the impact of CM aspiration on human ASM contractility and whether treatment with the inhaled corticosteroid (ICS) beclomethasone dipropionate (BDP) or the cromone sodium cromoglycate (SCG) may prevent the potential CM-induced AHR.

2. Materials and methods

2.1. Tissue collection and preparation

Regions of lungs were taken from uninvolved areas of neoplastic lesions and resected from 16 patients undergoing lobectomy surgery for lung cancer. Tissues were placed in Krebs-Henseleit (KH) buffer solution as previously described (Cazzola et al., 2011) and transported to the Laboratory of Respiratory Clinical Pharmacology at the University of Rome "Tor Vergata" (Italy) from a nearby hospital. None of the patients had been chronically treated with bronchodilators or corticosteroids, and serum immunoglobulin E (IgE) levels were in the normal range (<100 IU/ml), and no CM specific IgE were detected.

Preoperative lung function parameters were normal in all the patients who were not affected by chronic obstructive respiratory disorders. Detailed demographic and metric characteristics of donors are reported in Table 1.

In the laboratory, the airways were cut into rings (subsegmental bronchi: thickness 1–2 mm, diameter 4–6 mm) and transferred into a 10-ml High Tech 8 Channels Manual Compact Organ Bath system (Panlab Harvard Apparatus, Spain) containing KH buffer solution (37 °C) and aerated with O_2/CO_2 (95:5%). Tissues were allowed to equilibrate and the KH buffer solution was constantly changed.

2.2. Milk challenge

Human isolated bronchi were incubated for up to 150 min with different dilutions (v/v with KH buffer solution) of CM (carbohydrate 5.0 g/dl, lactose 4.0 g/dl, fat 3.6 g/dl, protein 3.4 g/dl; "Latte fresco Granarolo Alta Qualità", Granarolo S.p.A, Bologna, Italy), fat/lactose-free CM (carbohydrate 4.9 g/dl, lactose <0.1 g/dl, fat <0.1 g/dl, protein 3.1 g/dl; "Latte Accadì Senza Lattosio Senza Grassi", Granarolo S.p.A, Bologna, Italy), or human breast milk (HM; carbohydrate 7.2 g/dl, lactose 6 g/dl, fat 3.4 g/dl, protein 1.2 g/dl). HM was donated by a 34-year-old woman at the 4th month of lactation.

2.3. Transmural stimulation

Transmural stimulation, also called electrical field stimulation (EFS), was performed by placing tissues between two wire platinum electrodes (20 mm apart, Panlab Harvard Apparatus, Spain), connected to a 3165 multiplexing pulse booster stimulator (Ugo Basile, VA - Italy). Bronchial rings were contracted by EFS_{10Hz} (biphasic pulse with a constant current of 10 V, 0.5 ms, 10s) in order to stimulate the vagal nerve firing (parasympathetic pathway) observed in human in vivo via endogenous cholinergic contractile tone (Cazzola et al., 2011).

2.4. Preparation of drugs

The following drugs were used: BDP (Sigma-Aldrich, Milan, Italy) diluted in distilled dimethyl sulfoxide (DMSO), β-lactoglobulin (Sigma-Aldrich, Milan, Italy) diluted in KH buffer solution, and SCG (Sigma-Aldrich, Milan, Italy) diluted in water. Compounds were stored in small aliquots at −80 °C until their use.

2.5. Contraction measurement

Bronchial rings were connected to isometric force transducers Fort25 (WPI, UK). The signal was amplified by a Powerlab 8/36 and Octal Bridge Amp system (AD instruments, UK), recorded and analyzed via the LabChart 7 interface software (AD instruments, UK). Tissues were mounted on hooks and one was attached with thread to a stationary rod and the other hook tied with thread to an isometric force displacement transducer. Airways were allowed to equilibrate by flushing with fresh KH buffer solution. Passive tension was determined by gentle stretching of tissue (0.5–1.0 g) during equilibration. The isometric change in tension was measured by the transducer and the tissue vitality was assessed by EFS_{10Hz}. These procedures allowed the bronchial rings to be correctly positioned between the hooks. When the passive contractile tone reached the plateau, rings were washed three times with fresh KH buffer solution and allowed to equilibrate for further 45 min.

2.6. Experimental design

2.6.1. Study characteristics

This study was designed as ex vivo, prospective, randomized, negative- and positive-controlled, time-controlled, blinded, parallel-group.

2.6.2. Endpoints

The co-primary endpoints of this study were to investigate the impact of CM aspiration on ASM contractility and whether BDP and SCG may have a role in protecting human airways from the potential AHR induced by CM. Graphical examples of the experimental design and bronchi contractility is shown in Fig. 1.

