Relation of L-arginine to airway hyperreactivity

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Abstract. The deficiency or the decrease in the bioavailability in basic substrate for nitric oxide synthesis – L-arginine can be one of factors contributing to the airway hyperreactivity. We studied the influence of L-arginine supplementation on the experimental airway hyperreactivity induced in guinea pigs by exposure to toluene vapours. L-arginine was administered before exposure in a dose of 300 mg/kg b.w. intraperitoneally during 3 or 17 days. After that the airway reactivity changes to histamine or acetylcholine were studied in *in vitro* conditions. In addition to that the tissue strips from exposed animals were incubated with L-arginine in concentration 10^{-4} mol/l. The administration of L-arginine during 3 days decreased the airway reactivity increased by irritant exposure. We recorded the decrease in the airway reactivity in animals with bronchial hyperreactivity after incubation of tissue strips with L-arginine, too. The pre-treatment of animals with L-arginine during 17 days did not affect the airway smooth muscle reactivity in larger extent. The exogenous administration of L-arginine resulted in a protective effect under the conditions of experimental airway hyperreactivity. The effect of supplementation was different depending on airway level and pre-treatment duration. The results refer to the importance of optimal L-arginine level for the control of bronchomotoric tone.

Key words: L-arginine — Airway hyperreactivity — Exogenous irritant — Guinea pig

Introduction

L-arginine is an amino acid present in the proteins of all life forms. In mammals, it is classified as a semi-essential or conditionally essential amino acid because the ability of body to synthesize sufficient quantities to meet its needs varies according to developmental stage and the health status of the individual (Barbul 1986). L-arginine becomes essential amino acid e.g. under conditions involving the catabolic state such as sepsis, burn injury, trauma when the capacity of endogenous L-arginine synthesis is surpassed. L-arginine was recently shown to act as an important molecule that regulates essential cellular functions. It is one of the more metabolically versatile amino acids, however, its metabolism is complex and highly regulated. It gives rise not only to proteins but also to nitric oxide (NO), urea, citrulline, creatine, agmatine, glutamate, polyamines, proline. These metabolites can act as signalling molecules or as intermediates for another metabolic pathways (Wu and Morris 1998). Thus, L-arginine through its metabolites is involved in function control of different systems and organs including respiratory system (King et al. 2004; Morris 2004).

The interest in L-arginine metabolism has increased greatly over the past 15 years, it was triggered primarily by the discovery of NO synthesis in mammals and its remarkable biological roles. Most pharmacological actions of L-arginine are anyway attributed to NO (Nevin and Broadley 2002; Ricciardolo 2003). NO is a significant neurotransmitter of the inhibitory nonadrenergic noncholinergic nervous system in the airways. It 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.

NO is produced by constitutive isoforms (cNOS) of NO synthases (nNOS – neuronal and eNOS – endothelial) in the physiological conditions or by inducible NOS and thus it may contribute to development of various respiratory illnesses (asthma, chronic obstructive pulmonary disease, tuberculosis, tumours, virus infections, etc.) (Nevin and Broadley 2002; Ricciardolo 2003). We also demonstrated in our previous experiments the involvement of NO in the airway reactivity changes under the experimental conditions (Antošová et al. 2005a,b,c).

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L-arginine as a substrate is utilized and metabolized through another enzymes – arginase I and arginase II to L-ornithine and urea. Arginase I is a cytosolic enzyme present mainly in the liver. Arginase II is a mitochondrial enzyme that is functioning mainly in extrahepatic tissues (Wu and Morris 1998). Endogenous arginase activity is functionally involved in regulation of airway smooth muscle tone (Meuers et al. 2003; Antošová et al. 2005c) and can impair a neuronal NO-mediated airway smooth muscle relaxation in some conditions (Maarsingh et al. 2005, 2006). Both arginase isoforms are highly upregulated in asthmatic lung as well as NOS enzymes that can result in cross-interactions. Studies of these conditions have involved primarily the inducible NOS (iNOS) and arginase I isoenzyme. As both enzymes use the same substrate, one would anticipate that their simultaneous expression should result in the competition for substrate and can limit the availability of L-arginine for NO synthesis in optimal quantities. The L-arginine deficiency can result in the damage of bronchomotor tone control (Meuers et al. 2003; Maarsingh et al. 2005).

