

## EXPERIMENTAL STUDY

## Arginase in the airways hyperreactivity

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**Abstract: Background:** The interest of arginase action is increasing because limitation of L-arginine bioavailability by arginase for NO synthesis via constitutive NOS can contribute to airway hyperreactivity.

**Objectives:** We investigated the effect of intervention in the arginase activity in guinea pig model of experimental ovalbumin-induced airway hyperreactivity.

**Methods:** We analysed the response of tracheal and lung tissue smooth muscle strips to histamine or acetylcholine after in vitro administration of arginase in a dose of 75 UI or after administration of the non-selective inhibitor of arginase N<sup>o</sup>-hydroxy-L-arginine (NOHA) in a dose of 5 and 10 µmol. We used as well as the incubation of strips with the precursor of NO synthesis L-arginine in a dose of 10<sup>-4</sup> mol/l together with NOHA.

**Results:** We did not find any significant differences in the reactivity of tracheal and lung tissue smooth muscle if we applied arginase in a dose of 75 UI in vitro. NOHA in a dose of 5 and 10 µmol induced the decrease of tracheal and lung tissue smooth muscle reactivity overall. The decrease of the contraction amplitude was dose-dependent. The supplementation of NO synthesis precursor L-arginine in a dose of 10<sup>-4</sup> mol/l together with NOHA intensified the decrease of the airways reactivity induced by an arginase inhibition.

**Conclusion:** The results suggest that arginase is involved in the control of airways bronchomotoric tone and therefore modulation of arginase activity could be a useful tool for airway smooth muscle tone control in clinical conditions (Fig. 7, Ref. 33). Full Text (Free, PDF) [www.bmj.sk](http://www.bmj.sk).

Key words: airway hyperreactivity, ovalbumin, guinea pig, arginase, NOHA.

Airway diseases such as asthma and chronic obstructive pulmonary disease are characterized by airway hyperresponsiveness, airway inflammation and epithelial damage. The prevalence of these diseases is increasing on regardless of new therapeutic approaches – anti-leukotrienes, anti-IgE, immunotherapy or inhibitors of matrix metalloproteinase (1). New insights into the pathophysiology of airway diseases could lead to other effective therapeutic approaches for their treatment (2). Nitric oxide (NO) might be one of these “new” targets as an extraordinary important biomessenger for a number inter- and intracellular signalling pathways including function of respiratory system (3). It is an important unique endogenous biological messenger molecule that causes bronchodilation, vasodilation, it participates in the regulation of gas changes, blood flow, mucociliary transport, surfactant production and it represents an important non-specific defence mechanism in the airways. Nitric oxide is produced by family of enzymes NO synthase (NOS) isoforms that utilize the semi-essential amino acid L-arginine as a substrate for the NO and L-citrulline production (4). In the airways the constitutive NOS (cNOS) isoforms are mainly expressed in the inhibitory

nonadrenergic noncholinergic (iNANC) nervous system neurons (nNOS), in the endothelium (eNOS) and in the epithelium (nNOS and eNOS), whereas inducible NOS (iNOS) induced by proinflammatory cytokines during airway inflammation is expressed mainly in macrophages and epithelial cells (5).

The availability of the NO synthesis precursor L-arginine in the airways is also under control of arginase that hydrolyzes L-arginine to L-ornithine and urea. Arginase I is classically considered to be an enzyme of the urea cycle in the liver, but also occurs in extrahepatic tissues including the lung (6). It is a cytosolic isoform that is highly expressed mainly in the liver and constitutes more than 98 % of the total body arginase activity. Arginase II is a mitochondrial isoform that contributes to the remaining 2 % of the total body arginase activity. It is present in many non-hepatic tissues such as the lung, kidney, prostate, brain, intestine and mammary gland. Both arginase isoforms are constitutively expressed in the airways particularly in the bronchial epithelium and in fibroblasts from peribronchial connective tissue (7) although Carraway et al (8) consider location of activity isoform II predominantly to the lung. Endogenous arginase activity is functionally involved in both the neural and non-neural regulation of the airway smooth muscle tone (9, 10) and can impair a neuronal nitric oxide-mediated airway smooth muscle relaxation in some conditions (11, 12). Since NOS enzymes are expressed in the lung too, this can result in cross-interactions between both types of enzymes. Both enzymes use the same substrate therefore simultaneous expression of both enzymes should

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result in the competition for substrate and thus limit the availability of L-arginine for NO synthesis in optimal quantities. Although the affinity of L-arginine is much higher for NOS than for arginase, the maximum activity of arginase is more than 1000-fold that of NOS (6). These facts lead to an increasing interest in arginase activity and a potential relevance of competition between arginases and NOS for their common substrate in the airway physiology and pathophysiology, as well (13, 14).