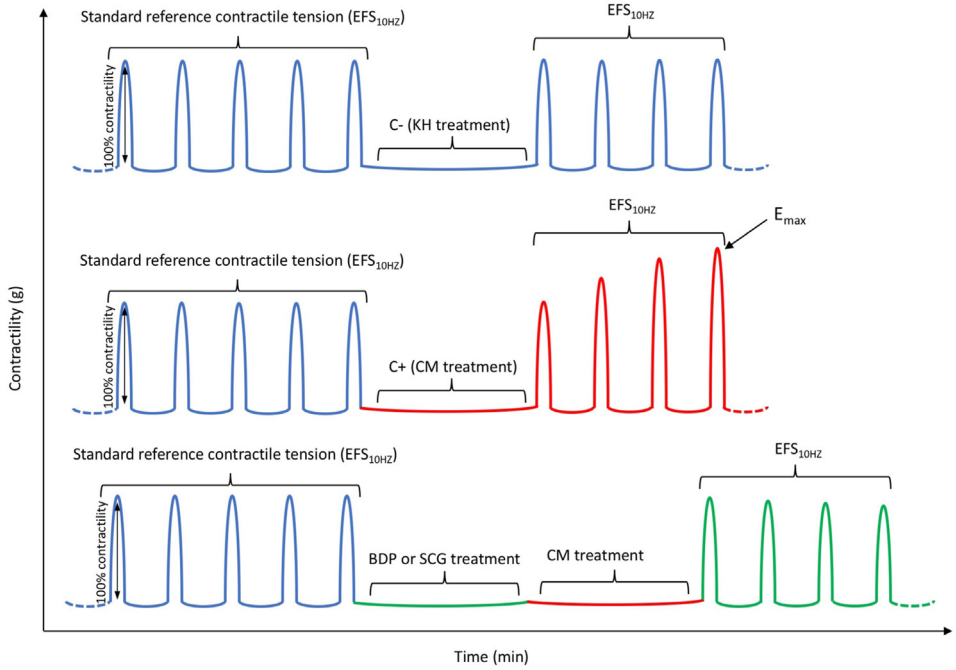

Fig. 1. Graphical examples of the experimental design and bronchi contractility in human isolated airways. BDP: beclomethasone dipropionate; C+: positive control (60-min challenge with CM, dilution 1:10 v/v); C-: negative control (KH buffer solution); CM: cow's milk; EFS: electrical field stimulation; KH: Krebs-Henseleit; SCG: sodium cromoglycate.

2.6.3. Study 1. effect of milk challenge on the contractile tone of human isolated bronchi induced by transmural stimulation

Isolated airways were challenged with different concentrations of CM (dilution 1:1000, 1:100, 1:10 v/v with KH buffer solution) and stimulated by EFS_{10Hz} trains delivered every 15 min for 1 h. After having identified the minimum concentration of CM that elicited significant AHR ($P < 0.05$ vs. negative control, C-), experiments were repeated for 150 min by delivering trains of EFS_{10Hz} each 15 min during the first hour, and then every 30 min. Isolated airways were also challenged with fat/lactose-free CM or HM at the same dilution of that of CM-inducing AHR. Experiments were also performed on bronchial rings incubated with KH buffer solution (C-). In further experiments, isolated airways were challenged with different concentrations of β-lactoglobulin diluted in KH buffer solution (2 μg/ml, 20 μg/ml, 0.2 mg/ml, 2 mg/ml) and stimulated by EFS_{10Hz} trains delivered every 15 min for 1 h. All the solutions were prepared to have the same isotonic osmolarity (290 mOsm).

2.6.4. Cytokines quantification

At the end of experiments, supernatants were collected for the quantification of interleukin (IL)-4, IL-13 and IL-33. The quantification of the compounds was performed by using RayBio® ELISA kits, in agreement with the manufacturer instructions. Briefly, the assays employed specific antibodies for human IL-4, IL-13, and IL-33 coated on 96-well plates. Standards and supernatants were pipetted into the wells and IL-4, IL-13, and IL-33 present in the samples were bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-human IL-4, IL-13, and IL-33 antibodies were added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted into the wells. The wells were again washed, tetramethylbenzidine substrate solution was added to the wells and colour developed in proportion to the amount of IL-4, IL-13, and IL-33 bound. The stop solution changed the colour from blue to yellow, and the intensity of the colour was measured at 450 nm.

2.6.5. Study 2. effect of BDP and SCG on CM hypersensitivity

Isolated airways were pre-incubated 1 h with BDP 0.1 μM–10 μM or SCG 0.1 μM–10 μM for 1 h. After that, bronchial tissue was challenged with CM at dilution inducing AHR and stimulated by trains of EFS_{10Hz} delivered each 15 min for 1 h. Experiments were also performed on positive control (C+), bronchial rings that were challenged with CM and not treated with BDP or SCG.

2.7. Analysis

2.7.1. Pharmacological analysis

The contractile response of isolated bronchial rings was expressed as a percentage of the standard reference contractile tension acquired at the end of equilibration time. The standard bronchial contractile tension was calculated as the mean of 5 consecutive EFS_{10Hz}, 3 min apart from each other. Maximal contractile tension (E_{max}) was identified as the highest contractile force induced by EFS stimulation. For every seven bronchial rings mounted in the isolated organ bath system, one served as a time control (Mercier et al., 2002).

2.7.2. Group size, randomization, blinding, data and statistical analysis

Since no published data are currently available concerning the potential AHR induced by CM on ASM and the possible protective role of BDP and SCG, in the present study it was not possible to calculate the sample size. However, we set $n = 5$ in agreement with the current pharmacological guidelines for preclinical studies, where n refers to independent values and not replicates (available at: https://bpspubs.onlinelibrary.wiley.com/hub/journal/14765381/author-guidelines.html). The total number of bronchial rings necessary to complete the study was $n = 120$, including C-, C+, and time control. Isolated airways were collected from at least $n = 5$ different donors. The number of samples from a subject permitted to test all the treatments in the same experiment, including controls. The impact of CM, fat/lactose-free CM or HM on the ASM contractility was compared with C-, whereas the effect of different concentrations of BDP and SCG against the potential AHR induced by CM was compared with C+.

