Bratisl Lek Listy 2008; 109 (5)
191 – 197

EXPERIMENTAL STUDY

Effect of NO-synthase and arginase inhibition in airway hyperreactivity

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Abstract: *Background:* The decreased L-arginine bioavailability, the basic substrate for nitric oxide synthesis, can be one of the factors contributing to the airways hyperreactivity.
Objectives: We investigated the effect of various inhibitors of the enzyme activities utilizing L-arginine in a guinea pig model of experimental ovalbumin-induced airway hyperreactivity.
Methods: We used the in vivo pre-treatment with non-specific inhibitor of NO synthase Nω-nitro-L-arginine metylester (L-NAME) and relatively specific inhibitor of inducible NO synthase – aminoguanidine. Inhibitors were administered in one-shot (on the 14th day, 30 minutes before the inhalation of ovalbumin) or in a long-time regime (during the whole period of sensibilization by ovalbumin – 14 days). We administered the inhibitor of arginase Nω-hydroxy-L-arginine (NOHA) to the tracheal and lung tissue smooth muscle strips from sensibilized animals.
Results: We observed an increase in the tracheal smooth muscle response to histamine in animals that received an inhalation dose of L-NAME (40 mg/kg b.w.) or aminoguanidine (50 mg/kg b.w.) 30 minutes before the inhalation of ovalbumin but did not evoke any significant difference in the reactivity of lung tissue smooth muscle. Tracheal smooth muscle responded with enhanced contraction amplitude to histamine after chronic pre-treatment with L-NAME or aminoguanidine. The inhibition of arginase with NOHA in vitro decreased the tracheal and lung tissue smooth muscle reactivity to histamine.
Conclusion: The results suggest that NO-synthase isoforms as well as arginase are involved in the production of NO and in the control of bronchomotoric tonus (Fig. 4, Tab. 2, Ref. 31). Full Text (Free, PDF) *www.bmj.sk.*
Key words: airway hyperreactivity, ovalbumin, guinea pig, NO synthases, arginase.

The regulation of physiological functions of the respiratory system is under control of different mediators that are also involved in the pathophysiology of the respiratory diseases. Some of these hallmarks (asthma, chronic obstructive pulmonary disease) are associated with increased airway reactivity to a variety of stimuli including allergens, chemical irritants, cold air and pharmacological agents such as histamine and metacholine (1). Endogenous nitric oxide (NO) plays an important role in the regulation of the airway tone as well as in the airway inflammation and may be importantly involved in the development of airways hyperreactivity (2).

Nitric oxide is a gaseous free radical that is generated by a family of NO synthase (NOS) isoforms that utilize the semi-essential amino acid L-arginine. In the airways, constitutive NOS (cNOS) isoforms – neuronal (nNOS) and endothelial NOS (eNOS) – are mainly expressed in inhibitory nonadrenergic noncholinergic (iNANC) neurons (nNOS), endothelium (eNOS) and epithelium (nNOS and eNOS) whereas inducible NOS (iNOS) which is induced by proinflammatory cytokines during airway inflammation is mainly expressed in macrophages and epithelial cells (3). The deficiency in cNOS-derived NO is involved in the development of the airways hyperreactivity (4, 5). It is interesting that the activities of individual NOS isofoms are dependent on the type of hyperreactivity-inducing factors. Jang et al (6) present that ozone exposure increases eNOS and nNOS activity and decreases iNOS activity. Other authors suppose the increase in the activity of iNOS synthase in the conditions of airways hyperreactivity (7). The questions as to which isoform is the most important in allergen-induced hyperreactivity and whether the inducible NO synthase plays a prominent role in the development of airway hyperreactivity are discussed.

Recent studies have indicated that alterations in L-arginine homeostasis and limitation of L-arginine availability to NOS play a major role in NO deficiency and airway hyperreactivity (8, 9). Thus, another enzyme utilizing L-arginine attracts attention in the resent years - arginase. The increased activity of arginase that hydrolyzes L-arginine into L-ornithine and urea may be an important mechanism involved in the reduced bioavailability of L-arginine in the airways (10). Arginase I is a cytosolic enzyme present mainly in the liver. Arginase II is a mitochondrial en-

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Acknowledgement: This work was supported by grants VEGA 1/3375/06 and MZ 2005/13-MFN-05.

zyme that is functioning mainly in extrahepatic tissues (11). Endogenous arginase activity is functionally involved in the regulation of airway smooth muscle tone (9, 12) and can impair a neuronal nitric oxide-mediated airway smooth muscle relaxation in some conditions (13). Both arginase isoforms are highly upregulated in asthmatic lung as well as NOS enzymes that can result in cross-interactions of both types of enzymes.