We were interested in the effect of modulation of arginase homeostasis in the conditions of the experimental allergen-induced airways hyperreactivity. We proposed that changed arginase activity could modulate the availability of L-arginine in these conditions as was documented in some paper (15). So, to test this hypothesis we used in our experiments the supplementation of the precursor of NO synthesis – L-arginine, the supplementation of arginase itself, the inhibition of arginase activity by N<sup>ω</sup>-hydroxy-L-arginine (NOHA) and the administration of L-arginine with NOHA. Experiments were realized in *in vitro* conditions using a method of organ baths.

## Material and methods

### Animals and agents

The pathogens-free adult male Trik strain guinea pigs weighing 200–300 g were used in our study approved breeding facility (Department of Toxicology and Breeding of Experimental Animals, Department of Experimental Pharmacology Slovak Academy of Sciences, Good Water, Slovak Republic). Animals were group-housed in individual cages in climate-controlled animal quarters and received water and food ad libitum. Room temperature was maintained at 21±1 °C and a 12/12 h light/dark regimen was maintained.

The study protocol was approved by the local Ethical Committee of Jessenius Faculty of Medicine. Animal care, procedures of allergen-induced airways hyperreactivity and euthanasia with small guillotine were performed in accordance with internationally accepted recommendations – the Helsinki Declaration World Medical Association, Direction of European Commission on the protection of animals used for experimental and other scientific purposes (86/609/EHS, 1986) and statute valid in Slovak Republic (Law No. 289/2003 Statute-book Regulation of Slovak Republic).

We used six groups of animals:

*Group 1* (n=8) – we administered L-arginase in a dose of 75 UI to strips of tracheal and lung tissue smooth muscle from animals with the ovalbumin-induced airway hyperreactivity placed in organ bath.

*Group 2* (n=8) – we administered N<sup>ω</sup>-hydroxy-L-arginine (NOHA) in a dose of 5 μmol/organ bath (30 ml) to strips of tracheal and lung tissue smooth muscle from animals with ovalbumin-induced the airways hyperreactivity.

*Group 3* (n=8) – we administered N<sup>ω</sup>-hydroxy-L-arginine (NOHA) in a higher dose of 10 μmol/organ bath (30 ml) into organ bath to strips of tracheal and lung tissue smooth muscle from animals with ovalbumin-induced the airways hyperreactivity.

*Group 4* (n=8) – we administered N<sup>ω</sup>-hydroxy-L-arginine (NOHA) in a dose of 10 μmol/organ bath (30 ml) to strips of tracheal and lung tissue smooth muscle from animals with ovalbumin-induced the airways hyperreactivity together with L-arginine in a dose of 10<sup>-4</sup> mol.l<sup>-1</sup>.

*The control groups* (n=8) were sensitized by ovalbumin but we added into organ baths to strips dissolving agent – aqua pro injectione in a volume 0.2 ml.

### Model of allergen-induced hyperreactivity

The modified method of Fraňová et al (16) with senzibilization of guinea pigs by allergen (ovalbumin) was used for the provocation of the airways hyperreactivity. An allergen solution containing 100 mol.ml<sup>-1</sup> of ovalbumin in saline was injected in exact time interval – at the 1st day 0.5 ml administered intraperitoneally and 0.5 ml subcutaneously, at the 3rd day 1 ml administered intraperitoneally and at the 14th day 1 ml of solution was nebulized and inhaled into the respiratory system. The inhalation of ovalbumin was performed in a body plethysmograph (Hugo Sachs Electronic, type 885, Germany) for rodents and small animals.

