INTRODUCTION

The respiratory system is open to the environment and exposed to potential hazards from the inspired air. Therefore, the respiratory system developed defensive mechanisms that remove noxious agents from airways (1). Both reflex and non-reflex "cleansing" mechanisms interact, usually complementing one another. In our previous studies we tested the effects of acute stimulation of nasal afferents or of allergic rhinitis (AR) and intranasal (i.n.) capsaicin challenge. Thirty male guinea pigs, sensitized to ovalbumin were used in the study. They were divided into 3 groups of 10 animals each: AR group (i.n. ovalbumin), capsaicin group (i.n. capsaicin 50 µM, 15 µl), and controls without any challenge. The animals were anesthetized with urethane (1.1 mg/kg) and allowed to breath spontaneously via tracheostomy. Metal canula was introduced into the right hemithorax to assess intrapleural pressure. ER was elicited by mechanical stimulation of the vocal folds using a thin nylon loop introduced upwards via tracheostomy. Maximal expiratory effort of ER (MEE) and the count of post-ER laryngeal coughs were evaluated. Mechanical stimulation of the vocal folds in controls produced isolated ER. They were followed by post-ER cough only in 11% of provocations. AR and capsaicin challenge increased MEE compared with that in controls (P<0.05). In these two groups of animals, the ER was followed by post ER-cough in 75% of provocations. The count of post-ER coughs in the group order control/AR/capsaicin was 0-2/2-4/1-3, respectively; P<0.05). The ER from the vocal folds is upregulated in a similar manner as is cough and sneeze. The central neuronal mechanisms are proposed to mediate this effect, but the spread of inflammation from upper airways to the larynx, verified histologically in the present study, may contribute as well.

Key words: allergic rhinitis, capsaicin, cough, expiration reflex, vocal cords

MATERIAL AND METHODS

The study was approved by the Ethic Committee of Jessenius Faculty of Medicine in Martin, Slovakia in compliance with all applicable laws and policies of the Slovak Republic under No: 1822/07-221. Thirty male TRIK strain guinea pigs (350-450 g) purchased from the Department of Experimental Pharmacology, Slovak Academy of Sciences (Dobra Voda, Slovakia) were used for the study. The animals were kept in the lower airways and lungs. The larynx may play a significant role in protection of lower airways. It has a unique anatomical and functional position with respect to the airway defense. A large variety of reflex behaviors, active, such as cough or expiration reflex, and passive, such is apnea, laryngeal closure, or laryngospasm, can be elicited from the larynx (8). The knowledge of nasal cough upregulation, particularly of cough of tracheobronchial origin, cannot be extended to the expiration reflex, because these two are different reflex behaviors. Therefore, the aim of the present study was to evaluate the changes in expiration reflex (ER) evoked from the vocal folds during experimental allergic rhinitis and stimulation of nasal afferents with capsaicin.
Central Animal Holding facility of JFM CU, in air-conditioned, humidified, temperature maintained environment, with 12 h light/dark cycle and access to water and standard animal food ad libitum.

All animals were sensitized with ovalbumin, i.p., (10 µg in 100 mg aluminum hydroxide in 1 ml of saline, Sigma-Aldrich, St. Louis, MO). Successful sensitization was confirmed 21 days later by skin prick tests with ovalbumin. The animals were then assigned to three groups, 10 animals each:

1. Controls, which received intranasal (i.n.) saline challenge (15 µl of buffered saline, warmed to body temperature, into both nostrils);
2. Capsaicin group, which received i.n. capsaicin (15 µl, 50 µM) to induce symptoms of rhinitis due to vascular and neuronal responses to capsaicin challenge as described previously (2);
3. Rhinitis group which received i.n. ovalbumin (0.5%, 15 µl, into each nostril) to induce allergic nasal inflammation in sensitized animals.

All groups underwent the same experimental procedures.