The changes in both enzymatic system – NO synthases and arginases can modify the NO availability, which might be of therapeutic interest. If NO participates in the allergen-induced airway reactivity changes, then a modulation of enzyme activity can be reflected in response. Therefore, in the present study we investigated whether the modification of the NO synthase and arginase activity will affect the experimental allergen-induced airway hyperreactivity. We used the in vivo pre-treatment of animals with non-specific NO synthase inhibitor Nω-nitro-L-arginine metylester (L-NAME) or with relatively specific inducible NO synthase inhibitor – aminoguanidine. These agents were used in different regimens of *in vivo* treatment – both acute and chronic. We suppressed the activity of arginase with Nω-hydroxy-L-arginine (NOHA) in *in vitro* conditions in the smooth muscles preparations from sensibilized animals.

Material and methods

Animals and agents

Pathogen-free adult male Trik guinea pigs weighing 250–350 g were used in our study. The 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 hrs light/dark regimen was maintained. We used 8 groups of animals:

Group 1 (n=8) inhaled Nω-nitro-L-arginine metylester (L-NAME) (subst. Sigma) in a dose of 40 mg/kg b.w. on 14th day, 30 minutes before the inhalation of ovalbumin.

Group 2 (n=8) inhaled aminoguanidine (subst. Sigma) in a dose of 50 mg/kg b.w. on 14th day, 30 minutes before the inhalation of ovalbumin.

Group 3 (n=8) was pre-treated with Nω-nitro-L-arginine metylester (L-NAME) (subst. Sigma) in a dose of 40 mg/kg b.w. intraperitoneally during the whole period of sensibilization by ovalbumin (14 days).

Group 4 (n=8) received aminoguanidine (subst. Sigma) in a dose of 50 mg/kg b.w. intraperitoneally during the whole period of sensibilization by ovalbumin (14 days).

In group 5 (n=8) we administered Nω-hydroxy-L-arginine (NOHA) into the bath with organ strips of tracheal and lung tissue smooth muscle from animals with ovalbumin-induced airways hyperreactivity in a dose of 5 μmol/organ bath (30 ml).

*The control group*s (one for the groups 1 and 2, second for the groups 3 and 4 with eight animals in each group) were sensitized by ovalbumin but administered with dissolving agent – aqua pro injectione in a dose of 1.0 ml on the 14th day, 30 minutes before the inhalation of ovalbumin or received aqua pro injectione in a dose of 1.0 ml during the whole period of sensibilization by ovalbumin (14 days). In the third control group (for the group 5) we added a dissolving agent – aqua pro injectione into the bath with organ strips in volume 0.2 ml.

Induction of allergen-induced hyperreactivity

The modified method of Fraňová et al (14) with sensibilization of guinea pigs by allergen (ovalbumin) was used in order to provoke the airways hyperreactivity. An allergen solution containing 100 $\mu mol.ml^{-1}$ of ovalbumin in saline was injected in exact time intervals, namely, 0.5 ml intraperitoneally and 0.5 ml subcutaneously on the 1st day, 1 ml intraperitoneally on the 3rd day and 1 ml by inhalation on the 14th day. The inhalation of ovalbumin was performed in a body plethysmograph (Hugo Sachs Electronic, type 885, Germany) for rodents and small animals.

Animals received humane care in compliance with the Guide for Care and Use of Laboratory Animals. The Ethical Committee of Jessenius Faculty of Medicine approved the study protocol.

