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EXPERIMENTAL STUDY

Antitussive activity of *Althaea officinalis* L. polysaccharide rhamnogalacturonan and its changes in guinea pigs with ovalbumine-induced airways inflammation

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Abstract: *Aim:* The presented studies were aimed on experimental confirmation of *Althaea officinalis* polysaccharide rhamnogalacturonan antitussive effect and its changes in conditions of allergic inflammation.
Methods: We have tested whether rhamnogalacturonan inhibits cough reflex and modulates airways reactivity of guinea pigs *in vivo*. The cough in guinea pigs was induced by 0.3 M citric acid (CA) aerosol for 3 min interval, in which total number of cough efforts (sudden enhancement of expiratory flow accompanied by cough movement and sound) was counted. Specific airway resistance and its changes induced by citric acid aerosol were considered as an indicator of the *in vivo* reactivity changes.
Results: 1) *Althaea officinalis* polysaccharide rhamnogalacturonan dose- dependently inhibits cough reflex in unsensitized guinea pigs. Simultaneously, plant polysaccharide shortened the duration of antitussive effect when it was been tested in inflammatory conditions. 2) Rhamnogalacturonan did not influence airways reactivity *in vivo* conditions expressed as specific resistance values neither sensitized nor unsensitized groups of animals. 3) The antitussive activity of codeine (dose 10 $mg.kg^{-1}$ b.w. orally) tested under the same condition was comparable to higher dose of rhamnogalacturonan in unsensitized animals. 4) The characteristic cellular pattern of allergic airways inflammation was confirmed by histopathological investigations.
Conclusion: Rhamnogalacturonan isolated from *Althaea officinalis* mucilage possesses very high cough suppressive effect in guinea pigs test system, which is shortened in conditions of experimentally induced airways allergic inflammation *(Tab. 1, Fig. 4, Ref. 25)*. Full Text in free PDF *www.bmj.sk.*
Key words: polysaccharides, rhamnogalacturonan, guinea pigs, antitussive activity, *Althaea officinalis.*

Althaea officinalis L. (*Malvaceae*) or marsh mallow is an erect perennial plant, which original habitat was in salty marshes or wet, brackish uncultivated ground in southern Europe. Now it is naturalized in North America and cultivated from Western Europe to Russia (Leung and Foster, 1996). The name Althaea is derived from the Greek *altho*, meaning to heal, and its medicinal qualities have been recognized since Ancient Egyptian times. Roots, leaves and flowers were used as crude drugs in treatment of respiratory system catarrhs, irritating cough, skin wounds, gastritis, ventricular ulcers, cystitis, urethritis and various inflammations of nasal and oral cavities (Blumenthal et al, 2000).

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A. officinalis belongs to the *Malvaceae* family and it is known to contain relatively high mucilage proportion which makes *A. officinalis* an excellent demulcent, emollient, expectorant and anti-inflammatory.

The mucilage, made up of complex polysaccharides, occurs in different compartments of the plant and may have various physiological functions, e.g. it may act as an energy reserve, storage of carbohydrates and protective colloid, may play a role in frost tolerance or water transport. These hydrocolloids constitute a structurally diverse class of biological macromolecules with a broad range of physicochemical properties, which are widely used for applications in pharmacy and medicine. The nature of the mucilage isolated from *A. officinalis* is well describes (Capek et al, 1997, 1999).

Previously, it has been considered that the main components responsible for therapeutic effect of *A. officinalis* mucilage are polysaccharides or polysaccharide-protein complexes originated from different parts of plant (Nosalova et al, 1992; Sutovska et al, 2007). Recently, Nosalova et al (2005) confirmed statistically significant ability of various isolated polysaccharide components of mucilage to reduce parameters of mechanically induced cough reflex in conscious cats. The best cough suppression was observed with administration of *A. officinalis* polysaccharide

rhamnogalacturonan (Nosalova et al, 2006). The authors showed that its antitussive effect was very close to, only by 12 % lower, to the activity of codeine. This drug belongs to group of centrally acting opioid agonists, which side effects occurring during antitussive treatment are well known. On the other hand, efficacy of codeine during experimentally- induced bacterial inflammation of the airways was not significantly changed in conscious cats test system (Strapková et al, 1982), which makes this drug still clinically mostly used for cough suppression.

