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Effects of Inhalation of STIM-Orai Antagonist SKF 96365 on Ovalbumin-Induced Airway Remodeling in Guinea Pigs

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Abstract

Airway remodeling (AR) consists of wall thickening and hyperreactivity. STIM (stromal interaction molecule) and Orai protein pathways mediate extracellular Ca^{2+} signals involved in AR. This study aims to define the effects on AR of the STIM-Orai antagonist SKF 96365 given by inhalation in three increasing doses in ovalbumin-induced AR. In the control group, the antiasthmatic budesonide and salbutamol were given in the same model. The airway structure was evaluated by histological and immunohistochemistry and reactivity by specific airway resistance, contraction strength of isolated airway smooth muscles, and mucociliary clearance expressed by ciliary beating frequency. The immuno-biochemical markers of chronic inflammation were evaluated by BioPlex and ELISA assays. The AR was mediated by inflammatory cytokines and growth factors. The findings show significant anti-remodeling effects of SKF 96365, which were associated with a decrease in airway hyperreactivity. The anti-remodeling effect of SKF 96365 was mediated via the suppression of IL-4, IL-5, and IL-13 synthesis, and IL-12–INF-γ–TGF-β pathway. The budesonide-related AR suppression had to do with a decrease in proinflammatory cytokines and an increase in the anti-inflammatory IL-10, with negligible influence on growth factors synthesis and mucous glands activity.

Keywords

Airway hyperactivity · Airway remodeling · Biomarkers · Cytokines · Inflammation · SKF 96365 · STIM-Orai pathway

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1 Introduction

Airway remodeling (AR) refers to structural changes resulting from repeated injury and repair processes. The AR in asthma consists of epithelial alterations and metaplasia, increased deposition of extracellular matrix components under the bronchial subepithelial basement membrane (Boulet 2018), airway smooth muscle (ASM) hypertrophy and hyperplasia (Munakata 2006), gland enlargement, and neovascularization (Bergeron et al. 2010). Immunologic and molecular mechanisms that drive the progression of asthma to AR are still incompletely understood. Studies of allergen-induced AR in transgenic mice suggest roles for cytokines, chemokines, and growth factors, such as transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), Th_2 cytokines—interleukin-5 (IL-5), IL-9, and IL-13, and epithelial-derived nuclear factor kappa B (NF-κB)-regulated chemokines (Doherty and Broide 2007). The release of TGF-β by injured epithelium results in the synthesis of extracellular matrix proteins by fibroblasts and myofibroblasts (Hostettler et al. 2008) and secretion of cytokines, chemokines, and growth factors by modulating autocrine proliferative responses of ASM, all of which driving the AR (Spinelli et al. 2012; Shore 2002). Some other cytokines affecting airway inflammation or structural cells such as tumor necrosis factor-alpha (TNF-α), IL-4, fibroblast growth factor (FGF), or epidermal growth factor (EGF) also have this potential (Busse et al. 2005; Kuperman et al. 2005).

Growth factors acting via tyrosine kinase receptors, inflammatory mediators, like histamine, acting via G protein-coupled receptors, and cytokines acting via cell surface glycoprotein receptors increase the cytoplasmic content of calcium ions (Ca^{2+}). A rise in Ca^{2+}, besides contraction and proliferation of ASM, mediates mucus production, ciliary beat frequency, or cytokine/chemokine synthesis in structural and inflammatory cells. A major conduit for Ca^{2+} influx in non-excitable cells is through plasma membrane store-operated channels (SOC) (Samanta and Parekh 2016) following the emptying of Ca^{2+} stores in the endoplasmic reticulum. In immune cells, calcium release-activated channels (CRAC), containing stromal interaction molecule (STIM) and Orai proteins in the endoplasmic reticulum, activate de novo synthesis and secretion of pro-inflammatory leukotrienes and increase expressions of c-Fos, nuclear factor of activated T-cells (NFAT), and various cytokines which orchestrate the subsequent inflammatory responses (Sutovska et al. 2016; Hogan et al. 2010; Vig et al. 2008). SOC-mediated Ca^{2+} entry also is involved in TGF-β-facilitated ASM cell proliferation (Gao et al. 2013) and IL-13/TNF-α induced airway hyperresponsiveness (Spinelli and Trebak 2016; Jia et al. 2013). The ASM remodeling in asthma is associated with changes in the expression of ion channels and pumps (Mahn et al. 2010; Perez-Zoghbi et al. 2009). The ASM cells from asthmatic mice display an increased expression of STIM-Orai proteins (Spinelli et al. 2012).

Targeting CRAC channels seems a promising approach to managing chronic airway diseases. The combined modulation of airway epithelial, ASM, and inflammatory cell activities by CRAC channel inhibitors could effectively dampen airway hyperreactivity, inflammatory, and remodeling components. We investigated this hypothesis in the present study in an experimental model of ovalbumin-induced airway remodeling in the guinea pig.