Measurement of airway responsiveness in vitro

The animals were killed 24 hours after last allergen provocation – on the 15th day. Strips from trachea and lung tissue (lower part of diaphragmatic lobe of the lungs) were prepared and placed into organ bath with Krebs-Henseleit solution (110.0 mol/l NaCl, 4.8 mol/l KCl, 2.35 mol/l $CaCl_2$, 1.20 mol/l $MgSO_4$, 1.20 mol/l $KHPO_4$, 25.0 mol/l $NaHCO_3$ and 4 g glucose in glass-distilled water). The solution was continuously oxygenated with mixture of 95 % O_2 and 5 % CO_2 at pH 7.5±0.1 and temperature 36±0.5 °C. One of the strip endings was connected to a force transducer (TSR 10G, Vývoj Martin, Slovakia) 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 tissue strips were exposed initially to the tension of 4 g (30 minutes – loading phase). Thereafter, the tension was readjusted to a baseline of 2 g (30 minutes – adaptation phase). The Krebs-Henseleit solution was changed every 10 minutes. Strips were contracted by cumulative doses of histamine (10^{-8}–10^{-3} mol/l) or acetylcholine (10^{-8}–10^{-3} mol/l).

Statistical analysis

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

Results

Figure 1 shows the changes in airway reactivity in guinea pigs sensitized with ovalbumin (grey columns) in comparison with the control animals to histamine (white columns) that have been recorded in our previous experiments. The control group received physiological salt solution instead of ovalbumin in an equal regimen. The sensibilization caused an increase in the reactivity of tracheal (10^{-5}–10^{-3} mol/l histamine – $p<0.01$) as well as lung tissue smooth muscle (10^{-6}–10^{-3} mol/l histamine).

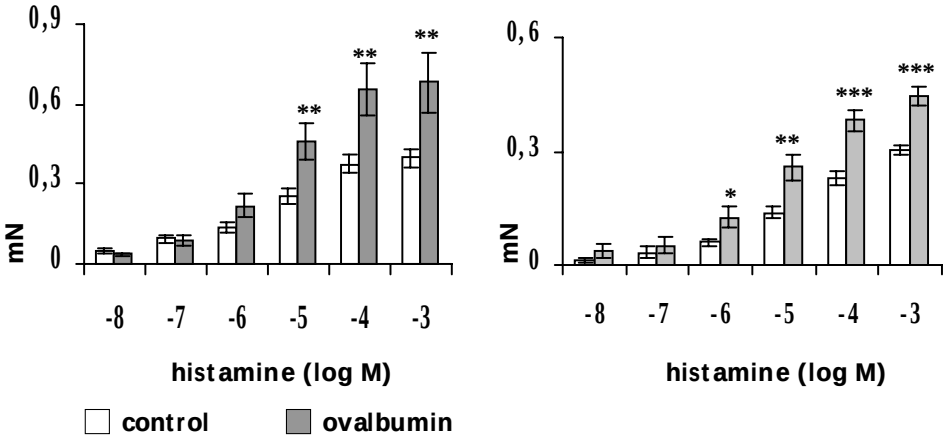

Fig. 1. The comparison of changes in airways reactivity in animals sensitized (grey columns) with ovalbumin and animals that received saline solution (white columns) to histamine. Sensitization with ovalbumine increased the amplitude of the contraction of tracheal (left) and lung tissue (right) smooth muscle. The columns represent the average values of the contraction amplitude with mean average ± S.E.M. Axis x – the concentration of histamine in log M, axis y – the amplitude of contraction in mN. * p<0.05, ** p<0.01, *** p<0.001.