### Airway responsiveness

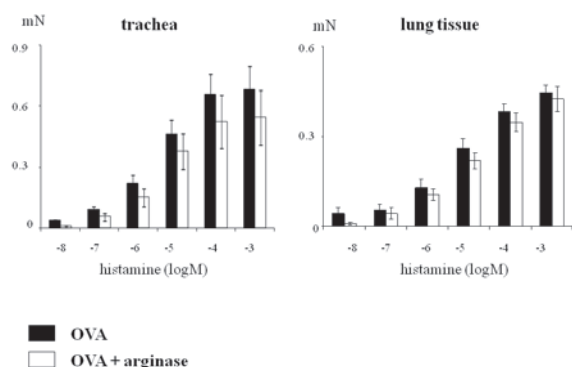
Animals were euthanized 24 hours after the inhalation of ovalbumin. Strips were prepared from trachea and lung tissue and placed into organ bath with Krebs-Henseleit solution (110.0 mol/l NaCl, 4.8 mol/l KCl, 2.35 mol/l CaCl<sub>2</sub>, 1.20 mol/l MgSO<sub>4</sub>, 1.20 mol/l KHPO<sub>4</sub>, 25.0 mol/l NaHCO<sub>3</sub> and 4g glucose in glass-distilled water). The solution was continuously aerated with mixture of 95 % O<sub>2</sub> and 5 % CO<sub>2</sub> at pH 7.5±0.1 and temperature 36±0.5 °C. One of the strip endings was connected to a force transducer (TSR 10 G, Vývoj Martin, Slovak Republic) and an amplifier (M1101 SUPR, Mikrotechna Praha, Czech Republic) and tension records were made on a Line Recorder TZ 4620 (Laboratorní přístroje Praha, Czech republic). The changes of tension were recorded on a computer with specific software (RES s.r.o, Martin, Slovak Republic). The tissue strips were exposed initially to the tension of 4 g (30 minutes – loading phase). Then, the tension was readjusted to a baseline of 2 g (30 minutes – adaptive phase). The Krebs-Henseleit solution was changed every 10 minutes. The strips were contracted by cumulative doses (10<sup>-8</sup>–10<sup>-3</sup> mol/l<sup>-1</sup>) of histamine or acetylcholine (substances Sigma Aldrich).

### Statistical analysis

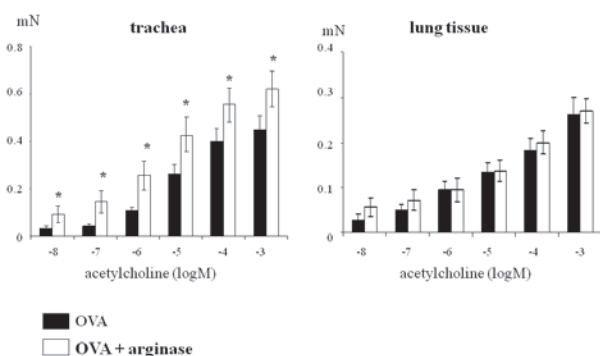
All data are expressed as the mean ±S.E.M. The significance of differences in values between the groups were analysed using the ANOVA test. p<0.05 was considered to be statistically significant.

## Results

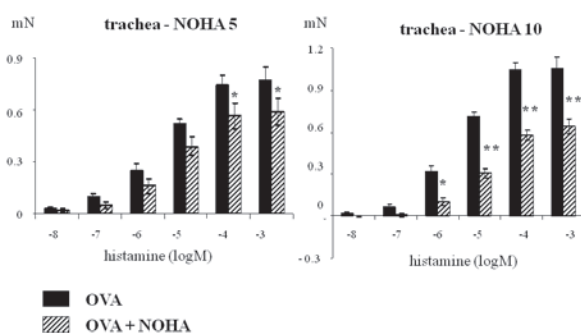
The difference in the airway reactivity between guinea pig sensitized with ovalbumin and nonsensitized control animals