2 Methods

2.1 Animals

A total of 70 adult guinea pigs, strain TRIK, weighing 150–300 g were used in the study. The animals were obtained from the Department of Experimental Pharmacology of the Slovak Academy of Sciences, in Dobra Voda, Slovakia, and underwent at least one-week adaptation period in the animal house, having commercial chow and water ad libitum. Additionally, they were adapted to the laboratory environment for

several days before experiments, with the ambient temperature of 21–24 °C and relative humidity of 55 ± 10%. There were 7 randomly selected groups consisting of 10 animals each. The airway inflammation was induced in six groups, with the remaining group serving as a healthy ovalbumin-negative (OVA-) control. All the remaining animals were sensitized by repetitive administration of the OVA allergen for 28 days. From the 14th day of sensitization on, the treatment by salmeterol, budesonide, and SKF 96365 (1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy]ethyl-1*H*-imidazole hydrochloride) was started in five experimental groups. The sixth group, OVA28, was exposed only to isotonic saline (5 min inhalations) and served as a sensitized negative control group. SKF 96365 groups were exposed for 5 min to different doses/concentrations of the compound by the inhalation route: SKF2.5 – 2.5 μM, SKF5.0 – 5.0 μM, and SKF10 – 10 μM. The tested SKF 96365 compound and two control drugs, salmeterol (SAL – 0.17 mM, 5 min inhalation) and budesonide (BUD – 1 mM, 5 min inhalation), were applied once daily for 14 days. The influence of SKF 96365 on airway defense mechanisms was evaluated in all three concentration groups receiving it. The immunohistochemistry was performed on specimens taken from the group of animals treated with the highest SKF 96365 concentration of 10 μM having the most pronounced effects.

2.2 Reagents

Chicken ovalbumin, histamine, salmeterol, and budesonide were purchased from Sigma-Aldrich (Bratislava, Slovakia), SKF 96365 from Tocris Bioscience (Bristol, Great Britain), aluminum hydroxide and Tween 80 from Centralchem (Bratislava, Slovakia), methacholine and saline (sodium chloride solution) from ApliChem (Darmstadt, Germany), RPMI 1640 medium from Gibco/Thermo Fisher Scientific (Waltham, MA).

SKF 96365 and salmeterol were dissolved in water for injection, budesonide in 1% Tween 80, and all other chemicals in saline. Solutions of all compounds were aerosolized by a PARI jet nebulizer (output 5 L/s, particles mass median diameter 1.2 μm; Paul Ritzau, Pari-Werk GmbH, Starnberg, Germany) and delivered to the head-out plethysmograph composed of separate nasal and body chambers (HSE type 855; Hugo Sachs Electronic, March, Germany) where the animals were placed. The experimental procedures were accomplished 24 h after the last drug application.

2.3 Ovalbumin Sensitization and Challenge Protocol

For the 28-day long sensitization, OVA adsorbed on aluminum hydroxide in saline, 5 mg OVA, and 100 mg $Al(OH)_3$ was administered parenterally as follows: Day 1 intraperitoneally and subcutaneously, Day 4 intraperitoneally, and Day 12 subcutaneously. Afterward, it was nebulized and the mist was introduced into the head chamber of a plethysmograph at Days 9, 15, 18, 20, 24, and 27. The OVA(−) group of animals (healthy negative control) was challenged for 28 days with isotonic saline only.

2.4 Morphology and Immunohistochemistry

Airway Reactivity In Vivo Specific airway resistance (sRaw) was used to evaluate the airway reactivity in vivo, mostly expressing the contractility of ASM cells. Conscious guinea pigs were individually placed in the head-out body plethysmograph. sRaw was calculated according to Pennock et al.'s (1979) method based on the airflow difference between the two chambers of the body plethysmograph. Changes in sRaw were recorded for 1 min after 30-s long histamine (10^{-5} M) and methacholine (10^{-5} M) inhalation. There was a 1 min interval between inhalations of bronchoconstrictor, with fresh air blowing into the head chamber.

Ciliary Beat Frequency (CBF) In Vitro A specimen of the ciliated epithelium was brush-taken from the trachea for an in vitro investigation. The

temperature of the nutritive RPMI 1640 medium for cilia was maintained at 37–38 °C using a PeCon Temp Controller 2000–2 (PeCon GmbH; Erbach, Germany). Specimens were put onto glass slides and evaluated under an inverted phase-contrast microscope (Zeiss Axio Vert. A1; Carl Zeiss AG; Jena, Germany). Impaired ciliated cells were discarded. Sequential 10-s video files were recorded at 1 min intervals for 15 min at a frame rate from 256 to 512 frames/s using a digital high-speed video camera (Basler A504kc; Basler AG; Ahrensburg, Germany). The CBF was evaluated using Ciliary Analysis software (LabVIEW™) to generate a ciliary region of interest (ROI) (Hargas et al. 2011).

Histological and Immunohistochemical (IHC) Analysis Histomorphological and IHC staining was performed on formalin-fixed paraffin-embedded tissue samples collected from guinea pigs' left lungs, serially cut at 4 μm thickness. Gömöri's staining was used for reticulin fibers, anti-alpha smooth muscle actin (SMA), and anti-mucin 5 AC (MUC5AC) antibodies (Abcam; Cambridge, UK) for immunohistochemistry. To achieve a better adherence of tissue sections for immunoreactions, we used silanized slides (DAKO; Glostrup, Denmark), which were baked for 2 h in an oven at 59 °C. Immunohistochemical staining was performed by the autostainer BenchMark ULTRA (Roche; Rotkreuz, Switzerland) with mouse monoclonal antibodies MUC5AC (MRQ 19 clone, the incubation time of 16 min) and SMA (1A4 clone, the incubation time of 28 min) using Kit ultraView DAB (Ventana Medical Systems Inc.; Munich, Germany). All sections were counterstained with hematoxylin (DAKO, Glostrup, Denmark). For negative controls, the primary antibody was omitted. Slides were viewed under the Olympus BX41 microscope (Olympus; Tokyo, Japan). The image capture and the measurement of the thickness of bronchi walls (μm) in 10–15 similar size random bronchi in each section were performed using Quick Photo Micro software v2.2 (Olympus, Tokyo, Japan). The degree of MUC5AC positivity was semi-quantitatively determined by two independent observers under a dual-head microscope and grading system as previously described (Sutovska et al. 2015). Each specimen processed was evaluated as negative (degree 0 and 1) or positive (degree 2 and 3).