The airway inflammation is associated with allergen-induced early and late inflammatory reactions and airway hyperresponsiveness to a variety of stimuli resulting in invariable obstruction of the airways and cough (Holgate and Davies, 2009). The development of airway hyperresponsiveness is importantly determined by changes in the neurogenic and non-neurogenic control of airway smooth muscle as well as by physical alterations in the airways, such as epithelial damage, mucosal swelling and remodeling of the airway wall that is characterized by thickening of the basement membrane, subepithelial fibrosis and increased airway smooth muscle mass (Aoshiba and Nagai, 2008). The airways inflammation is almost invariably accompanied by a pathological cough, which represents serious medical problem. Furthermore, inflammatory conditions of the airways could finally result in changed efficacy of drugs used to suppress cough.

According to the above mentioned, we decided to confirm efficacy of most active plant polysaccharide rhamnogalacturonan to influence the following airways reflexes in allergic inflammatory conditions:

i) cough reflex experimentally induced by **citric acid**, which is very relative to natural conditions of cough reflex onset during airways inflammation;

ii) airways smooth muscle reactivity *in vivo.*

The conditions of allergic inflammation in airways were induced by ovalbumine and verified by histopathological investigation of tracheal and pulmonary sections. The guinea pigs were selected and used as animal experimental models.

Material and methods

Animals

All experiments were approved by the local Ethics Committee of the Jessenius Faculty of Medicine in accordance with the revised Declaration of Helsinki from 1983 and follow the criteria of experimental animal's well fare. Animals used in the studies - adult male TRIK strain guinea pigs, weighing 150–350 g – were obtained from the Department of Experimental Pharmacology, Slovak Academy of Sciences, Dobra Voda, Slovakia and were housed in approved animal holding facility. The animals in total number 56 were used in experiments after one-week adapting period and after adaptation of guinea pigs to experimental conditions were selected according to response to tussigen (non-responders and hyporesponders were excluded).

The guinea pigs were divided into 7 groups, each consisting of 8 animals. The airways hyperresponsiveness was induced by ovalbumine in 3 groups of guinea pigs and 4 groups of animals were unsensitized as follows:

A – "two *negative controls*" both ovalbumine-sensitized and unsensitized groups received *solvent* – water for injection in the dose 1 ml. kg^{-1} b.w. orally;

B – "two *positive controls*" both ovalbumine-sensitized and unsensitized group received *codeine* in the dose 10 mg. kg^{-1} b.w. orally;

C – polysaccharide *rhamnogalacturonan* from *Althaea officinalis* was administered to "experimental groups" of guinea pigs as water solution orally in the dose 25 and 50 mg. kg^{-1} b.w.; both doses were used in unsensitized guinea pigs, higher dose of polysaccharide in ovalbumine-sensitized animals.

The influence on citric acid induced cough reflex as well as was airways smooth muscle reactivity *in vivo* and *in vitro* conditions were tested on all above mentioned groups.

Plant material

Roots of *Althaea officinalis* L. var. Robusta were obtained from the Centre for the Cultivation of Medicinal Plants, Faculty of Medicine, J. E. Purkyne University, Brno, Czech Republic.

The roots of the plant were crushed and a crude mixture of polysaccharides was obtained by water extraction (complex water extract), followed by ethanol precipitation of the water extract and dialysis of the precipitate (mucilage). The three main polysaccharide components were isolated from the mucilage by various purification techniques, i.e. a highly branched 1,5-a-L-arabinan, a linear 1,6-a-D-glucans, and a branched *rhamnogalacturonan.* It's main backbone is composed of alternating sequences of 1,4-linked a-D-GalA (galacturonic acid) and 1,2-linked a-L-Rha (rhamnose) residues, bearing on O-3 of GalA monomeric GlcA (glucuronic acid) and on O-4 of Rha galactose residues or short chains of 1,4-linked galactose units.

Chemicals

Ovalbumine, citric acid and contractive mediators' acetylcholine and histamine were obtained from Sigma-Aldrich (Germany). Codeine was purchased from Lachema (Czech Republic). Codeine, rhamnogalacturonan and citric acid were dissolved in water for injection and all other drugs in 0.9 % saline.