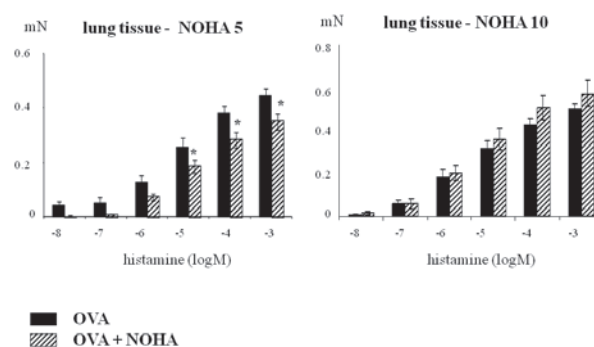
**Fig. 1.** Effect of arginase (white columns) in a dose of 75 UI on the reactivity of tracheal and lung tissue smooth muscle to histamine *in vitro* in animals with ovalbumin-induced airway hyperreactivity compared with response of animal without arginase (black columns). The columns represent the average values of the contraction amplitude with mean average  $\pm$  S.E.M. Axis x – the concentration of histamine in log M, axis y – the amplitude of contraction in mN.



**Fig. 2.** Effect of arginase (white columns) in a dose of 75 UI on the reactivity of tracheal and lung tissue smooth muscle to acetylcholine *in vitro* in animals with ovalbumin-induced airway hyperreactivity compared with response of animal without arginase (black columns). \*  $p < 0.05$ .



**Fig. 3.** Effect of N<sup>ω</sup>-hydroxy-L-arginine pre-treatment (NOHA) in a dose of 5 µmol (NOHA 5) or 10 µmol /organ bath (NOHA 10) *in vitro* (cross-hatch columns) compared with control group (black columns) on the reactivity of tracheal smooth muscle to histamine. \*  $p < 0.05$ , \*\*  $p < 0.01$ .



**Fig. 4.** Effect of N<sup>ω</sup>-hydroxy-L-arginine pre-treatment (NOHA) in a dose of 5 µmol (NOHA 5) or 10 µmol /organ bath (NOHA 10) *in vitro* (cross-hatch columns) compared with control group (black columns) on the reactivity of lung tissue smooth muscle to histamine. \*  $p < 0.05$ .

challenged with histamine were described in our previous papers (17). The sensitization with ovalbumin caused the statistically significant increase of the tracheal as well as lung tissue smooth muscle reactivity (figure is not showed).

Figure 1 shows the comparison of the airway reactivity changes in sensitized animals without arginase (black columns) and in the presence of arginase *in vitro* (white columns). We can see certain a tendency for the decrease of the amplitude of tracheal (mainly) and lung tissue smooth muscle contraction to histamine but that was statistically non significant if arginase was added directly to the strips in the organ bath in a dose of 75 UI.

The picture of airway smooth muscle response to acetylcholine was different. We observed the increase of tracheal smooth muscle reactivity that was statistically significant in all concentration of histamine ( $p < 0.05$ ) at the incubation of strips with arginase in a dose of 75 UI (white columns) in the comparison with the response of strips without arginase (black columns). We did not observe statistically significant changes in the response of lung tissue smooth muscle in the equal conditions (Fig. 2).

Figure 3 shows the effects of incubation of tracheal smooth muscle with arginase inhibitor – NOHA in a dose of 5 µmol or 10 µmol/organ bath (cross-hatch columns) in comparison the group of animal without NOHA (black columns). Both groups were sensitised with ovalbumin. We recorded the decrease of tracheal smooth muscle reactivity at the concentration  $10^{-4}$ – $10^{-3}$  mol/l of histamine ( $p < 0.05$ ) in a dose of NOHA 5 µmol. The increase of the dose to a 10 µmol intensified the fall of the tracheal smooth muscle response to histamine. The response was statistically significant at the concentration  $10^{-6}$  ( $p < 0.05$ ) and  $10^{-5}$ – $10^{-3}$  mol/l of histamine ( $p < 0.01$ ) in comparison to control group (black columns).

It is interesting that a statistically significant response of lung tissue smooth muscle (Fig. 4) in the concentration  $10^{-5}$ – $10^{-3}$  mol/l of histamine ( $p < 0.05$ ) is visible in a dose of NOHA 5 µmol (cross-hatch columns) in comparison the group of animal without NOHA (black columns). The higher dose of NOHA (10 µmol) did not evoke the more expressive response.