Values are presented as mean ± SEM of $n = 5$ bronchi from different subjects, the statistical significance was assessed by the Student's *t*-test and analysis of variance (ANOVA), and the level of statistical significance was defined as $P < 0.05$. The data and statistical analysis comply with the

recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Data were collected and managed in order to perform pre-specified statistical analysis; any so called "p-hacking" were avoided in order to prevent that any potential bias could affect the robustness of results, as reported by Head and colleagues (Head et al., 2015).

Each bronchial ring was randomly assigned to a specific treatment by using a computer-generated sequence. All the study procedures were performed under blinded condition, in which both the operator and data analysis were blinded.

All data analysis was performed using computer software GraphPad Prism 5 (GraphPad Prism, La Jolla, CA, USA).

2.8. Ethics approval and consent to participate

Ethical approval (R.S. 37/20, 2020; Independent Ethical Committee, Fondazione PTV Policlinico Tor Vergata) and informed consent were consistent with the National Committee of Bioethics and Committee for Bio-safety, Biotechnology and Life Sciences (available at: http://old.iss.it/binary/eric/cont/Informed_consent.pdf), the recommendations on the collection of biological samples for research purposes (available at: https://search.coe.int/cm/Pages/result_details.aspx?ObjectID=09000016805d84f0), the ethical and legal recommendations concerning the biobank and the research biorepository (available at: https://www.oeci.eu/Documents/OECI_Biobank.pdf), and the Comitato Nazionale per la Biosicurezza, le Biotecnologie e le Scienze per la Vita (Raccolta di campioni biologici a fini di ricerca, consenso informato, 2009; available at:http://bioetica.governo.it/media/3457/p2009-misto-2-raccolta-di-campioni-biologici-a-fini-di-ricerca-consenso-informato-it.pdf).

3. Results

3.1. Baseline characteristics of isolated airways

No significant difference ($P > 0.05$) was detected between the wet weight of the human isolated bronchial rings used in the different treatments arms (overall 89.31 ± 7.61 mg), regardless of the smoking habit of donors. The contractile tension induced by trains of 10 Hz EFS in control bronchi and before treatments was 408.76 ± 66.96 mg. The amount of tissues available to complete this study permitted to use the same tissue for all conditions (C+, C-, different concentrations of treatments) in each experiment.

3.2. Impact of CM and β-lactoglobulin on the ASM contractility

A 60 min-challenge with CM 1:1000 and 1:100 v/v elicited no effect ($P > 0.05$) on human transmural-induced ASM contractility compared to C-. Conversely, treatment with CM 1:10 v/v significantly enhanced ASM contractility (E_{max} vs. C-: +67.04 ± 17.08%, $P < 0.05$), as well as β-lactoglobulin administered at 0.2 mg/ml and 2 mg/ml (overall E_{max} vs. C-: +52.82 ± 13.85%, $P < 0.05$) (Fig. 2A). The transmural-induced AHR induced by CM 1:10 v/v remained stable for 150 min post-treatment ($P < 0.001$ vs. C-; Fig. 2B). Neither CM nor β-lactoglobulin significantly altered the passive contractile tone of human bronchial rings ($P > 0.05$ vs. pre-challenge, data not shown).

3.3. Impact of fat/lactose-free CM and HM on ASM contractility

Fat/lactose-free CM 1:10 v/v induced significant AHR (E_{max} vs. C-: +77.91 ± 1.34%, $P < 0.05$) after 60 min of treatment, and the increased transmural-induced ASM contractility remained stable for 150 min post-treatment ($P < 0.001$ vs. C-) (Fig. 3). The extent and time-course of AHR induced by fat/lactose-free CM 1:10 v/v was not significantly different ($P > 0.05$) that than elicited by CM 1:10. On the contrary, HM 1:10 v/v did not significantly ($P > 0.05$) alter the ASM contractility compared to C- (Fig. 3).

3.4. Protective effect of BDP and SCG on the AHR induced by CM

Both BDP and SCG prevented the AHR elicited by 60 min-challenge with CM 1:10 v/v in a concentration-dependent manner. BDP 1 μM and 10 μM, but not BDP 0.1 μM, induced a significant reduction of AHR elicited by CM, compared with C+ (−52.49 ± 10.97% and −66.98 ± 7.90% respectively, $P < 0.05$). In the same manner, also SCG 1 μM and 10 μM, but not SCG 0.1 μM, significantly inhibited the AHR induced by CM, compared with C+ (−59.03 ± 9.24% and −73.52 ± 7.41% respectively, $P < 0.05$). No difference was detected between BDP and SCG with respect to the prevention of AHR ($P > 0.05$), and both the compounds abolished the AHR mediated by CM when administered at higher concentrations (Fig. 4). BDP and SCG did not significantly alter ($P > 0.05$) the ASM contractility in not-challenged airways (data not shown).

3.5. Cytokine release

The challenge with CM 1:10 v/v induced a significant ($P < 0.05$) release of IL-4 and IL-13 in human isolated bronchial tissue (+117.30 ± 7.00% and +85.04 ± 11.93% vs. C-, respectively). Also β-lactoglobulin 0.2 mg/ml significantly increased the release of IL-4 and IL-13 (+85.04 ± 11.93% and +66.96 ± 8.20% vs. C-, respectively). Neither CM 1:10 v/v nor β-lactoglobulin significantly ($P > 0.05$) modulated the