2.5 Biochemistry

Cytokines Animals were killed by transversal interruption of the cervical spinal cord and respiratory tract organs were removed. Bronchoalveolar lavage (BALF) was obtained by application of 10 mL/kg of 0.9% saline at 37 °C BBC Breaking News @BBCBreaking· Mar 4 Prince Philip has had "successful procedure" for pre-existing heart condition and will stay in hospital for number of days, Buckingham Palace says into a ligated left lung. The supernatant was acquired by centrifugation at 1500 rpm for 2 min. The Bio-Plex® 200 System and Bio-Plex Pro™ Human Cytokine Th1/Th2 Assay (Bio-Rad, Hercules, CA) were used to assess the content of cytokines and chemokines involved with the allergic inflammation of airways, such as IL-4, IL-5, IL-10, IL-12, IL-13, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon-gamma (INF-γ). The assay is based on the sandwich ELISA design using magnetic beads. The measured analyte is bound between the capture and detection antibodies. The capture antibody-coupled beads were first incubated with antigen standards, samples, or controls, followed by the incubation with biotinylated detection antibodies and the reporter streptavidin-phycoerythrin (S-P) conjugate. Then, the beads were passed through the Bio-Plex 200 suspension array reader, equipped with two lasers of 532 nm and 635 nm excitations to measure the fluorescence of beads and bound S-P. A high-speed digital processor managed the data output, and the Bio-Plex Manager™ v6.0 software presented the concentration results in pg/mL.

Enzyme-Linked Immunosorbent Assay (ELISA) and Specific Reagents Supernatants from BALF and lung tissue homogenates were used for the quantification of growth factors. Lung tissue homogenates were prepared by sonication with a power output of 700 W (Stuart homogenizer in

SHM/STAND; Ecomed, Bratislava, Slovakia) in 1 mL of lysis solution (Tissue Extraction Reagent I Invitrogen™, ThermoFisher Scientific, Waltham, MA) containing a protease inhibitor cocktail (Sigma Aldrich Chemicals, St. Louis, MO). Samples were centrifuged at 10,000 rpm for 5 min at 4 °C (MICRO 220R Centrifuge, Hettich GmbH, Tuttlingen, Germany). BALF and homogenate supernatants were collected into sterile tubes and frozen at −80 °C for further analysis.

The level of TGF-β was determined in the supernatant from lung homogenates using an ELISA kit for transforming growth factor beta-1 (USCN Life Science Inc., Houston, TX) and EGF in the BALF supernatant using a kit for guinea pig total epidermal growth factor (MyBioSource, San Diego, CA) according to the manufacturer's instructions. The absorbance was measured spectrophotometrically at a wavelength of 450 nm and output data were assessed by SkanIt Software for Varioskan® Flash v2.4.5 (ThermoFisher Scientific, Waltham, MA). The detection levels were 5.7 pg /mL for TGF-β and 0.1 ng/mL for EGF.

2.6 Statistical Elaboration

Data on cytokines, growth factors, and sRaw were expressed as means ±SE. Data on the thickness of the SMA/collagen III layer were expressed as medians of representative 12–15 bronchial walls assessed in each guinea pig. CBFs were expressed as a median value (Hz) for each ROI, followed by the calculation of the arithmetic mean in each microscopic preparation. Student's *t*-test or one-way ANOVA with *post-hoc* Bonferroni test was selected as appropriate to test for statistically significant intergroup differences. Fisher's exact test was selected to evaluate the findings in the immunohistochemical analysis of MUC5AC. A p-value <0.05 defined significant differences. The analysis was performed using a commercial statistical package of GraphPad Prism software v6.01 (San Diego, CA).

3 Results

3.1 Effects of SKF 96365 on Airway Defense Mechanisms

Specific Airway Resistance (sRaw) Consistent with the histomorphological part of the study that clearly showed an increase in the ASM mass in the OVA28 group, there were significant differences in the basal, histamine-induced, and methacholine-induced sRaw when compared to OVA(−) group (Fig. 1). In vivo contractility of airways, whether in basal conditions or induced by either bronchoconstrictor, was significantly reduced in all experimental, with SKF 96365, and positive control, with salmeterol, groups of guinea pigs. SKF 96365 is a nonspecific SOC inhibitor causing effective and relatively selective CRAC channel inhibition when it is used in a concentration from 2 μM (Singh et al. 2010) to 12 μM (Spinelli and Trebak 2016; Chung et al. 1994), and salmeterol is a well-known classic asthma reliever. The CRAC channel blocker showed a dose-dependent suppression of mediator-induced airway hyperreactivity. Further, 14-day-long daily inhalation of 10 μM SKF 96365 exceeded the impact of the long-acting β-agonist salmeterol.

Ciliary Beating Frequency (CBF) We assessed the CBF as a key determinant of inflammation-induced AR in the ovalbumin-sensitized animals and the influence of CRAC channel inhibition on mucociliary clearance. The AR was associated with a negligible decrease in CBF. However, long-term treatment by SKF 96365 in the lowest tested concentration of 2.5 μM significantly inhibited the motion of cilia, as opposed to the control drugs budesonide and salmeterol or SKF 96365 in higher doses, all of which did not further suppress the CBF (Fig. 2).