We were surprised by the response of tracheal smooth muscle to acetylcholine (Fig. 5). The incubation of tracheal smooth

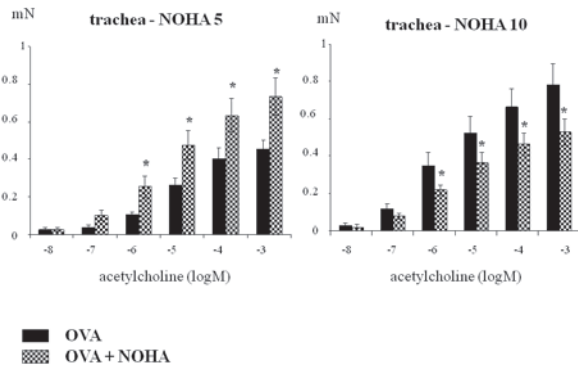


Fig. 5. Effect of N<sup>o</sup>-hydroxy-L-arginine pre-treatment (NOHA) in a dose of 5 µmol (NOHA 5) or 10 µmol /organ bath (NOHA 10) in vitro (cross-hatch columns) compared with control group (black columns) on the reactivity of tracheal smooth muscle to acetylcholine. \* p<0.05.

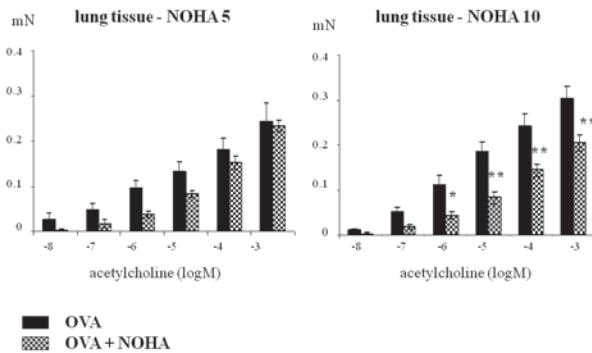


Fig. 6. Effect of N<sup>o</sup>-hydroxy-L-arginine pre-treatment (NOHA) in a dose of 5 µmol (NOHA 5) or 10 µmol /organ bath (NOHA 10) in vitro (cross-hatch columns) compared with control group (black columns) on the reactivity of lung tissue smooth muscle to acetylcholine. \* p<0.05, \*\* p<0.01.

muscle with arginase inhibitor – NOHA in a dose of 5 µmol induced the statistically significant increase of the amplitude of contraction in concentration of acetylcholine 10<sup>-6</sup>–10<sup>-3</sup> mol/l. NOHA in a dose of 10 µmol evoked in these concentrations statistically significant reversed effect – a decrease of the contraction amplitude (cross-hatch columns).

It is evident that higher dose of NOHA (10 µmol) had the more expressive effect if was administered to the lung tissue preparations (Fig. 6) and if was followed the response to acetylcholine. The fall of the contraction amplitude was statistically significant in acetylcholine concentration 10<sup>-6</sup> mol/l (p<0.05) and 10<sup>-5</sup>–10<sup>-3</sup> mol/l (p<0.01).

Figure 7 shows the comparison of the tracheal smooth muscle response from animals with ovalbumin-induced airways hyperreactivity (black columns) with response if we added into organ bath NOHA in a dose of 10 µmol (cross-hatch columns) and combination of L-arginine (10<sup>-4</sup> mol/l) with NOHA in a dose of 10 mol (grey columns). NOHA (cross-hatch columns) in a dose of 10 µmol decreased the contraction amplitude significantly in

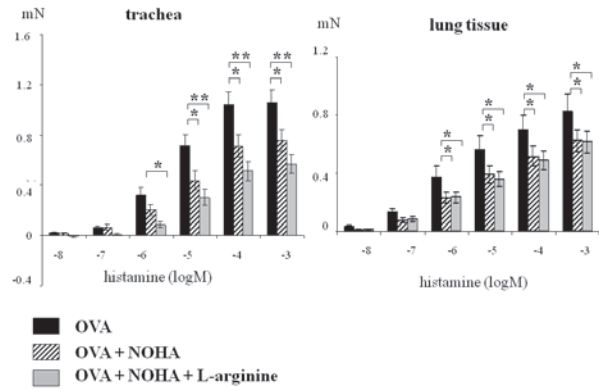


Fig. 7 and 6. Effect of N<sup>o</sup>-hydroxy-L-arginine pre-treatment (NOHA) in a dose of 10 µmol /organ bath (cross-hatch columns) and combination of L-arginine (10<sup>-4</sup> mol/l) with NOHA in a dose of 10 µmol (grey columns) in vitro (cross-hatch columns) compared with control group (black columns) on the reactivity of tracheal and lung tissue smooth muscle to histamine. \* p<0.05, \*\* p<0.01.