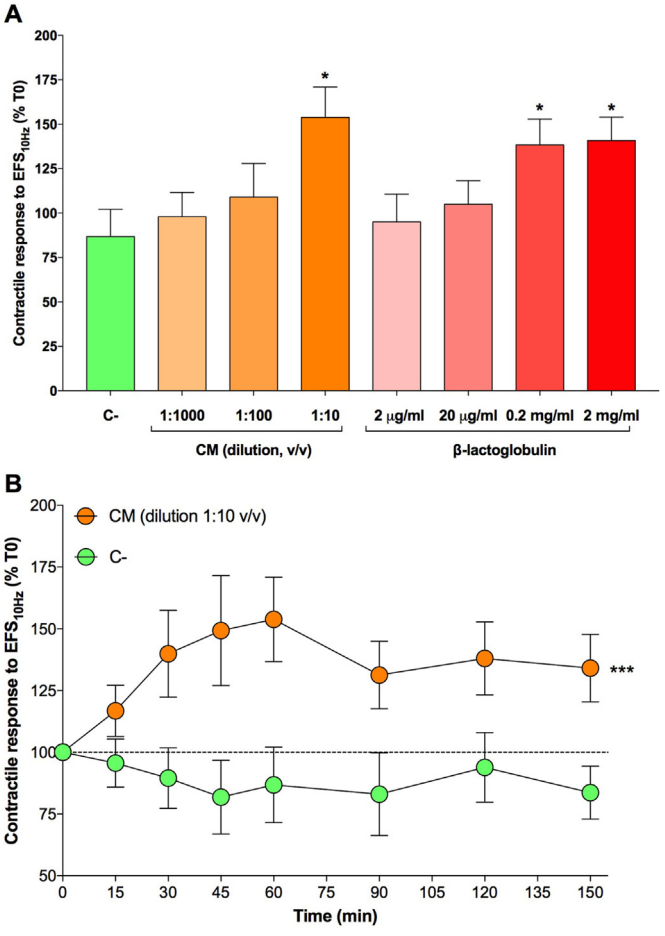

Fig. 2. Concentration-response of 60 min-challenge with CM (A) and effect of CM 1:10 v/v over 150 min of incubation (B) in human isolated bronchi stimulated by EFS_{10HZ}. *$P < 0.05$ (statistical analysis assessed by Student's t-test) and ***$P < 0.001$ (statistical analysis assessed by two-way ANOVA) vs. C-; data represent the mean ± SEM of n = 5 bronchial tissue from different subjects. C-: negative control (KH buffer solution) CM: cow's milk; EFS: electrical field stimulation; KH: Krebs-Henseleit.

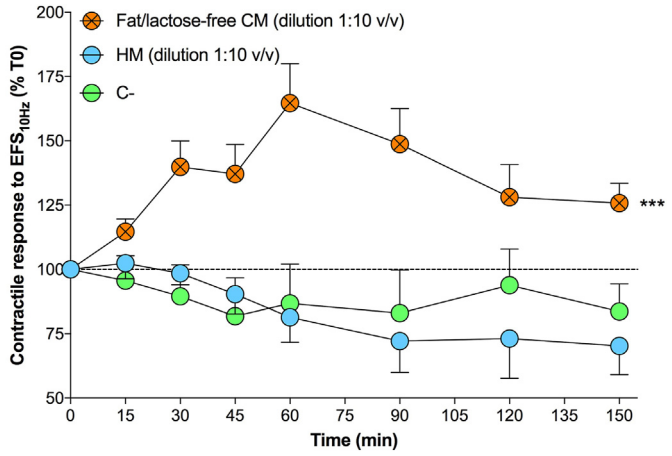

Fig. 3. Impact of fat/lactose-free CM and HM on contractile tone induced by EFS_{10HZ} in human isolated bronchi. ***P < 0.001 (statistical analysis assessed by two-way ANOVA) vs. C-; data represent the mean ± SEM of n = 5 bronchial tissue from different subjects. C-: negative control (KH buffer solution); CM: cow's milk; EFS: electrical field stimulation; HM: human milk; KH: Krebs-Henseleit.

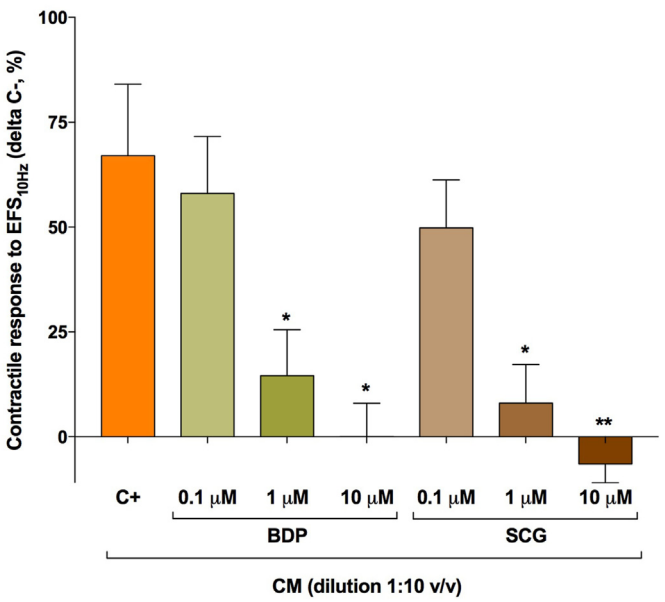

Fig. 4. Protective effect of BDP and SCG against the AHR induced by CM in human isolated bronchi stimulated by EFS_{10HZ}. *P < 0.05 and **P < 0.01 vs. C+ (statistical analysis assessed by Student's t-test); data represent the mean ± SEM of n = 5 bronchial tissue from different subjects. AHR: airway hyperresponsiveness; BDP: beclomethasone dipropionate; C+: positive control (60-min challenge with CM, dilution 1:10 v/v); C-: negative control (KH buffer solution); CM: cow's milk; EFS: electrical field stimulation; KH: Krebs-Henseleit; SCG: sodium cromoglycate.

concentrations of IL-33 vs. C-. Both BDP and SCG significantly (P > 0.05) reduced the increased levels of IL-4 (−65.44 ± 5.29% and −73.27 ± 7.54% vs. CM 1:10 v/v, respectively) and IL-13 (−91.31 ± 13.31% and −83.13 ± 11.06% vs. CM 1:10 v/v, respectively). No effect of BDP and SCG was detected on IL-13. Detailed information on the modulation of the levels of IL-4, IL-13, and IL-33 is shown in Fig. 5.