**Experimental protocol**

Guinea pigs were anaesthetized by i.p. injection of urethane (1 mg kg⁻¹, Biosynth, Riedel de Haen AG, Germany). The animals were then placed in the supine position on the heated operating pad. Body temperature was continually monitored and maintained at 37-38°C. The larynx and trachea were surgically exposed in the midline, avoiding damage to the vagus and laryngeal recurrent nerves. A plastic cannula (3 mm of external diameter) was introduced into the trachea between the 7-9th tracheal rings allowing spontaneous breathing. A metal intrapleural cannula was introduced into the right hemithorax via the incision of the anterior thoracic wall at the 5th intercostal space. Electromanometer (Electromanometer HSE, Hugo Sachs Electronic) was used for recording of intrapleural pressure.

The animals of all groups were challenged i.n. with solutions listed above (saline, capsaicin, and ovalbumin as per group assignment). The saline challenged animals were free of nasal symptoms, the capsaicin and ovalbumin challenged ones developed reproducible nasal symptoms which involved sneezing, nasal obstruction, and discharge resulting in nasal acoustic phenomena, nasal rubbing, and crackles.

The animals with nasal symptoms were kept in the head down position. Defensive airway reflexes were induced by mechanical stimulation of the larynx with a thin loop of nylon fiber. Laryngeal cough was induced by repetitive cranial to caudal movements of the fiber in the larynx, pushing gently the fiber tip against the mucosa. ER was induced by mechanical stimulation of the medial margin of the vocal folds with a nylon loop introduced from the tracheal side. ER was elicited during the late inspiratory phase or early-middle expiratory phase of the tidal breathing cycle.

**Evaluation of the reflex behaviors**

The intensity of the airway protective reflexes was evaluated from the trace of the intrapleural pressure. The following parameters were quantified for laryngeal cough: number of cough efforts (NE), strength of maximum expiratory effort (MEE) in the attack, average expiratory effort (EE), and the sum of all positive expiratory deflections (total expiratory activity - TEA) in one cough attack. During the ER, the MEE was assessed. Because the ER is sometimes followed by single or multiple coughs, consisting of several forceful inspiratory-expiratory efforts, the number of these coughs (post-ER coughs) was counted.

**Tissue processing**

At the end of experiment, the animals were euthanized with an overdose of anesthetics. The samples of nasal and laryngeal mucosa were taken in all three experimental groups. Tissue specimens were fixed in formaldehyde. Paraffin embedded slices were processed and stained with the hematoxylin-eosin stain.

**Data evaluation**

Maximum and total expiratory effort data were expressed as means±SE, the number of post ER coughs was expressed as median and interquartile range. Significance of differences among the three experimental groups was analyzed with one-way ANOVA, followed by post hoc Tukey’s test. P<0.05 was considered to indicate statistical significance. Statistical analysis was performed with a commercial Systat ver. 11 software package (Systat Software, Richmond, CA).

**RESULTS**

**Reflex behaviors**

The intensity of laryngeal cough differed significantly between the experimental groups. Cough was enhanced regarding NE, MEE, and TEA in the groups with allergic rhinitis and intranasal capsaicin challenge, compared with the control group (Table 1). However, the average intensity of the expiratory effort was not affected.

Mechanical stimulation of the vocal folds in controls regularly produced isolated ER, which was followed by post-ER cough in 11% of provocations. Allergic rhinitis and i.n. capsaicin challenge increased the maximum expiratory effort during the ER compared with controls; the respective values were 11.8 ±0.4, 10.9 ±0.2, and 6.9 ±0.5 kPa, P<0.05 (Fig. 1). In the rhinitis and capsaicin challenged animals, the ER was followed by post ER-cough in up to 75% provocations; the average count of post-ER coughs in the control/rhinitis/capsaicin animals was 1.0 (0-2)/3.5 (2-4)/2.5 (1-3), P<0.05, respectively (Fig. 2).