the concentration of histamine 10<sup>-5</sup>–10<sup>-3</sup> mol/l (p<0.05). We recorded the intensification of the response if we added the combination of precursor with arginase inhibitor (grey columns) into organ bath that was statistically significant in concentration 10<sup>-6</sup> mol/l (p<0.05) and 10<sup>-5</sup>–10<sup>-3</sup> mol/l (p<0.01). The response of tracheal smooth muscle to acetylcholine was similar but less expressive (figure is not showed).

The response of lung tissue smooth muscle in the same conditions (ovalbumin – black columns, ovalbumin+NOHA – cross-hatch columns, ovalbumin+L-arginine+NOHA – grey columns) was less expressive but statistically significant in the concentration of histamine 10<sup>-6</sup>–10<sup>-3</sup> mol/l (p<0.05).

## Discussion

The interest in arginase is growing because limitation of L-arginine bioavailability by arginase for NO synthesis from constitutive NOS can contribute to airway hyperreactivity. However, majority of studies is directed to the arginase activity resp. modulation of NOS activity by this enzyme in the vascular system. Here, arginase inhibits the production of NO via several potential mechanisms, including competition with NOS for the substrate L-arginine, uncoupling of NOS resulting in the generation of NO scavenger, superoxide, and peroxynitrite, repression of the translation and stability of inducible NOS protein, inhibition of inducible NOS activity via the generation of urea, and by sensitization of NOS to its endogenous inhibitor (13).

Upregulation of arginase inhibits endothelial NOS-mediated NO synthesis and may contribute to endothelial dysfunction in hypertension, aging, ischemia-reperfusion. Arginase also redirects the metabolism of L-arginine to L-ornithine and the formation of polyamines and L-proline that are essential for smooth muscle cell growth and collagen synthesis (18). The different isoforms of arginase are induced by lipopolysaccharides and arginase activity can be markedly inhibited during cytokine induction of iNOS because of NOHA formation. The inhibition of

arginase activity that occurs by NOHA during marked iNOS induction may be a mechanism to ensure sufficient arginine availability for high-output production of NO (19).

The role of arginase in the airway is not completely clear. It is known that in addition to the NO synthases some of the cells of respiratory system can express one or both of the arginase isoenzymes (I and II) that represent a major alternate route of arginine metabolism (6). A growing number of studies have found induction of arginase activity and its expression in a variety of inflammatory conditions suggesting that the arginase may play important roles in the pathophysiology of inflammatory diseases. Several reports have demonstrated the significant increase in both isoenzymes arginase activity and expression in animal model of asthma and in human asthmatics (in BAL, lung biopsies).

Arginase has been implicated in the pathogenesis of asthma in several ways: first, by reducing L-arginine availability it can limit NO production. Second, the ornithine produced by arginase can be converted to polyamines and/or proline, thus providing support for cell proliferation and collagen synthesis, both of which are important processes in airway remodelling in asthma and other pulmonary diseases (15). High levels of arginase can be associated with interleukin-4 and interleukin-13 over-expression, suggesting that arginine pathways are critical in the pathogenesis of asthma (20). In addition, reduced L-arginine availability for inducible NO synthase by arginase may lead to an increased production of peroxynitrite, contributing to increased airway smooth muscle contractility, airway inflammation and cell damage in this disease (21).