Morphological Features of Airways Immunohistology was used to evaluate lung tissue specimens for the inflammation-induced structural changes in the ovalbumin-sensitized animals and the subsequent effects of long-term treatment

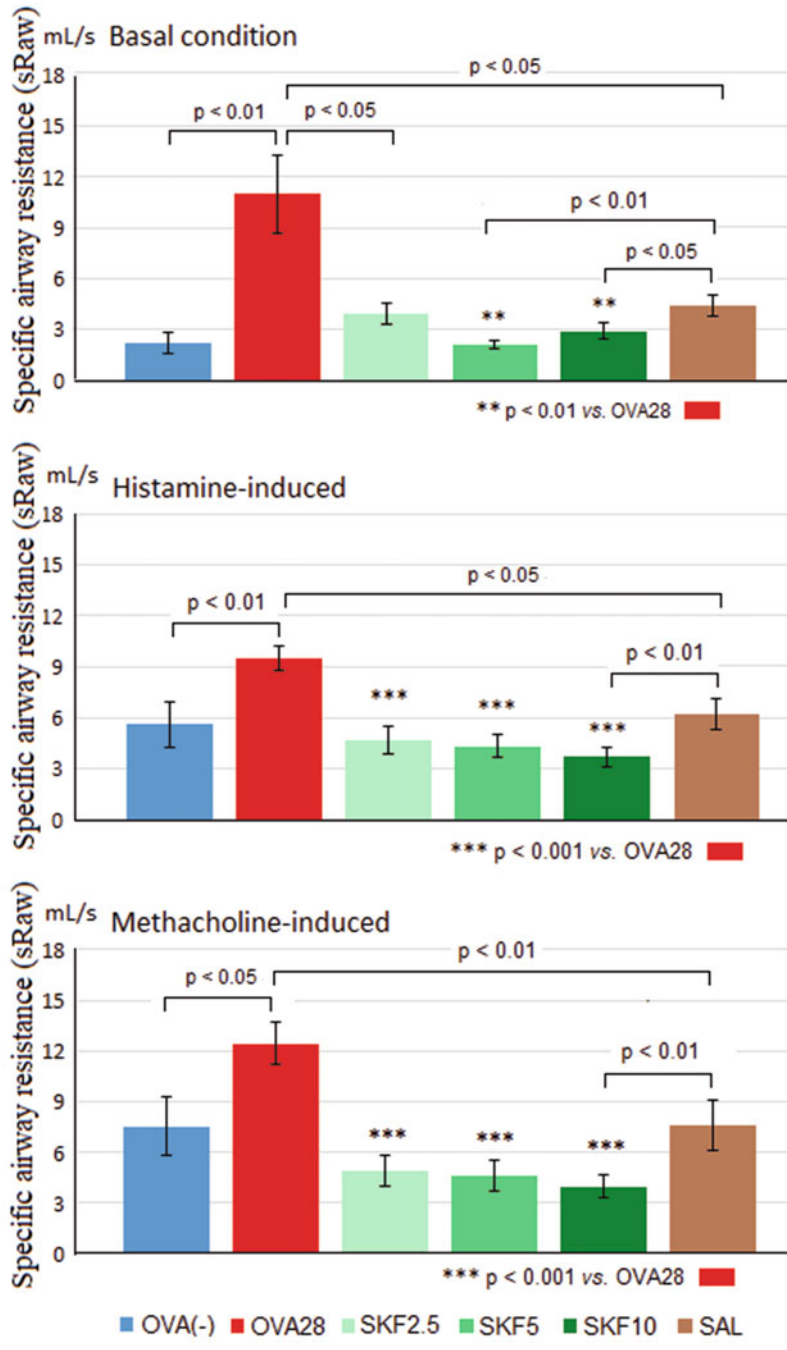

Fig. 1 Effects of inhalation of SKF 96365 in increasing concentrations on airway hyperreactivity assessed from the contractile response on the background of the ovalbumin (OVA)-sensitized guinea pigs. Top panel—basal condition—OVA sensitization and inhalations of isotonic saline only; middle panel—histamine inhalation in OVA-sensitized animals; bottom panel—methacholine inhalation in OVA-sensitized animals. The contractile response was expressed as the specific airway resistance (sRaw). OVA(−), unsensitized control; OVA28, sensitized negative control group; SKF2.5, 5, and 10 sensitized and treated with inhalation of SKF 96365 in the successively doubled concentrations of 2.5, 5, and 10 μM; and SAL, sensitized positive control group treated with inhalation of beta-2 agonist salmeterol (one-way ANOVA and post hoc Bonferroni test)

by the highest concentration of SKF 96365 as well as the classical corticosteroid anti-asthmatic budesonide. For comparison, specimens from saline-exposed animals were used (OVA-). Specimens from the sensitized negative controls (OVA28) showed the remodeling features such as subepithelial fibrosis, goblet cell hyperplasia, and increased ASM mass (Fig. 3). These changes were partially reverted in specimens from both SKF 96365 and budesonide-treated animals.