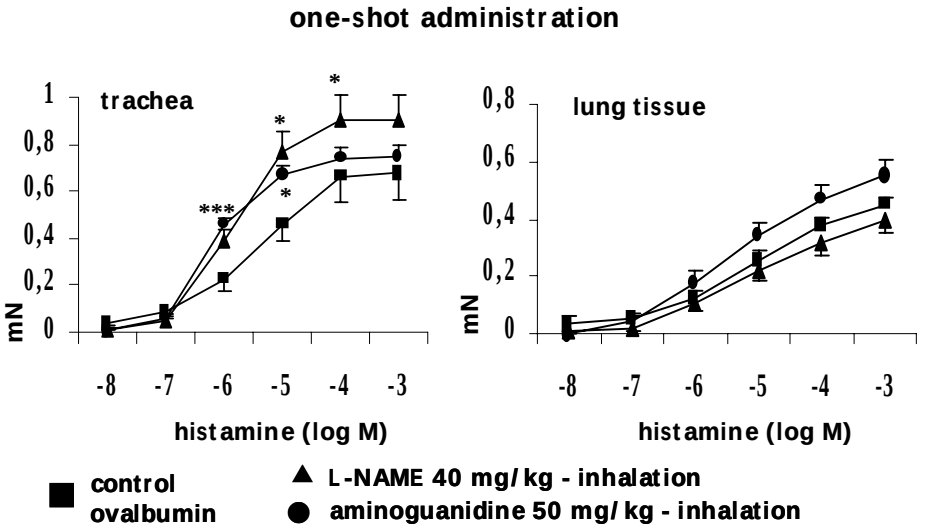

Fig. 2. Effect of pre-treatment with L-NAME (▲) or aminoguanidine (●) administered by inhalation 30 minutes before the last inhalation of ovalbumin on the reactivity of tracheal (left) and lung tissue (right) smooth muscle to histamine compared with control group (■). Pre-treatment with NOS inhibitors significantly enhanced the response of tracheal smooth muscle. Data are the mean±S.E.M. *p<0.05, ***p<0.001.

The effect of L-NAME in a dose of 40 mg/kg b.w. (▲) or aminoguanidine (●) in a dose of 50 mg/kg b.w. that were administered inhalation on the 14th day, 30 minutes before the inhalation of ovalbumin on the reactivity of tracheal and lung tissue smooth muscle to histamine is compared with the control group (■) (Fig. 2). We observed an increase in tracheal smooth muscle response to histamine. The amplitude of contraction was significantly enhanced ($p<0.05$) at the concentration of 10^{-5} and 10^{-4} mol/l of histamine in the case of L-NAME inhalation (▲). We recorded similar changes with aminoguanidine pre-treatment (●) but the significance of the changes was $p<0.05$ at the concentration of 10^{-5} mol/l and $p<0.001$ at the concentration of 10^{-6} mol/l of histamine.

Administration of NOS inhibitors in the equal regime did not evoke significant difference in the reactivity of lung tissue smooth muscle when we compare the changes with the control group, although showed is a tendency of an increase in the contraction amplitude in the animals that received inhalation doses of aminoguanidine before the last administration of ovalbumin (Fig. 2).

Tracheal smooth muscle strips responded by raising the contraction amplitude to histamine in case that the animals received the 14-days pre-treatment with L-NAME in a dose of 40 mg/kg

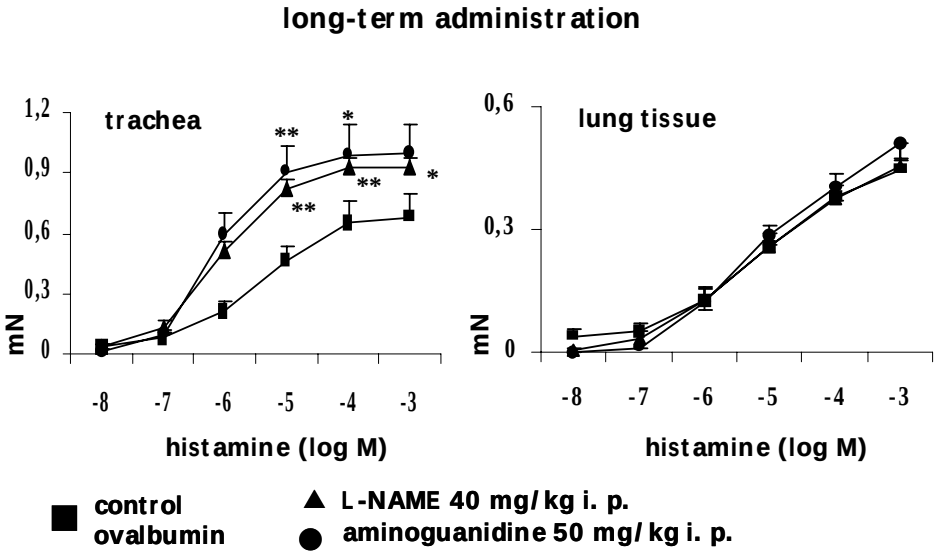

Fig. 3. Effect of pre-treatment (14 days) with L-NAME (▲) or aminoguanidine (●) intraperitoneally (i.p.) on the airways reactivity changes. The response of tracheal smooth muscle to histamine was increased in pre-treated animals. Data are the mean ± S.E.M. *p<0.05, **p<0.01.