The activity and role of arginase in the airway (in the health or in the diseases) is thus constantly discussed. Therefore, we aimed to investigate a participation of arginase in the allergen-induced bronchial hyperreactivity because is few of studies studying the arginase activity in the airway smooth muscles. We used arginase alone and arginase inhibitor N<sup>ω</sup>-hydroxy-L-arginine (NOHA) in two doses that were administered into organ baths with strips of airway specimens removed from of animals with allergen-induced hyperreactivity. The inhibition activity of arginase provoked the dose-dependent effect that was displayed by the changes of the contraction amplitude of tracheal or lung tissue strips. N<sup>ω</sup>-hydroxy-L-arginine (NOHA) is an intermediate in the biosynthesis of nitric oxide by NO synthases and has been found to be a physiological competitive inhibitor of arginase catalysed conversion of L-arginine to L-ornithine (22, 23, 24). We recorded more expressive response of airway strips if we used arginase supplementation except the response of tracheal smooth muscle to acetylcholine that was increased in the comparison to histamine. This different response to used bronchoconstrictive mediators can be related to the different participation of both mediators in the neural reflex mechanisms. If NO serves as a braking mechanism for cholinergic mechanism so competition of arginase with NOS could result to the its weakening. The blockade of arginase activity by NOHA produced expected effect – the decrease of ovalbumin-induced airways hyperreactivity. This was dose-dependent in the whole when higher dose of arginase inhibitor NOHA provoked more expressive effect.

The competition NO synthase with arginase in the vascular system has been studied in response to inflammatory cytokines when the iNOS produces high NO amount. Under such conditions the substantial amounts of NOHA produced by iNOS inhibited the arginase activity (19). There are several possible mechanisms for decrease in NO bioavailability in the airway dysfunction that can be similar to endothelial dysfunction. They include modulation of NOS activity by arginase. The inhibition of arginase can restore NO signalling role and airway tone by regulating of L-arginine availability for NO synthases activity, cGMP production and smooth muscle relaxation (25). The results of our experiment – the enhanced relaxant response of the airways smooth muscle to arginase inhibition as well as a potentiation of this response by L-arginine confirms this thesis. Topal et al (26) propose that endothelial NO synthesis depends on the activity of cell membrane L-arginine carriers and mitochondrial arginase II through two types of L-arginine pools. They suggest that the freely exchangeable pools are caveolae whereas the nonexchangeable ones are mitochondriae. Can we propose similar situation in the respiratory system, too?

We recorded the difference in the used agents action at different levels of the airway. In the whole, tracheal smooth muscle responses we can assess as to be more expressive. We assume that the different localization of enzymes utilizing L-arginine as well as the antioxidant mechanisms in the upper and lower airway can be a cause resulting in the different response of these areas (27). This result can be connected with another fact that NOS neurons utilizing L-arginine are higher in number in proximal than distal airways and NO acts as bronchodilator mainly in proximal airways (28). It is probably a reason for the finding that NO prevents more the contraction of the large airways than small ones (29, 30). It is necessary to take into consideration the participation of vessels in case of lung tissue (31). It is possible to suppose increased L-arginine uptake from the vascular space in the lung tissue or the increase in L-arginine uptake involved increased CAT-1 or CAT-2 transporter density or activity (32).

It is interesting the difference in the response of the airways to used mediators of bronchoconstriction – histamine or acetylcholine. This difference is likely due to differences in the contribution of a neural reflex mechanism. Histamine causes bronchoconstriction not only directly by inducing the contraction of airway smooth muscle through its receptors but also indirectly via the excitation of a cholinergic pathway by neural reflex. Acetylcholine, in contrast, is less effective in eliciting bronchoconstriction by neural reflex. We can suppose that differences in the localization of different receptors for these mediators or an alternative nervous system of airway (iNANC nervous system with vasoactive intestinal polypeptide, sympathetic nervous system etc.) could due these responses (33).

Our experiments fill in the information in effects of the modulation of arginase activity in the conditions of ovalbumin-induced airway hyperreactivity that are unsatisfactory. They confirm also in accord with literature that arginase may be one of important factors influencing the airway reactivity. We suppose that L-arginine level in our model of the bronchial hyperreactivity can decrease by higher activity of arginase. This confirms the results that show the decrease

of ovalbumin-induced hyperreactivity after inhibition of arginase activity with NOHA or favourable effect of supplementation of L-arginine on the airway smooth muscle responses in the conditions of the airway hyperreactivity. Arginase thus offers another possibility for therapeutic intervention in diseases connected with this symptom.

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