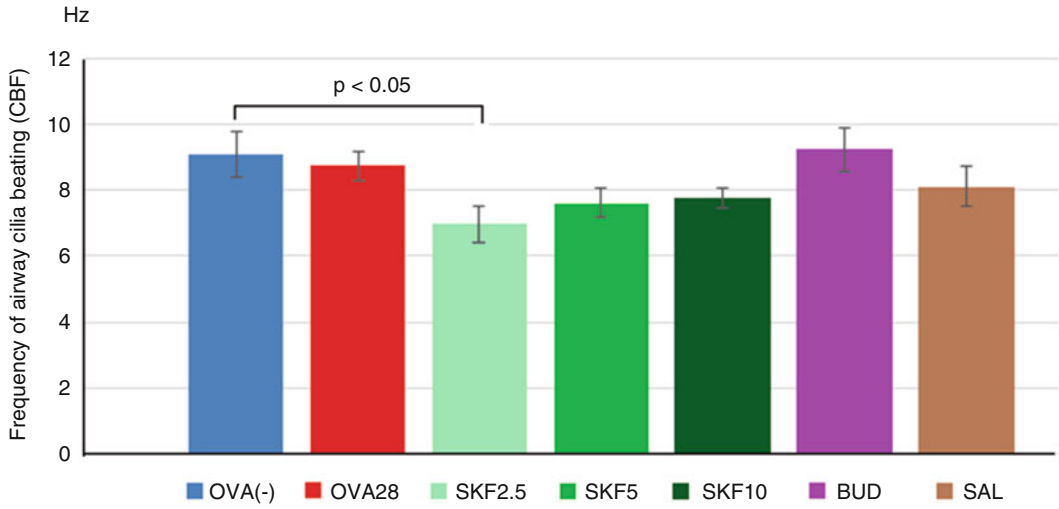

Fig. 2 Cilia beating frequency (CBF) in the investigated groups of guinea pigs: OVA(−), unsensitized control group; OVA28, ovalbumin-sensitized negative control group treated with isotonic saline; SKF2.5, 5, and 10, sensitized and treated with inhalation of SKF 96365 in the successively doubled concentrations of 2.5, 5, and 10 μM; and BUD and SAL, sensitized positive control groups treated with inhalations of budesonide and salmeterol, respectively (one-way ANOVA and post hoc Bonferroni test)

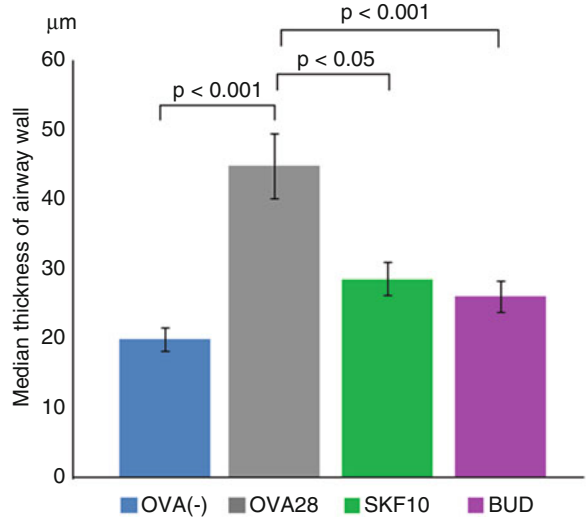

Fig. 3 Thickness of smooth muscle actin (SMA) in airway walls visualized with monoclonal SMA (1A4 clone) antibody and chromogen (3,3′-diaminobenzidine). OVA (−), unsensitized guinea pigs; OVA28, ovalbumin-sensitized negative control group treated with isotonic saline; SKF10, sensitized and treated with inhalations of 10 μM SKF 96365; and BUD, sensitized positive control group treated with budesonide (see Methods for details) (one-way ANOVA and post hoc Bonferroni test)

Specimens from the OVA(−) animals showed no signs of remodeling. Likewise, immunohistochemistry for SMA (Fig. 4) and MUC5AC showed significant expression enhancements in the sensitized negative controls (OVA28). Only was the SKF 96365 capable of reducing these AR

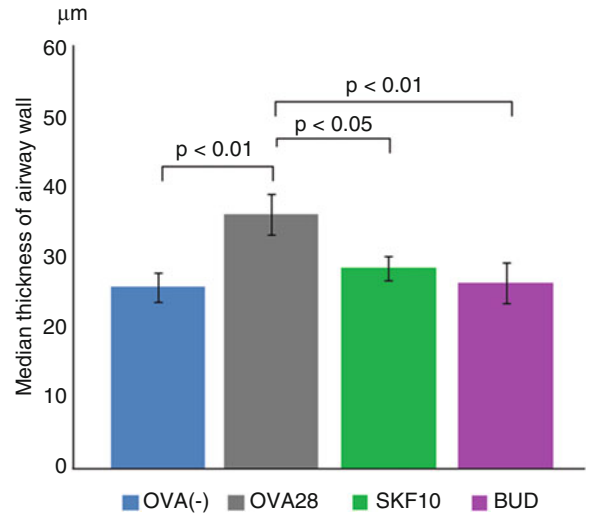

Fig. 4 Thickness of reticulin fibers (collagen III) in airway walls visualized with Gömöri's staining. OVA(−), unsensitized guinea pigs; OVA28, ovalbumin-sensitized negative control group treated with isotonic saline; SKF10, sensitized and treated with inhalation of 10 µM SKF 96365; and BUD, sensitized positive control group treated with budesonide (one-way ANOVA and post hoc Bonferroni test)

Table 1 Semi-quantitative analysis of anti-mucin 5 AC (MUC5AC) antibody positivity. The number of negative (infiltration score 0 and 1) and positive (infiltration score 2 and 3) samples in ovalbumin-unsensitized – OVA(−) guinea pigs, ovalbumin-sensitized negative controls treated with isotonic saline (OVA28), and ovalbumin-sensitized guinea pigs treated with inhalation of SKF 96365 in a concentration of 10 µM (SKF10), and ovalbumin-sensitized positive controls treated with inhalation of budesonide (BUD)

	Negative samples	Positive samples
OVA(−)	4	4
OVA28	0	10*
SKF10	5	3**
BUD	3	6

*p < 0.05 versus OVA(−); **p < 0.01 versus OVA28 (Fisher's exact test)

features, including goblet cell hyperplasia and overproduction of mucin. Budesonide effectively suppressed ASM hyperplasia and prevented fibrotic changes but failed to influence the MUC5AC enhancement (Table 1).