**Histology**

Nasal mucosa of the rhinitis animals was infiltrated with eosinophils (data not shown). Laryngeal mucosa at the time of reflex provocations did not show the signs of eosinophilic inflammation. In the rhinitis animals, we observed a massive

| Table 1. Intensity of laryngeal cough induced by mechanical stimulation of the laryngeal mucosa in guinea pigs under urethane anesthesia. |
|---------------------------------|--------|-----------|-----------|
|                                | Controls| Allergic rhinitis | Capsaicin |
| NE (coughs)                    | 1 (0-2) | 4 (2-6)²⁺      | 3 (2-4)²  |
| MEE (kPa)                      | 6.9 ±0.6| 10 ±1.7⁻      | 9.3 ±0.9  |
| EE (kPa)                       | 6.5 ±0.5| 7.3 ±0.9     | 7.5 ±0.5  |
| TEA (kPa)                      | 13.3 ±0.8| 24.8 ±1.2⁻    | 27 ±1.8   |

NE - number of cough efforts; MEE - maximum expiratory effort; EE -average expiratory effort; TEA - sum of expiratory efforts in an attack. Data for the number of cough efforts are expressed as a median and interquartile range, data for the strength of expiratory efforts are expressed as means ±SE, *P<0.05, “P<0.001.
eosinophilic migration onto the endothelial surface of the vessel (Fig. 3A). Some of the eosinophils were crossing the vessel wall per diapedesis (Fig. 3B). Only sporadic eosinophils were found in the tissue just beneath and within the most basal epithelial layer. In contrast, laryngeal specimens from the control and capsaicin group showed no pathological changes.

DISCUSSION

The present study shows that the expiration reflex elicited from the vocal folds was enhanced in animals with experimental allergic rhinitis and in animals whose nasal afferents were stimulated with capsaicin. These findings support the hypothesis that upper airway disorders per se may increase readiness of reflex airway defensive mechanisms to respond. The purpose of this upper airway originated modulation is likely to increase lower airway defense in upper airway disorders and consequently to diminish the possibility of spreading the pathological process from the upper airways to more distal parts of the respiratory tract.

The expiration reflex was previously studied under the conditions of local laryngeal inflammation induced by croton and turpentine oil application (9). It has been found that as opposed to the mildly inflammatory croton oil, turpentine, a severely inflammatory agent, reduces the intensity of the expiration reflex by about 50%. Local inflammation makes the expiration reflex easier inducible and stronger in humans. Suppression of the reflex can be seen in patients with laryngeal tumors, possibly due to local tissue destruction (10).

We had previously reported that the cough response is enhanced in allergic rhinitis or during stimulation of nasal afferents, and we discussed this phenomenon as a potential natural reaction of the organism to support lower airway defenses (2-6). To test the hypothesis whether the expiration reflex is influenced under similar experimental conditions we now employed acute intranasal administration of capsaicin or experimentally induced allergic process, both of which are more complex nasal sensory activation than simple superficial application of histamine. Both stimuli, i.e., intranasal capsaicin and allergic rhinitis, cause short-lasting, 15-30 min, nasal symptoms due to sensory stimulation (2, 11).

Afferent sensory endings activators applied into the nose (histamine and capsaicin) sensitize the cough reflex in humans, cats, and guinea pigs (2-4). Local anesthesia of related nasal afferents completely abolishes this sensitization (12). Histamine is an archetype mediator of nasal allergic inflammation which directly stimulates a subset of sensory endings (13). The transient receptor potential cation channel, subfamily V, member 1 (TRPV1) selective agonists also are effective activators of nasal afferents. A large proportion of TRPV1 positive trigeminal nerve endings in the nose express a variety of receptors sensitive to stimuli associated with inflammation, such as histamine H1 or leukotriene cysteinyl-LT1 receptors (14).

Local regional and reflex effects of the sensory nerve activation with histamine and capsaicin may generate additional stimuli and further sensitize nasal sensory nerves (7). Both
agents when applied into the nose cause sensitization of the cough reflex. It is unlikely that the aspiration of nasal content into the lower airways accounts for the sensitization of cough reflex after intranasal sensory nerve activation, although this possibility cannot be entirely ruled out in animals and human subjects whose airways were not disengaged. It is important to note that during rhinitis, both allergic and capsaicin-induced, bits of nasal secretion can spread into lower airways, especially into the larynx, and stimulate laryngeal afferent nerve endings. Several precautions, head down position of the animals and tracheostomy, were taken in our experimental setup to minimize and eliminate such mechanisms as postnasal dripping or aspiration of aerosolized nasal content into the larynx.