4. Discussion

The results of this research indicate that the acute challenge with CM elicits AHR in human isolated bronchi, by increasing ≃70% the parasympathetic contractile response induced by EFS_{10Hz} when diluted 1:10 with KH buffer solution. Conversely, at higher dilutions CM did not alter the human ASM contractility. Interestingly, it has been demonstrated via semi-quantitative analysis that the positivity of milk in the airways of sudden death cases in infants ranged from 0.3% to 86.4%, with a mean value of 18.0% (Iwadate et al., 2001). Such an evidence endorses the translational applicability of our ex vivo model of milk aspiration.

Considering the consolidated translational impact resulting from experiments performed in isolated bronchial tissue (Calzetta et al., 2015, 2017, 2019; Cazzola et al., 2015; Rogliani et al., 2015, 2019), in this study we provide for the first time a suitable ex vivo model of CM aspiration that may be applied to understand the mechanisms leading to AHR and the potential pharmacological interventions to prevent such a detrimental condition.

Since high concentrations of some disaccharides such as glucose may induce AHR in human bronchial tissue (Cazzola et al., 2012; Rogliani et al., 2016), we tested whether the concentration of lactose in the CM could lead to AHR by challenging the isolated airways with fat/lactose-free CM. This experimental approach permitted also to assess the potential role of the fat component of CM in the modulation of ASM contractility. In this respect, we have demonstrated that neither the fat nor lactose constituents of CM were responsible of AHR, because fat/lactose-free CM induced the same increase in ASM contractile response as that elicited by CM.

Indeed, this evidence supports the hypothesis that probably the protein component of CM may be the cause of AHR of human ASM. Interestingly, since HM did not induce AHR in human isolated bronchi, we have hypothesized that some specific protein(s) of CM, and not of HM, could be responsible for the increased contractility in human airways. Thus, considering the differences in the protein constitution between HM and CM, probably β-lactoglobulin, the predominant protein in bovine whey that is completely absent from human whey, could be the main candidate leading to the AHR in human ASM. In this study we have proven such a hypothesis, as β-lactoglobulin induced AHR in human isolated bronchi not only when it was diluted in KH buffer solution at the same concentration of normal CM composition (2 mg/ml) (Franzoi et al., 2019), but also when the concentration of β-lactoglobulin was consistent with that of CM diluted 1:10 (0.2 mg/ml) used in our experiments. In any case, we cannot exclude that also caseins of CM may have a role in increasing the contractility of human bronchi. In fact, while α-S1 caseins represent the largest casein fraction in CM, β-caseins by far dominate in HM. However, β-lactoglobulin remains one of the major CM allergens (Cortes-Perez et al., 2009; Adel-Patient et al., 2001). In this respect, we have found that not only CM but also β-lactoglobulin increase the release of IL-4 and IL-13 from human bronchial tissue, with no effect on IL-33. This phenomenon may explain why CM and β-lactoglobulin induce AHR to EFS_{10Hz} but do not alter the baseline passive contractile tone of human isolated airways. In fact, it has been demonstrated that IL-4 and IL-13 do not increase ASM contractility per se, but they elicit AHR to different stimuli via the activation of type II IL-4R (IL-4Rα/IL-13Rα1) expressed on ASM (Gour and Wills-Karp, 2015; Manson et al., 2020; Brightling and Bradding, 2005). Moreover, the data of this study allow ruling out the possibility that CM and β-lactoglobulin may induce AHR due to the activation of an IL-33/IL-13 axis, as conversely previously reported (Kaur et al., 2015).

Certainly, the proposed model of CM aspiration allows excluding that the AHR is mediated by an allergic response since bronchial tissues were not previously sensitized to CM, and none of the donors were positive for CM specific IgE. Thus, the evidence provided by our results suggests that β-lactoglobulin is the main protein responsible for the EFS-induced AHR in human airways.

In this study, we have also investigated whether treatment with either BDP or SCG might prevent the CM-induced AHR. BDP and SCG prevented the increased ASM contractility elicited by CM in a concentration-response manner, although both the agents resulted effective only when administered at concentrations ≥1 μM. Interestingly, the level of