3.2 Cytokines and Growth Factors

Cytokine Content To gain insight into the mechanisms of AR, we quantitatively evaluated the content of key cytokines and growth factors in the lung tissue. IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, TNF-α, GM-CSF, and INF-γ were examined in control OVA-sensitized untreated and treated with SKF 96365 and anti-inflammatory budesonide. IL-4, IL-5, IL-13, and IL-10 were significantly elevated in BALF collected from sensitized negative controls (OVA28) when compared to healthy unsensitized animals. SKF 96365 and budesonide significantly reduced the content of cytokines characteristic for chronic allergic inflammation and AR, such as IL-4, IL-5, and IL-13. Further, SKF 96365 enhanced the synthesis of the anti-inflammatory IL-12 and INF-γ. Unlike the SKF 96365, budesonide failed to affect the synthesis of IL-12 and INF-γ but induced a release of the anti-inflammatory IL-10. This effect, however, was significant only when compared to that in OVA(−) but not sensitized negative controls (OVA28) (Fig. 5).

Epidermal Growth Factor (EGF) and Transforming Growth Factor-Beta (TGF-β) The lung tissue EGF and TGF-β were significantly elevated after 28-day long sensitization (Fig. 6). Only SKF 96365 treatment was capable of reducing the synthesis and release of TGF-β,

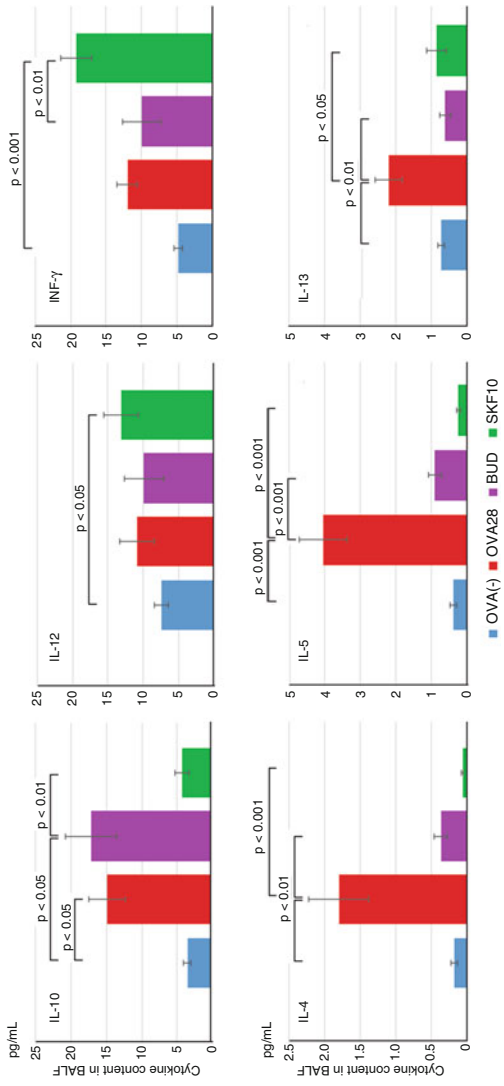

Fig. 5 Cytokine content in BALF. IL, interleukins, INF-γ, interferon-gamma; OVA (−), unsensitized guinea pigs; OVA28, ovalbumin-sensitized negative control group treated with isotonic saline; SKF10, sensitized and treated with inhalation of 10 μM SKF 96365; and BUD, sensitized positive control group treated with budesonide (one-way ANOVA and post hoc Bonferroni test)

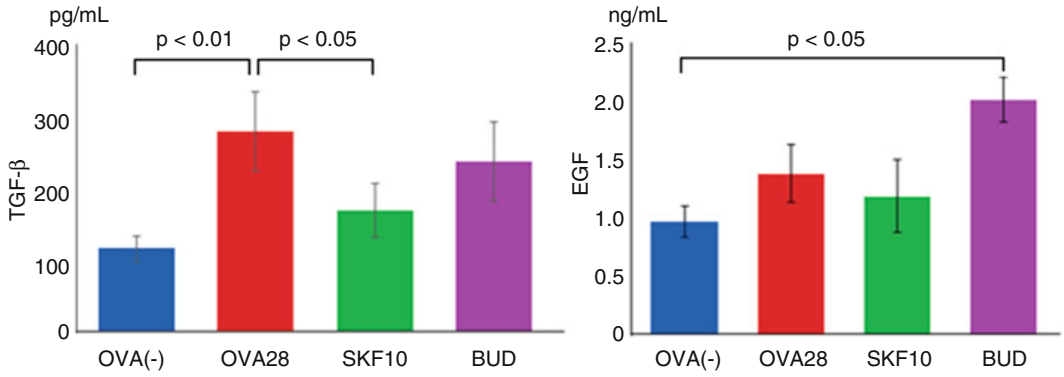

Fig. 6 Growth factors engaged in airway remodeling: transforming growth factor-beta (TGF-ß) and epidermal growth factor (EGF), evaluated in the supernatant of lung tissue homogenate. OVA(−), unsensitized guinea pigs; OVA28, ovalbumin-sensitized negative control group treated with isotonic saline; SKF10, sensitized and treated with inhalation of 10 μM SKF 96365; and BUD, sensitized positive control group treated with budesonide (one-way ANOVA and post hoc Bonferroni test)

which corresponded to an increase in IL-12 and INF-γ (BioPlex analysis), confirming the activation of the IL-12–INF-γ–TGF-β anti-remodeling pathway. However, SKF 96365 failed to significantly affect the EGF content that remained elevated. Budesonide failed to reduce either TGF-β or EGF. Since TGF-β is believed to be causally associated with goblet cells hyperplasia and mucus hyperproduction in asthma, these results are in line with the histomorphological findings outlined above.