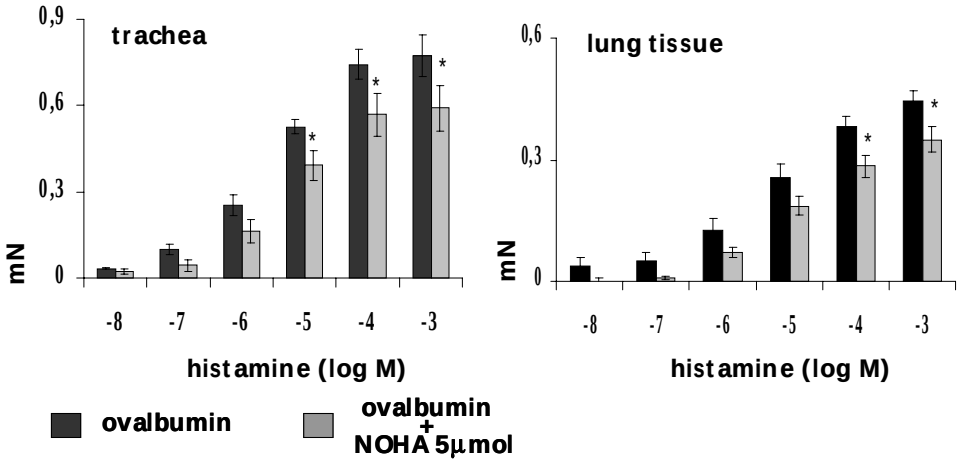

Fig. 4. Effect of N$^{\omega}$-hydroxy-L-arginine pre-treatment (NOHA – grey columns) in vitro compared with control group (black columns) on the reactivity of tracheal and pulmonary smooth muscle to histamine. Arginase inhibitor decreased the amplitude of contraction in tracheal (left) and pulmonary (right) smooth muscle. The columns represent the average values of the contraction amplitude with mean average ± S.E.M. Axis x – the concentration of histamine in log M, axis y – the amplitude of contraction in mN. *p<0.05.

b.w. intraperitoneally (i.p.) (▲) or aminoguanidine (●) in a dose of 50 mg/kg b.w. i.p. The amplitude of tracheal smooth muscle contraction was statistically significant. It increased with the concentration of histamine 10^{-5}–10^{-3} mol/l (L-NAME) and 10^{-5}–10^{-4} mol/l (aminoguanidine). However, the effects of both inhibitors were the same in whole (Fig. 3).

Figure 3 shows also that long-term pre-treatment (14 days) with L-NAME in a dose of 40 mg/kg b.w. i.p. (▲) or aminoguanidine (●) in a dose of 50 mg/kg b.w. i.p. did not evoke any statistically significant changes in the reactivity of lung tissue smooth muscle to histamine in comparison to the control group (■). We obtained an almost identical picture of changes in reactivity to acetylcholine, therefore we do not show the figures.

The results of experiments are summarized in Table 1. It is obvious that the difference in the effects of NOS inhibitors depends on the airways level, i.e. the effect is more expressive in trachea than lung tissue. We can see an increase in tracheal smooth muscle reactivity after one-shot and long-term administration of both used NOS inhibitors. The duration of the pre-treatment does not affect the airways reactivity changes.

Tab. 1. Changes in airway reactivity after pre-treatment with L-NAME or aminoguanidine (I – one-shot therapeutic regimen, II – long-term therapeutic regimen).

		L-NAME		Aminoguanidine	
		I	II	I	II
Trachea	histamine	↑	↑	↑	↑
	acetylcholine	↑	↑	↑	↑
Lung	histamine	–	–	–	–
	acetylcholine	–	–	–	–

↑ increase of reactivity, – unaltered

Tab. 2. Changes in airway reactivity after pre-treatment with NOHA in vitro.