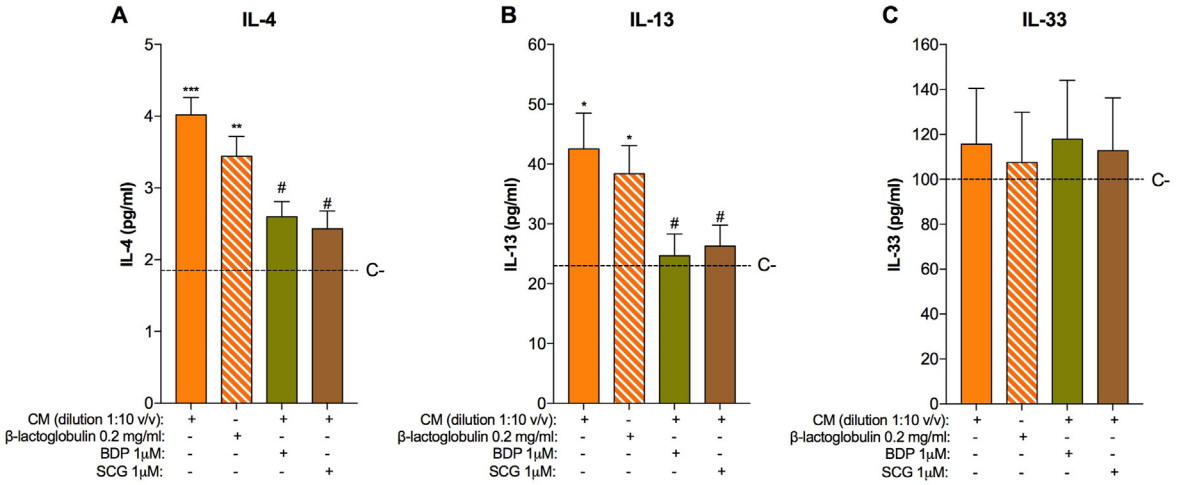

Fig. 5. Impact of CM, β-lactoglobulin, BDP, and SCG on the release of IL-4 (A), IL-13 (B), and IL-33 (C) in human isolated bronchi. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. C-; #P < 0.05 vs. CM (statistical analysis assessed by Student's t-test); data represent the mean ± SEM of n = 5 bronchial tissue from different subjects. BDP: beclomethasone dipropionate; C-: negative control (KH buffer solution); CM: cow's milk; IL: interleukin; KH: Krebs-Henseleit; SCG: sodium cromoglycate.

efficacy detected for both BDP and SCG in this research was greater than that previously reported in other studies performed in isolated ASM (Rogliani et al., 2020; Cazzola et al., 2016; Calzetta et al., 2018; Stewart and Fennessy, 1983; Lin et al., 2011), probably due to differences in the nature of the stimuli applied to the isolated airways and/or species-specific responses to the investigated treatments.

The rapid protective impact of BDP detected in this study is consistent with that resulting from previous experiments and is mainly mediated by non-genomic effect (Cazzola et al., 2016). Such an observation gives reason to believe that the CM-induced AHR may be related with an impairment of the $G_{s\alpha}$ subunit of G protein activity and or an alteration of the cyclic adenosine monophosphate-dependent protein kinase A axis. Moreover, the protective effect of SCG against AHR suggests that in human isolated airways challenged with CM the increased contractility to EFS may be also related with the degranulation of mast cells. In fact, it has been demonstrated that mast cells are located in close proximity to parasympathetic neurons (Powers et al., 2001; Weigand et al., 2009) and it is well known that SCG is a mast cell–stabilizing agent characterized by a very rapid onset of action and that prevents the release of mast cell mediators such as histamine, cysteinyl leukotrienes, prostaglandin (PG) D_2, PGE_2, and the prostacyclin metabolite 6-keto $PGF_{1\alpha}$ (Säfholm et al., 2019; Anderson et al., 2010). This may suggest an interaction between mast cells and ASM, a proved critical condition for the development of asthma (Brightling and Bradding, 2005).

Unexpectedly, in the present investigation BDP and SCG resulted to be equally effective at inhibiting CM-induced AHR. To date it is extensively recognized that cromones are characterized by a superior safety profile than ICSs, particularly in children (Kannisto et al., 2002). As a matter of fact, the overall beneficial therapeutic action of ICSs may be limited by the risk of adverse events (AEs) such as gastroesophageal reflux disease that, in turn, may facilitate CM aspiration, although the currently available inhaled formulations have less systemic AEs compared to oral ones (Schäcke et al., 2002). In fact, in children ICSs are reported to increase the risk of diabetes, osteopenia, and growth impairment (Hossny et al., 2016). On the other hand, SCG may be safely administered to children via nebulization (Food and Drug Administration (FDA), 2003).

This study has certainly some limitations, mainly related with the intrinsic characteristics of the ex vivo model. Specifically, since the bronchial rings were obtained from random areas of the lungs, it is not possible to predict whether these areas would have been those exposed to CM aspiration. Furthermore, the model used in this study cannot take into consideration the role of airway mucus in the exposure to CM.

5. Conclusion

The evidence raised from our research along with those currently available in the literature suggests that preventive treatment with SCG may be indicated especially in infants weaned off breast-feeding and switched to CM or toddlers fed with CM experiencing GER. In this light, further specific studies are needed to confirm the translational evidence concerning the effect of CM aspiration in human airways and the protective effect of inhaled therapy in children with GER.

CRediT authorship contribution statement

Beatrice Ludovica Ritondo: Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Paola Rogliani:** Conceptualization, Data curation, Writing - original draft, Visualization, Project administration, Funding acquisition. **Francesco Facciolo:** Methodology, Investigation, Writing - original draft, Writing - review & editing. **Silvia Falco:** Writing - original draft, Writing - review & editing. **Aurora Vocale:** Writing - original draft, Writing - review & editing. **Luigino Calzetta:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paola Rogliani reports grants and personal fees from Boehringer Ingelheim, grants and personal fees from Novartis, personal fees from AstraZeneca, grants and personal fees from Chiesi Farmaceutici, grants and personal fees from Almirall, grants from Zambon, personal fees from Biofutura, personal fees from GlaxoSmithKline, personal fees from Menarini, personal fees from Mundipharma.