4 Discussion

This study investigated whether blocking the CRAC channel activity could retard the airway remodeling (AR) induced on the background of chronic allergic inflammation induced by ovalbumin (OVA) sensitization in the guinea pig. The guinea pig model of allergic inflammation closely resembles the asthmatic process in the human airways. Allergen sensitization and repeated aerosolized allergen challenges are the accepted way of inducing AR, which is routinely assessed by histological examination of paraffin-embedded specimens of the airway tissue (McGovern and Mazzone 2014). Although the challenges are recommended for 12 weeks, the modified 28-day long model of chronic inflammation we used in the present study showed key AR features such as mucus hypersecretion evidenced by the enhanced MUC5AC protein expression, subepithelial fibrosis evidenced by an increase in the mass of reticulin fibers in Gömöri's staining, and ASM hyperplasia evidenced by the enhanced expression of SMA in bronchial walls. Further, we found a close association of histomorphological features of AR with the impaired airway defense mechanisms and immuno-biochemical changes, pointing to fully developed allergic inflammation, a major sign of which was a significant increase in in vivo basal and bronchoconstrictor-induced hyperreactivity-sensitized animals compared to healthy controls challenged with saline only. The CBF, a key predictor of mucociliary clearance, decreased. The AR in our model corresponded to the increased content of markers of remodeling such as the volume of elastic fibers and actin in the airway and lung tissue exposed to cumulative doses of OVA twice a week for 4 weeks (Pigati et al. 2015) or and to allergic rhinitis induced by repeated OVA intraperitoneal administration followed by intranasal challenges within 3 weeks (Chen et al. 2017) in the guinea pigs. These changes also correspond well to the epithelial airway dysfunction typical of AR in

chronic asthma reported in humans where subepithelial fibrosis and mucus hypersecretion commonly result in impaired mucociliary clearance (Burgess et al. 2016; Sedaghat et al. 2016; Borish 2002; Durrani et al. 2011; Al-Muhsen et al. 2011).

In the present study, the content of IL-4, IL-5, IL-10, and IL-13 increased in BALF after 28-day long OVA sensitization in healthy guinea pigs. The IL-13, particularly, is one of the most crucial mediators of allergic inflammation. There is evidence that blocking the IL-13 in the airways alone is sufficient to prevent airway inflammation, hyperreactivity, ciliated cell loss, and mucus overproduction induced by allergen challenges in animal models of asthma (Erle and Sheppard 2014; Ford et al. 2001). IL-13-induced stimulation of the airway epithelium activates hypersecretory MUC5AC-expressing mucus cells. IL-4 appears to have effects akin to those of IL-13, as both share the common receptor on club cell, type II IL-4R whose signaling plays a role in allergen-induced mucus hyperproduction (Kuperman et al. 2005). ASM cells in asthmatic airways proliferate in response to IL-4, IL-13, and IL-5 which is a key activator of eosinophils (Halwani et al. 2010; Doherty and Broide 2007). Contrarily, the synthesis of IL-10, a prototype anti-inflammatory cytokine, decreases in asthmatic airways (Jahromi et al. 2014; Ogawa et al. 2008). Unlike the clinical condition, where the development of asthma is often associated with the immune-related predisposition, in the present experimental study we used healthy guinea pigs and repeatedly exposed them to the OVA allergen mimic allergic airway inflammation and AR. The premise was that OVA would induce chronic airway inflammatory and remodeling changes, without much immune involvement. In fact, the stimulation of protective mechanisms driven by IL-10 and IL-12 in OVA28 animals, appearing with AR, was statistically insignificant when compared to the OVA(−) group. On the other hand, 10 μM concentration of SKF 96365 not only decreased the content of the asthma-related proinflammatory cytokines production IL-4, IL-5, and IL-13 but also significantly countered the increase in the anti-inflammatory IL-12–IFN-γ pathway. The Th1 cytokines IL-12 and IFN-γ form a natural counterbalance to Th2 cytokines, driving protective cell-mediated immunity (Biedermann et al. 2004). IFN-γ secreted by Th1 cells activates macrophages and dendritic cells to produce IL-12 which, in turn, decreases the antigen-induced bronchial hyperresponsiveness, eosinophilia, and mucus goblet cell hyperplasia induced by Th2 cells (Barnes 2008; Caramori et al. 2008).

Airway structural changes are variably influenced by Th2 cytokines and growth factors. TGF-β, produced in large quantities by immune cells, fibroblasts, and epithelial cells, regulates the proliferation of immune cells and their recruitment into tissues (Bush 2019; Pakyari et al. 2013). Prochazkova et al. (2012) have shown that IL-12 is a cytokine having the ability to skew the ongoing TGF-β-dependent differentiation into Th1-like direction. IL-12 and IFN-γ production in asthmatics is often on the low side. The IL-12–INF-γ axis is considered a key downstream pathway in Th2-dependent asthma (Kim et al. 2010). Noteworthy, IL-12 activity may be countered by IL-4 action (Wong et al. 2001).

In the present study, AR features got retracted during the long-term administration of SKF 96365 in the concentration needed to selectively inhibit CRAC channels. The corticosteroid budesonide, tested in parallel for comparison, significantly inhibited ASM proliferation and increase in reticulin fiber mass but was unable to reduce mucus hypersecretion by the airway epithelium. This finding is consistent with literature data that steroids have limited effects on airway mucus hypersecretion (Shen et al. 2012). SKF 96365 exhibited similar or greater suppression of airway hyperreactivity than the classic bronchodilator salmeterol and, except for the lowest concentration tested, bronchodilatory effects were not accompanied by an adverse influence of airway cilia. Increases in the anti-inflammatory IL-12 and INF-γ caused by SKF 96365, a CRAC channel blocker, points to the possible mechanism of its anti-remodeling effect. Jia et al. (2013) have shown that mainly IL-13 enhances cytoplasmic puncta formation of ectopically

expressed fluorescently tagged STIM1 and increased store-operated Ca^{2+} entry (SOCE) in ASM cells, suggesting that proinflammatory cytokines might contribute via CRAC channels to airway hyperresponsiveness. In line with this suggestion, Jairaman et al. (2016) have provided evidence that bronchial epithelial cells sense some allergens through the activation of cell surface protease-activated receptor type 2, which leads to the opening of store-operated CRAC channels. These channels are thus posed to have a central role in allergen signaling in the airway epithelium.