Antigen-induced airway hyperresponsiveness

Sensitization of animals by the antigen ovalbumine, which causes airway reactivity changes on immunological base, was performed during 21 days. The allergen (ovalbumine in $c = 10^{-5}$ $mol.l^{-1}$)) was administered on the 1st day of sensitization intraperitoneally (0.5 ml) and subcutaneously (0.5 ml), on the 4th day intraperitoneally (1 ml), and on the 18th and 20th days only by inhalation during 2 min time interval. Inhalation was performed in double chamber whole bodypletysmograph box for small laboratory animals (HSE type 855, Hugo Sachs Elektronik, Germany) and ovalbumine aerosol generated by jet nebulizer (PARI jet nebulizer, Paul Ritzau, Pari-Werk GmbH, Germany, output 5 $l.s^{-1}$, particles mass median diameter 1.2 μm) was delivered to head chamber of the bodyplethysmograph.

Citric acid-induced cough reflex

The method of chemically induced cough reflex was described in detail before (Sutovska et al, 2009a). Briefly, conscious guinea pigs were individually placed in a bodypletysmograph box and were exposed to citric acid aerosol generated by a jet nebulizer in concentration 0.3 M for 3 min.

The following two methods for detection of cough were used to distinguish the cough efforts from sneezing and movements:

i) The changes of the expiratory airflow interrupting the basic respiratory pattern during cough effort were measured by pneumotachograph connected to the head chamber of bodyplethysmograph.

ii) The typical cough reflex movements and sounds were recognized by trained observer.

The number of coughs was evaluated on the basis of sudden enhancement of expiratory flow accompanied by a typical cough movement and sound during 2 min inhalations of the tussigen. The cough response was measured before administration of any agents (baseline measurement; N value in graphs) and then after their application in confirmed time intervals (0.5, 1, 2 and 5 h).

Since generally accepted time window of a protectionof cough receptors against adaptation to that kind of irritation is two hours, the following sequence was used in the experiments : the cough response on agent measured after 0.5 and 5 h on the 1st day, after 1 h on the 2nd day and after 2 h on the 3rd day.

The airway smooth muscle reactivity, in vivo conditions

In vivo airway smooth muscle reactivity was evaluated using double chambers bodyplethysmograph box for laboratory animals consisting of head and body chambers. The specific airway resistance values calculated by Pennock (1979) and their changes were regarded as indicator of *in vivo* airways reactivity. The specific airway resistance is proportional to phase difference between nasal and thoracic respiratory airflow, which means the bigger the phase difference the higher the value of specific airway resistance and also more significant degree of bronchoconstriction. This noninvasive plethysmograph technique is commonly used for evaluation of bronchoactive substances effect (Tohda et al, 2000).

The specific airway resistance was measured consecutively after the citric acid exposure and cough response registration during a 1 min interval. Their intensity before solvent, codeine and both doses rhamnogalacturonan application was considered as baseline (N value in graphs). The next were measured in 0.5, 1, 2 and 5 hours time intervals.

Histopathological investigation of tissue samples

Tracheal and pulmonary tissue samples of ovalbumine- sensitized control group of guinea pigs (cca 7x7x5 mm, hematoxylin- eosin stain, magnification x 100) were evaluated by two independent investigators using four- degree scale in which degree 0 means none, 1 mild, 2 middle and 3 distinct changes.

The following were common criteria for evaluation of tracheal and pulmonary sections: hyperemia, infiltration by eosinophils and mast cells; infiltration by lymphocytes of pulmonary tissue samples was only studied.

Statistics

All obtained data were statistically evaluated using the ANOVA and Student˘s t-test. Asterisks mark statistically significant results. The 0.05 (*) and lower level of probability were considered as significant.