Luigino Calzetta reports grants and personal fees from Boehringer Ingelheim, grants and personal fees from Novartis, nonfinancial support from AstraZeneca, grants from Chiesi Farmaceutici, grants from Almirall, personal fees from ABC Farmaceutici, personal fees from Edmond

Pharma, grants and personal fees from Zambon, personal fees from Verona Pharma, personal fees from Ockham Biotech.

Acknowledgments

We would like to thank dr. Sveva Remedia and her family, whose substantial contribution made it possible to carry out this study.

References

Adel-Patient, K., Creminon, C., Boquet, D., Wal, J.-M., Chatel, J.-M., 2001. Genetic immunisation with bovine β-lactoglobulin cDNA induces a preventive and persistent inhibition of specific anti-BLG IgE response in mice. Int. Arch. Allergy Immunol. 126 (1), 59–67.

Anderson, S.D., Brannan, J.D., Perry, C.P., Caillaud, C., Seale, J.P., 2010. Sodium cromoglycate alone and in combination with montelukast on the airway response to mannitol in asthmatic subjects. J. Asthma 47 (4), 429–433.

Brightling, C.E., Bradding, P., 2005. The re-emergence of the mast cell as a pivotal cell in asthma pathogenesis. Curr. Allergy Asthma Rep. 5 (2), 130–135.

Calzetta, L., Matera, M.G., Cazzola, M., 2015. Pharmacological interaction between LABAs and LAMAs in the airways: optimizing synergy. Eur. J. Pharmacol. 761, 168–173.

Calzetta, L., Rogliani, P., Facciolo, F., Rendina, E., Cazzola, M., Matera, M.G., 2017. Pharmacological characterization of the interaction between umeclidinium and vilanterol in human bronchi. Eur. J. Pharmacol. 812, 147–154.

Calzetta, L., Matera, M.G., Facciolo, F., Cazzola, M., Rogliani, P., 2018. Beclomethasone dipropionate and formoterol fumarate synergistically interact in hyperresponsive medium bronchi and small airways. Respir. Res. 19 (1), 65.

Calzetta, L., Pistocchini, E., Ritondo, B.L., Roncada, P., Cito, G., Britti, D., Matera, M.G., 2019. Isolated airways in equine respiratory pharmacology: they never lie. Pulm. Pharmacol. Therapeut. 59, 101849.

Cazzola, M., Calzetta, L., Page, C.P., Rinaldi, B., Capuano, A., Matera, M.G., 2011. Protein prenylation contributes to the effects of LPS on EFS-induced responses in human isolated bronchi. Am. J. Respir. Cell Mol. Biol. 45 (4), 704–710.

Cazzola, M., Calzetta, L., Rogliani, P., Lauro, D., Novelli, L., Page, C.P., Kanabar, V., Matera, M.G., 2012. High glucose enhances responsiveness of human airways smooth muscle via the Rho/ROCK pathway. Am. J. Respir. Cell Mol. Biol. 47 (4), 509–516.

Cazzola, M., Calzetta, L., Ora, J., Puxeddu, E., Rogliani, P., Matera, M.G., 2015. Searching for the synergistic effect between aclidinium and formoterol: from bench to bedside. Respir. Med. 109 (10), 1305–1311.

Cazzola, M., Calzetta, L., Rogliani, P., Puxeddu, E., Facciolo, F., Matera, M.G., 2016. Interaction between corticosteroids and muscarinic antagonists in human airways. Pulm. Pharmacol. Therapeut. 36, 1–9.

Colombo, J.L., Hallberg, T.K., 2000. Airway reactivity following repeated milk aspiration in rabbits. Pediatr. Pulmonol. 29 (2), 113–119.

Cortes-Perez, N.G., Ah-Leung, S., Bermúdez-Humarán, L.G., Corthier, G., Langella, P., Wal, J.-M., Adel-Patient, K., 2009. Allergy therapy by intranasal administration with recombinant Lactococcus lactis producing bovine β-lactoglobulin. Int. Arch. Allergy Immunol. 150 (1), 25–31.

Curtis, M.J., Alexander, S., Cirino, G., Docherty, J.R., George, C.H., Giembycz, M.A., Hoyer, D., Insel, P.A., Izzo, A.A., Ji, Y., 2018. Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. Br. J. Pharmacol. 175 (7), 987–993.

Food and Drug Administration (Fda), 2003. Intal® Nebulizer Solution (cromolyn sodium inhalation solution, USP). https://www.accessdata.fda.gov/drugsatfda_docs/label/2004/18596slr030_intal_lbl.pdf. Last accessed August 31, 2020.

Franzoi, M., Niero, G., Visentin, G., Penasa, M., Cassandro, M., De Marchi, M., 2019. Variation of detailed protein composition of cow milk predicted from a large database of mid-infrared spectra. Animals 9 (4), 176.

Gina, Global Initiative for Asthma (Gina), 2020. Global strategy for asthma management and prevention. https://ginasthma.org/wp-content/uploads/2020/04/GINA-2020-full-report_-final-_wms.pdf. Last accessed September 4, 2020.

Gold, Global Initiative for Chronic Obstructive Lung Disease, 2020. Global strategy for diagnosis, management, and prevention of COPD – 2020 Report. https://goldcopd.org/wp-content/uploads/2019/12/GOLD-2020-FINAL-ver1.2-03Dec19_WMV.pdf. Last accessed September 4, 2020.

Gour, N., Wills-Karp, M., 2015. IL-4 and IL-13 signaling in allergic airway disease. Cytokine 75 (1), 68–78.