Corticosteroid drugs are essential anti-inflammatory drugs in the treatment of asthma. In a guinea pig asthma model of Pigati et al. (2015), dexamethasone inhibited the secretion of the pro-inflammatory Th2 cytokines IL-4, IL-5, IL-13, and TNF-α, as well as INF-γ. In this study, we used budesonide as a positive control drug and noticed the effects on cytokines in BALF akin to those exerted by dexamethasone above quoted. Moreover, budesonide tended to increase the content of IL-10, which could have a mitigating influence on AR.

The present findings also show that TGF-β and EGF increased in BALF from the OVA28 group of animals, which corresponded to histomorphological alterations. It is generally accepted that TGF-β plays a regulatory role in AR. The evidence to support the contribution of TGF-β to AR in asthma is derived from several murine and human studies (Miller et al. 2006; Flood-Page et al. 2003). TGF-β induces the apoptosis of airway epithelial cells and increases goblet cell proliferation, which suggests its role in mucus hyperproduction. TGF-β also increases fibroblast proliferation with their differentiation into myofibroblasts and the synthesis of extracellular matrix protein in subepithelial fibrosis facilitating ASM contractility and airway narrowing (Ojiaku et al. 2017; Makinde et al. 2007). Further, agonists of airway bronchoconstriction, like methacholine, enhance the release of TGF-β and other mediators from ASM cells, which augment airway narrowing and remodeling (Oenema et al. 2013; Grainge et al. 2011). Thus, TGF-β represents a bonding link for airway remodeling and hyperreactivity in asthma. The finding of the present study is that SKF 96365, inhibiting the STIM-Orai pathway, but not budesonide, significantly decreased TGF-β content in BALF. This result is in line with the study of Gao et al. (2013) where TGF-β promoted the rat ASM cell proliferation increasing STIM-Orai1 and SOCE activities, which was partially reduced by SKF-96365. While corticosteroid therapy reduces airway inflammation in asthma, the role of glucocorticoids concerning TGF-β synthesis is contentious. Some murine studies show that dexamethasone, budesonide, and fluticasone inhibit TGF-α (Takami et al. 2012) but fail to affect TGF-β (Halwani et al. 2011), while others show the anti-remodeling effect of corticosteroids mediated through a decrease in TGF-β expression (Doherty and Broide 2007; Miller et al. 2006; McMillan et al. 2005). No effects on TGF-β or inhibition of collagen deposition and mucus hyperproduction in human asthma have yet been reported (Chakir et al. 2003).

The EGF upregulates MUC5AC gene transcription by acting as a ligand for the epithelial growth factor receptor (EGFR) (Chen et al. 2016). Histamine and TNF-α act in airways by enhancing the EGFR-induced goblet cell hyperplasia (Hirota et al. 2012; Nadel 2001). The EGFR pathway ultimately activates the nuclear factor (NF)-κB, elevating the expression of genes encoding proinflammatory cytokines. The EGF also stimulates ASM hyperplasia and subepithelial fibrosis. Asthmatic airways show an increase in EGF and EGFR immunoreactivity not only in the bronchial epithelium but also in the airway glands, smooth muscles, and the thickened basement membrane (Amishima et al. 1998). Samanta et al. (2014) have shown that Ca^{2+} entry through Orai protein in human bronchial epithelial cells stimulates EGF gene expression together with c-fos and NFAT transcription factors as well as NFAT-driven gene expression. The activation of c-Fos and NFAT pathways is prevented by pre-exposure of bronchial epithelial cells to the CRAC channel blockers Synta66 and 3,5-bis-trifluoromethyl-pyrazole (BPT2). However, detectable basal EGF transcription is present in the absence of Orai-STIM pathway

stimulation, which fits into the known physiological role of EGF in airway function. In the present study, neither budesonide nor SKF 96365 significantly inhibited the synthesis and release of EGF in the OVA-challenged guinea pig, the content of which was close to the level present in healthy non-sensitized animals. There is a biological plausibility that the EGFR-signaling pathway, but not EGF synthesis per se, is inhibited.

In conclusion, a 28-day-long OVA sensitization followed by inhalation challenges in the guinea pig is a valid model of asthma-like airway remodeling, in which the proinflammatory cytokines IL-4, IL-5, and IL-13, as well as TGF-ß and EGF, are involved. The structural airway alterations combined with the inflammatory process related to the magnitude of airway hyperactivity, expressed by increases in basal and constriction-induced sRaw and dysfunctional mucociliary clearance. The findings indicate that corticosteroids might have a mitigating role in remodeling, possibly through increased synthesis of IL-10. Further, we identified CRAC channels, forming an essential route of Ca^{2+} entry into airway epithelial cells, as a driving force of chronic inflammation, airway hyperactivity, and remodeling based on the reversal of the changes by the administration of a specific CRACK inhibitor, SKF 96365, in OVA-sensitized animals. The IL-12–INF-γ–TGF-β pathway activation is liable to be the underlying molecular mechanism suppressing the development of airway remodeling in response to chronic inflammation. Thus, targeting CRAC channels might offer promise as a novel therapeutic approach for managing chronic airway inflammatory disorders including advanced asthma and possibly also chronic obstructive pulmonary disease.

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Conflicts of Interest The authors declare no conflicts of interest concerning this article.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animals were treated in accord with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press) and the EU and Slovakian legislation regulating the welfare of experimental animals. This study was approved by a local Ethics Committee of the Jessenius Faculty of Medicine in Martin, Slovakia (permit EK 40/2018), registered by the Institutional Review Board/Ethics Board Office.

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