Results

Rhamnogalacturonan administered perorally (in the dose 25 and 50 mg.kg^{-1}) dose-dependently decreased the number of citric acid induced coughs (NE) in unsensitized guinea pigs (Fig. 1): lower dose (Rh 25 line) 1, 2 and 5 hours ($p<0.05$), and higher dose (Rh 50 line) 1 hour ($p<0.05$), 2 and 5 hour ($p<0.01$) measurements. It is also distinct that onset of the action is slower in comparison with the effect of orally administered codeine. We found very similar character and intensity of the NE decrease when compared to the higher dose of rhamnogalacturonan and codeine effects. Moreover, we found more intensive suppression of citric acid induced cough on rhamnogalacturonan opposite to codeine in measurement 2 hours after application of that dose of plant polysaccharide.

The values of specific airways resistance were significantly uninfluenced by rhamnogalacturonan administration in conscious guinea pigs. It is evident that changes of RxV in unsensitized negative control group and both experimental groups of animals (Rh 25, Rh 50) are very similar. Mild significant increase of specific airway resistance was found in the group of guinea pigs receiving codeine in measurements after 0.5 from application of the agent (Fig. 2).

Sensitization of guinea pigs by ovalbumine resulted in inflammatory changes of tracheal and pulmonary tissue samples presented in Table 1. It is manifest that tracheal sections were distinctly hyperemic and infiltrated by eosinophils. The pulmonary sections showed only signs of mild hyperemia and infiltra-

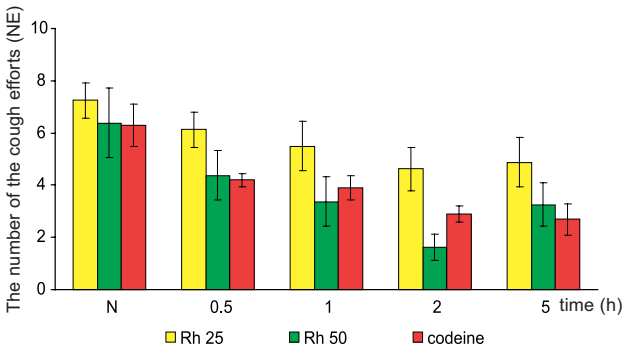

Fig. 1. The influence of rhamnogalacturonan on the number of citric acid- induced cough (NE). The antitussive effects were assessed 0.5, 1, 2 and 5 h after orally administered plant polysaccharide in two doses: 25 mg.kg^{-1} (Rh 25 line) and 50 mg.kg^{-1} (Rh 50 line). The comparison of effects with activity of codeine (10 mg.kg^{-1} b.w. orally). The values labeled as N represents baseline measurements result before any agents application. * $p<0.05$; ** $p<0.01$ vs N values (T-test, ANOVA).

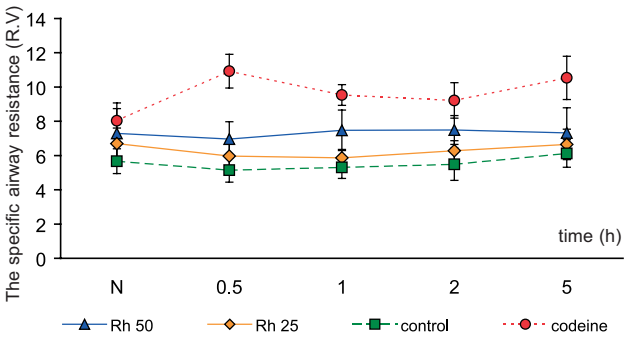

Fig. 2. The changes of specific airway resistance (R.V) *in vivo* conditions after *Althaea officinalis* polysaccharide rhamnogalacturonan administration (Rh 25, Rh 50). Bronchoconstriction induced by citric acid aerosol and compared with values recorded in positive (codeine) and negative (control) control groups of guinea pigs. * p<0.05; ** p<0.01 vs N values (t-test, ANOVA).

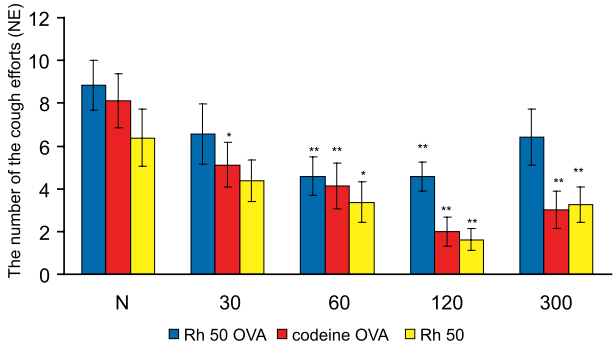

Fig. 3. The antitussive activity of rhamnogalacturonan in ovalbumine-sensitized guinea pigs (Rh 50 OVA) compared with codeine tested under the same conditions (codeine OVA) and effect of identical rhamnogalacturonan dose in unsensitized group of animals (Rh 50). * p<0.05; ** p< 0.01 vs N values (t-test, ANOVA).

tion by eosinophils, middle mast cells infiltration and furthermore, confirmed infiltration by lymphocytes is evidence of advanced allergic inflammatory response to ovalbumine.