Head, M.L., Holman, L., Lanfear, R., Kahn, A.T., Jennions, M.D., 2015. The extent and consequences of p-hacking in science. PLoS Biol. 13 (3), e1002106.

Hossny, E., Rosario, N., Lee, B.W., Singh, M., El-Ghoneimy, D., Soh, J.Y., Le Souef, P., 2016. The use of inhaled corticosteroids in pediatric asthma: update. World Allergy Organ. J. 9, 26.

Iwadate, K., Doy, M., Ito, Y., 2001. Screening of milk aspiration in 105 infant death cases by immunostaining with anti-human alpha-lactalbumin antibody. Forensic Sci. Int. 122 (2–3), 95–100.

Janahi, I.A., Elidemir, O., Shardonofsky, F.R., Abu-Hassan, M.N., Fan, L.L., Larsen, G.L., Blackburn, M.R., Colasurdo, G.N., 2000. Recurrent milk aspiration produces changes in airway mechanics, lung eosinophilia, and goblet cell hyperplasia in a murine model. Pediatr. Res. 48 (6), 776–781.

Kannisto, S., Voutilainen, R., Remes, K., Korppi, M., 2002. Efficacy and safety of inhaled steroid and cromone treatment in school-age children: a randomized pragmatic pilot study. Pediatr. Allergy Immunol. 13 (1), 24–30.

Kaur, D., Gomez, E., Doe, C., Berair, R., Woodman, L., Saunders, R., Hollins, F., Rose, F., Amrani, Y., May, R., 2015. IL-33 drives airway hyper-responsiveness through IL-13-mediated mast cell: airway smooth muscle crosstalk. Allergy 70 (5), 556–567.

Lin, Y.-Y., Chou, Y.-L., Chu, Y.-H., Wu, C.-C., Wang, J.-Y., Wang, H.-W., 2011. Effects of cromolyn sodium on isolated rat's trachea. Allergy Rhinol. 2 (2), e46–e50.

Manson, M.L., Säfholm, J., James, A., Johnsson, A.-K., Bergman, P., Al-Ameri, M., Orre, A.-C., Mårdh, C.K., Dahlén, S.-E., Adner, M., 2020. IL-13 and IL-4, but not IL-5 nor IL-17A, induce hyperresponsiveness in isolated human small airways. J. Allergy Clin. Immunol. 145 (3), 808–817.e2.

Mercier, F.J., Naline, E., Bardou, M., Georges, O., Denjean, A., Benhamou, D., Advenier, C., 2002. Relaxation of proximal and distal isolated human bronchi by halothane, isoflurane and desflurane. Eur. Respir. J. 20 (2), 286–292.

Orenstein, S.R., Orenstein, D.M., 1988. Gastroesophageal reflux and respiratory disease in children. J. Pediatr. 112 (6), 847–858.

Powers, M.J., Peterson, B.A., Hardwick, J.C., 2001. Regulation of parasympathetic neurons by mast cells and histamine in the Guinea pig heart. Auton. Neurosci. : Basic Clin. 87 (1), 37–45.

Rogliani, P., Calzetta, L., Ora, J., Lipsi, R., Segreti, A., Matera, M.G., Cazzola, M., 2015. Pharmacological assessment of the onset of action of aclidinium and glycopyrronium versus tiotropium in COPD patients and human isolated bronchi. Eur. J. Pharmacol. 761, 383–390.

Rogliani, P., Calzetta, L., Capuani, B., Facciolo, F., Cazzola, M., Lauro, D., Matera, M.G., 2016. Glucagon-like peptide 1 receptor: a novel pharmacological target for treating human bronchial hyperresponsiveness. Am. J. Respir. Cell Mol. Biol. 55 (6), 804–814.

Rogliani, P., Matera, M.G., Calzetta, L., Hanania, N.A., Page, C., Rossi, I., Andreadi, A., Galli, A., Coppola, A., Cazzola, M., Lauro, D., 2019. Long-term observational study on the impact of GLP-1R agonists on lung function in diabetic patients. Respir. Med. 154, 86–92.

Rogliani, P., Matera, M.G., Facciolo, F., Page, C., Cazzola, M., Calzetta, L., 2020. Beclomethasone dipropionate, formoterol fumarate and glycopyrronium bromide: synergy of triple combination therapy on human airway smooth muscle ex vivo. Br. J. Pharmacol. 177 (5), 1150–1163.

Rybak, A., Pesce, M., Thapar, N., Borrelli, O., 2017. Gastro-esophageal reflux in children. Int. J. Mol. Sci. 18 (8), 1671.

Säfholm, J., Manson, M.L., Bood, J., Al-Ameri, M., Orre, A.-C., Raud, J., Dahlén, S.-E., Adner, M., 2019. Mannitol triggers mast cell–dependent contractions of human small bronchi and prostacyclin bronchoprotection. J. Allergy Clin. Immunol. 144 (4), 984–992.

Schäcke, H., Döcke, W.D., Asadullah, K., 2002. Mechanisms involved in the side effects of glucocorticoids. Pharmacol. Ther. 96 (1), 23–43.

Stewart, A.G., Fennessy, M.R., 1983. Dual modes OF action for disodium cromoglycate IN inhibition OF antigen-induced contractions OF Guinea-pig isolated airways smooth muscle. Clin. Exp. Pharmacol. Physiol. 10 (5), 595–603.

Weigand, L.A., Myers, A.C., Meeker, S., Undem, B.J., 2009. Mast cell-cholinergic nerve interaction in mouse airways. J. Physiol. 587 (Pt 13), 3355–3362.