	Mediator	NOHA
Trachea	histamine	↓
	acetylcholine	↑
Lung	histamine	↓
	acetylcholine	↓

↑ increase of reactivity, ↓ decrease of reactivity

In Figure 4 we illustrate the effects of incubation of tracheal and lung tissue smooth muscle with arginase inhibitor – NOHA in a dose of 5 μmol/organ bath with volume of 30 ml (grey columns). We recorded a decrease in tracheal smooth muscle reactivity at the concentration 10^{-5}–10^{-3} mol/l of histamine ($p<0.05$) and a decrease in lung tissue reactivity at concentration 10^{-4}–10^{-3} mol/l of histamine in comparison to the control group (black columns). The results summarized in Table 2 show the decrease in the airways reactivity in the tissue with NOHA incubation with the exception of tracheal smooth muscle reactivity to acetylcholine.

Discussion

Some of respiratory diseases including asthma and chronic obstructive pulmonary diseases are attended by the airways hyperreactivity. There are multiple mechanisms contributing to this symptom, including the interference in NO homeostasis (2). As for the possible importance of modulation of NO synthases and arginase pathways, we aimed to investigate the effect of a modification of the activity of these enzymes in a model of experimental ovalbumin-induced airway hyperreactivity. In the present study we demonstrated that the inhibition of NO synthases activity by L-NAME and aminoguanidine increased the tracheal smooth muscle reactivity to histamine and acetylcholine in one shot as well as after long-term pre-treatment, but evoked no reactivity changes in lung tissue smooth muscle. The administration of arginase inhibitor – NOHA caused a decrease in the airways reactivity on the whole. The expected response of tracheal smooth muscle to acetylcholine may be associated with the different localization of enzymes utilizing L-arginine in the upper and lower airways or with the differences in the contribution of 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 the cholinergic pathway by neural reflex. As opposed to the latter, acetylcholine, is less effective in eliciting bronchoconstriction by neural reflex. We can suppose that differences in the localization of different receptors for these mediators could cause these responses. It is necessary to take into consideration the participation of vessels in the case of lung tissue.

The idea of utilizing the inhibitors of NO synthase in order to modify the NO formation emerged together with the discovery of the physiological NO effects (15, 16). It is assumed that a deficiency in cNOS-dependent NO release takes place in parallel with excessive production of NO by iNOS in allergen-induced airway hyperresponsiveness (17). The normal expressions and activities of eNOS and nNOS were recorded in bronchial hyperreactivity in other studies (7, 18) but the iNOS expression and activity were increased. cNOS and iNOS are both differently regulated and involved in the regulation of different processes. Although this notion may suggest that these enzymes function independently, the growing experimental evidence has led to the development of a theory on cross-talk between cNOS and iNOS (19). The expression of iNOS is a NO-regulated process. Low amounts of exogenous as well as endogenous NO inhibit NF-kB activation and suppress iNOS expression in cell types that express cNOS. Generation of NO by cNOS may represent a limiting factor for iNOS expression (20). Nitric oxide produced mainly by eNOS controls the smooth muscle tone (5) but NO produced by nNOS is a functional antagonist of excitatory cholinergic neurotransmission as well. The increase in iNOS expression and activity is considered in a majority of studies as the crucial trigger of hyperreactivity. These facts were the reason for using both agents – L-NAME and aminoguanidine in our study. The effect of both inhibitors on the airways response was similar in our study and we are not able to determine which of the isoforms plays the most important role in our experimental conditions. For this purpose more specific NO synthase inhibitors need to be used.

It is interesting that in our previous experiments we found an opposite effect with NOS inhibitors pre-treatment. L-NAME and aminoguanidine showed a mainly beneficial effect in the airways hyperreactivity induced by other trigger the exogenous irritant – toluene vapour (21). The inhibitors decreased the hyperreactivity of the tracheal and lung tissue smooth muscle that had been evoked by toluene. The decrease was dependent on the duration of their administration and on the type of inhibitor. Short-term administration of inhibitors was more effective than long-term administration. A more significant effect was recorded after the pre-treatment with non-selective inhibitor L-NAME (12) that gives a hypothesis for the participation of constitutive NO synthases mainly.