Ovalbumine induced allergic inflammation of the airways, which significantly decreased the duration of rhamnogalacturonan antitussive effect applied in the peroral dose 50 mg.kg^{-1}. The first statistically significant drop of cough efforts number (1 h measurement; p<0.01) pointed on the identical onset of the effect after 1 h from application of polysaccharide agent, followed by the next significant measured interval (2 h measurement; p<0.01). The last record of cough efforts number showed only non-significant decrease referred to compressed duration of cough suppressive effect (Fig. 3). The comparison with codeine tested under inflammatory conditions certified constant antitussive effect of opioid agonist despite of the airways allergic inflammation.

The values of specific airways resistance in ovalbumine- induced hyperresponsiveness of the airways were unaffected by rhamnogalacturonan. Similarly, codeine in guinea pigs with ovalbumine- induced airways allergic inflammation did not change the registered values of specific airways resistance (Fig. 4).

The values of NE and RxV registered in negative control groups of animals, both sensitized and unsensitized, receiving solvent (water for injection in the dose 1 ml. kg^{-1} b.w. orally) are expected to remain almost unchanged, therefore they are not graphically represented (except for RxV measured in the unsensitized negative control group) (Fig. 2).

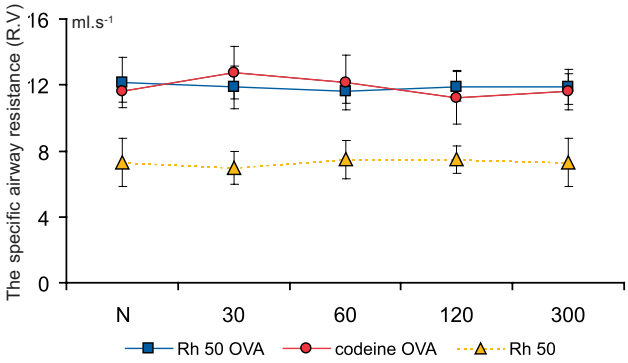

Fig. 4. The influence of orally administered *Althaea officinalis* polysaccharide rhamnogalacturonan on values of specific airways resistance measured in ovalbumine- sensitized group (Rh 50 OVA) and unsensitized group (Rh 50). The comparison with changes of *in vivo* airways reactivity indicator after orally applied codeine in sensitized guinea pigs (codeine OVA).

Discussion

The medicinal herbs represent potential sources of antitussive active constituents, e.g. polysaccharides with high cough suppressive efficiency and with minimal side effects. A number of them has been reported to possess antitussive effect similar to or prolonged as theperipherally acting antitussives (Nosalova et al, 2006, Sutovska et al, 2007). The results of presented experiments confirmed efficacy of polysaccharide rhamnogalacturonan,

Tab. 1. Observed histopathological changes of tracheal and pulmonary sections induced by ovalbumine in 21-days sensitization in control group of animals.

Tracheal sections			Pulmonary sections			
H	Eo	Mc	H	Eo	Mc	Ly
2.5±0.27	2.36±0.26	0.43±0.19	0.88±0.29	0.63±0.26	1.75± 0.16	0.75±0.31

the main constituent of *Althaea officinalis* mucilage, to suppress number of citric acid induced cough efforts in dose-dependent manner. Furthermore, this effect expressed as character of decline curve was comparable to centrally acting codeine antitussive activity. Codeine, the most active cough suppressive agent used in clinical practice, is used as a reference drug for any antitussive activity tests.