The present study results are highly indicative that nasal sensory nerves are the neural pathways involved in sensitization of the expiration reflex. We propose a central mechanism for the sensitization of both cough and expiration reflex, resulting from the stimulation of nasal sensory afferents. In this scenario, afferent input from the nose to the central regulatory circuits of the cough reflex renders the cough and expiration reflexes hyperresponsive. It has been demonstrated that the cough reflex is sensitized by the stimulation of vagal afferent neurons in the respiratory tract and esophagus, possibly interacting at the brainstem level. The sensitization of the cough and expiration reflexes via nasal trigeminal sensory pathways is perhaps more complex, since the trigeminal and the cough/expiration reflexes triggering vagal sensory nerves terminate in different areas within the brainstem (1).

We propose that some reflex behaviors might be suppressed by the nasal stimulation. Aspiration reflexes, which could be easily elicited from the pharynx (particularly nasopharynx), representing forceful spasmodic inspiratory efforts, may enhance the propagation of the nasal pathological processes, such as post nasal dripping or microaspiration, into lower airways (11). Therefore, enhancement of the aspiration reflex under the condition of rhinitis would be very unwanted. Unfortunately, we could not test whether this reflex was reduced in the present study, since it can hardly be elicited in the guinea pig (10).

In the present study, we successfully induced clinical signs of rhinitis in our animals, either by intranasal ovalbumin challenge or capsaicin application. The clinical consequences of both interventions were very similar, although each intervention is underlain by a different pathomechanism. Intranasal capsaicin enhances cough and expiration reflex predominantly via neuronal activation of TRPV1 and the mechanisms of neurogenic inflammation. Specimens of nasal mucosa obtained from the capsaicin group showed vasodilatation, with no signs of blood cell migration. In rhinitis, the symptoms represent an early stage of allergic response to antigenic challenge observed in sensitized animals (15). Animals sneeze, have nasal discharge, nasal rubbing, crackles, and progressively worsened nasal breathing. The symptoms are accompanied with eosinophilic infiltration of nasal mucosa, as reported elsewhere (16, 17). In our present model, the symptoms lasted up to 1 h. The expiration reflex was provoked approximately 20 min after the onset of symptoms. Interestingly, laryngeal mucosa did not reveal typical histological signs of eosinophilic inflammation. There was a clear sign of eosinophilic mobilization, yet they did not clearly infiltrate the tissue, although they migrated onto the intima of laryngeal vessels, which was in contrast to the number of eosinophils within nasal mucosa in the same experimental model. A progressive decrease in the count of eosinophils from the nose, a site of antigen challenge, downward to the larynx, trachea, and bronchi, with the sporadic presence of eosinophils in lung tissue has been reported (16, 17). A question may be raised of whether enhancement of cough and expiration reflex is caused by sensitization per se or by clinical symptoms of an ensuing disorder. It has been demonstrated that the sensitization alone, without clinical symptoms of rhinitis, does not enhance airway reflex responses. They are exaggerated only in the presence of clinical nasal symptoms (7).

The neuronal mechanisms of cough and expiration reflex enhancement due to nasal stimulation are unknown. Stimulation of nasal afferents can produce sneeze, another reflex behavior with a powerful expiratory component. Stimulation of nasal trigeminal afferents increases neuronal activity including that of expiratory units within the ventral respiratory group in anesthetized cats (18). This group is considered to contain crucial elements for generation of forced expiratory behaviors, such as cough or expiration reflex. According to the concept of multilevel and multifunctional arrangement of central neuronal control of airway defense (1), we can speculate that expiratory efforts might be augmented by convergent inputs to these neuronal mechanisms. Single neuron recording in the ventral respiratory group in cats during expiration reflex exposed vigorous, but short-lasting, burst of spontaneous activity of expiratory neurons, with concomitant recruitment of silent expiratory units (19). Both spontaneously active and recruited neurons could receive excitatory drive originating from the multiple afferent pathways, resulting in enhanced expulsive respiratory activity.

In conclusion, the expiration reflex is enhanced during stimulation of afferents by pathological processes in the nasal cavity, which likely reflects central and peripheral neuronal plasticity.

Conflict of interests: None declared.

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