The different effects of L-NAME were described in various experimental studies. L-NAME inhibits all types of NO synthase with subsequent inhibition of NO release by various cells including the airway smooth muscle cells. Mehta et al (4) described an increase in the contractile responses to histamine after the inhibition of NO production with L-NAME. They reported that L-NAME enhanced bronchial reactivity to histamine in control group but not in antigen-challenged guinea pigs suggesting a defect in bronchodilation activity of NO in the challenged animals. This defect probably did not arise from decreased NO production because the exhaled concentrations were the same in both control group and challenged animals. This fact suggests that the effect of L-NAME depends more on doses or different mechanisms of action than on NO synthases inhibition.

Prado et al (22) tested the differences between chronic and acute nitric oxide inhibition by L-NAME treatment in lung mechanisms, inflammation and airway remodelling in guinea pigs. They found out that both acute and chronic L-NAME treatments reduced the exhaled NO in sensitized animals. They showed that acute inhibition of NO production resulted in an increase in respiratory system elastance, whereas chronic inhibition resulted also in an increase in baseline respiratory system resistance. They considered chronic bronchodilator effects of NO to be related to actions predominantly in the larger airways. This supports our results as well.

The deficiency in NO may be caused by limited amount of L-arginine as a substrate for NO synthases. The enhanced competition between NO synthases and arginase for substrate leads to the negative cross-talk between these enzymes. This could be another mechanism of hyperreactivity (23, 24). High arginase activity may contribute to low circulating L-arginine levels, thereby to the limitation of L-arginine bioavailability and to NO deficiency that induces hyperreactivity of the airways (25). Although the affinity of L-arginine is much higher for NOS than for arginase, the maximum activity of arginase is more than 1000-fold of that of NO synthases suggesting similar rates of substrate utilization at physiological L-arginine concentrations. The arginase-mediated removal of L-arginine inhibits the expression of iNOS that may inhibit iNOS-mediated NO production. These findings suggest that arginase down-regulates the NO formation via multiple mechanisms. Some reports suggest (26) that limited L-arginine availability also promotes uncoupling of eNOS resulting in oxygen free radical formation (27) that can directly inactivate NO and incur damage of airways functions. Arginase-mediated impairment of NO synthesis has been implicated in asthma, cystic fibrosis etc. (9, 28). Interestingly, NOHA, which is an intermediate form produced during the catalysis of L-arginine by NOS, is a potent inhibitor of arginase, suggesting that NO synthase may also influence the arginase activity. We recorded a beneficial effect of NOHA on the responsiveness of the airways tissue in sensitized animals that was manifested by the decrease in amplitude contraction. We propose that arginase participates and has more important roles in our conditions of the airways hyperreactivity.

In addition, arginases are constitutively expressed in the airways, particularly in the bronchial epithelium and in peribronchial connective tissue fibroblasts. Arginase also redirects the metabolism of L-arginine to L-ornithine and the formation polyamines and L-proline that are essential for smooth muscle growth and collagen synthesis. Therefore, the induction of arginase may also promote the remodelling of airways (29) that can change the situation in the airways and may modify the airways response.

We recorded a difference in the action inhibitors at different levels of the airway. We assume that the different localization of enzymes utilizing L-arginine as well as the localization of antioxidant mechanisms in the upper and lower airway can be the cause resulting in different responses in these areas. This result can be connected with the fact that the number of NO synthases neurons utilizing L-arginine in the proximal airways are higher than that in distal airways. This is probably the reason for the finding that the prevention of contraction in the large airways by NO is more pronounced than in small ones. It is necessary to take into consideration the participation of vessels in case of lung tissue (30).

In conclusion, our experiments confirm that L-arginine/NO pathway may be one of the factors influencing the airway reactivity via the enzymes utilizing this substrate, namely NO synthase and arginase.

Based on our results together with other studies (22, 29, 31) we can propose that both types of enzyme but mainly arginase might be a highly attractive therapeutic target of modifying the response of airways smooth muscle to the detrimental insults. The continued development of specific molecules directed to the activity modulation of enzymes utilizing L-arginine represents a promising area of research that may lead to new therapeutic interventions in the treatment of respiratory diseases.

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Received October 26, 2007.
Accepted March 28, 2008.