The details of plant polysaccharides mechanisms of antitussive action are still not completely understood. Previously, we showed that antitussive effect of rhamnogalacturonan was very probably accompanied with some of peripheral and reflexive mechanisms, in which nasopharyngeal neurons and their 5-HT_2 receptors are involved. K^+_{ATP} ion channels, which substantial role in defense reflexes of the airway is undisputable, do not participate on rhamnogalacturonan mechanism of cough suppression. These mechanisms are discussed in details elsewhere (Sutovska et al, 2009b). Bronchodilation represents one of the possible peripheral mechanisms, which could participate on the antitussive effect of agents acting peripherally. This assumption is supported by more authors, which demonstrated cough suppressive activity of drugs with bronchodilatory properties (Belvisi, 2003; Chung, 2005). Evaluated results of *in vivo* experiments showed that rhamnogalacturonan did not change airways smooth reactivity. Therefore antitussive activity of rhamnogalacturonan, a polysaccharide originating from *Althaea officinalis* observed in our conditions in guinea pigs is not accompanied by bronchodilation.

Inflammatory disease of the airways is mainly characterized by representative histopathological features associated with typical clinical symptoms, e.g. reversible airway obstruction, bronchial hyperresponsiveness (BHR) and cough. The histopathologically evaluated section of airway allergic inflammation showed cellular infiltration, epithelial sloughing, basement membrane thickening, edema and hyperplasia of mucus-secreting glands, and hypertrophy of bronchial smooth muscle (Jeffery, 1992). The cellular pattern of allergic airways inflammation in presented studies was characterized by a high number of eosinophils, mast cells and lymphocytes. These findings are in accordance with Amin et al (2000) who showed very similar bioptic changes in airways of asthmatic patients. Furthermore, these authors showed that eosinophils and mast cells were the source of IL-4, IL-5 and IL-8 occurring in increased levels in patients with asthma. IL-4 and IL-5 produced by T helper-2 cells and mast cells stimulate B cell growth and increase immunoglobulin secretion. They are also a key mediator in eosinophils activation. IL-8 is a chemoattractant and is also a potent angiogenic factor produced by macrophages and other cell types such as epithelial cells (Levings et al, 2002). Epithelial damage by activated eosinophils and lymphocytes has been proposed as one of the major pathophysiological mechanisms in asthma (Venge et al, 1988). Activated eosinophils release cationic granule proteins, which are highly toxic to the respiratory epithelium. Lymphocytes are the source of tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) that have been shown to cause damage to bronchial epithelial cells cultivated in vitro (Kampf et al, 1999) and are involved in the attraction and activation of eosinophils. All these structural changes resulted in functional abnormalities that could possibly influence the effects of the drugs used to suppress respiratory diseases symptoms, which mechanisms of action is accompanied with airways wall constituents. Presented experiments tested if demonstrated antitussive activity of plant polysaccharide rhamnogalacturonan could be changed by conditions of allergic airways inflammation. It was shown that a shortened duration of cough suppressive effect supported peripheral mechanism of herbal polysaccharides action. Antitussive activity of codeine acting centrally was influenced by allergic inflammatory conditions only insignificantly. Previously, Strapkova et al (1984) followed changes in efficacy of different cough suppressants during inflammatory conditions in conscious cats. They found relatively unchanged antitussive effects of codeine, which correlated with results of presented experiments despite of the different animal model used. We preferred guinea pigs, because their airways receptors distribution, proportion and function are mostly similar to human airways (Muccitelli et al, 1987).

Cellular infiltration of the airways mentioned above, especially by mast cells, is strongly associated with BHR. In presented studies we recorded significantly higher contractile response to bronchoactive mediator citric acid in sensitized guinea pigs compared to unsensitized control group. This result corresponded with Lúdvíksdottir et al (2000). They found that patients with asthma had an increased responsiveness to bronchoconstricting mediator AMP. According to Polosa and Holgate (1997) bronchoconstriction induced by AMP is primarily mediated by mast cells.

In conclusion, polysaccharide isolated from medicinal plant Althaea officinalis possesses dose- dependent cough suppressive effect in unsensitized guinea pigs. Bronchodilation is not involved in mechanism of antitussive action. Allergic airways inflammation, confirmed by histopathological evaluation, shortens the duration of the rhamnogalacturonan antitussive effect.

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