Majority of studies are concerned with the role of L-arginine in the cardiovascular system (Solomonson et al. 2003). It has not yet been established the real role of L-arginine in the airway hyperreactivity although there was an effort to use L-arginine therapeutically in respiratory diseases with this symptom (Kharitonov 2005). Therefore, we studied whether and how the supplementation of L-arginine can influence the experimental airway hyperreactivity or if it has beneficial effects on reactivity changes evoked by exogenous irritant.

Materials and Methods

Animals and agents

Eight groups of pathogens-free male Trik guinea pigs (250–350 g) were used in our study. The animals were housed in individual cages in climate-controlled animal quarters and received water and food ad libitum. We used the pre-treatment with L-arginine in following regimens:

Group 1 (n = 8) received L-arginine (Sigma) in a dose of 300 mg/kg intraperitoneally (i.p.) in each of three consecutive days, 30 min before the provocation of the airway hyperreactivity by toluene. The administration of agent during 3 days was considered to be a short-term administration.

Group 2 (n = 8) was treated with L-arginine in a dose of 300 mg/kg i.p. in each of 17 consecutive days before the provocation of the airway hyperreactivity. Animals received L-arginine 30 min before toluene exposure during last 3 days. The administration of agent during 17 days was considered to be a long-term administration.

In Group 3 (n = 8) we administered L-arginine to strips of tracheal and lung tissue smooth muscle from animals with the airway hyperreactivity in organ bath in a dose of 10^{-4} mol/l.

Group 4 (n = 5) was treated with L-arginine in a dose of 300 mg/kg i.p. during 3 days without the provocation of the airway hyperreactivity.

The control group (n = 8) for each of experimental groups was without of L-arginine pre-treatment and received dissolving agent – aqua pro injectione in a dose of 0.3 ml i.p.

Toluene exposure

In our experiment we used the method of *in vivo* exposition to the toluene described by Strapková et al. (1995). The animals were spontaneously breathing toluene vapours in a special exposure chamber made of Plexiglas. The chamber consists of compressor, flow-meter, vaporizer and exposure cage. The device was situated in the fume-cupboard at 22°C. Toluene vapours were delivered into cage with constant flow of 4 l/min. The average concentration of toluene was 6 mg/l (1600 ppm). The duration of exposure was 2 h in each of three consecutive days.

Airway responsiveness

Animals were sacrificed 24 h after the last toluene exposure. The Ethical Committee of Jessenius Faculty of Medicine approved the study protocol. Strips from trachea and lung tissue 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 aerated 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 min – loading phase). Thereafter, the tension was readjusted to a baseline of 2 g (30 min – adaptation phase). The Krebs–Henseleit solution was changed every 10 min. The strips were contracted by cumulative doses of histamine or acetylcholine (10^{-8}–10^{-3} mol/l).

Statistical analysis

Statistical analysis was performed using ANOVA test. Differences were considered statistically significant when p-value was below 0.05. All results are expressed as mean ± SEM.

Results

We compared the response of tracheal smooth muscle to histamine or acetylcholine in the healthy animal with healthy

animals that received L-arginine in a dose of 300 mg/kg i.p. in the 3 consecutive days and animal with toluene-induced airway hyperreactivity with animal received L-arginine in a dose of 300 mg/kg i.p. administered 30 min before the provocation of the airways hyperreactivity by toluene exposure in the 3 consecutive days (Fig. 1).

We observed the decrease in the contraction amplitude of tracheal smooth muscle that was statistically significant in the concentration of histamine 10^{-4} – 10^{-3} mol/l ($p < 0.05$) (Fig. 1A) and of acetylcholine 10^{-4} – 10^{-3} mol/l ($p < 0.01$) and 10^{-5} mol/l ($p < 0.05$) (Fig. 1B) in the animals with hyperreactivity that received L-arginine. It is interesting that we recorded the increase in the contraction amplitude of the tracheal smooth muscle statistically significant in the concentration of histamine 10^{-5} – 10^{-3} mol/l ($p < 0.05$) or 10^{-4} – 10^{-3} mol/l ($p < 0.05$) of acetylcholine in animals without airway hypereactivity that received L-arginine.

The administration of L-arginine in the equal regime did not evoke statistically significant difference in the reactivity of lung tissue smooth muscle to histamine when we compare the changes to animals inhaling irritant without pre-treatment with L-arginine, although it is showed that a tendency of the fall in contraction amplitude (Fig. 2A). We recorded a very expressive statistically significant decrease in lung

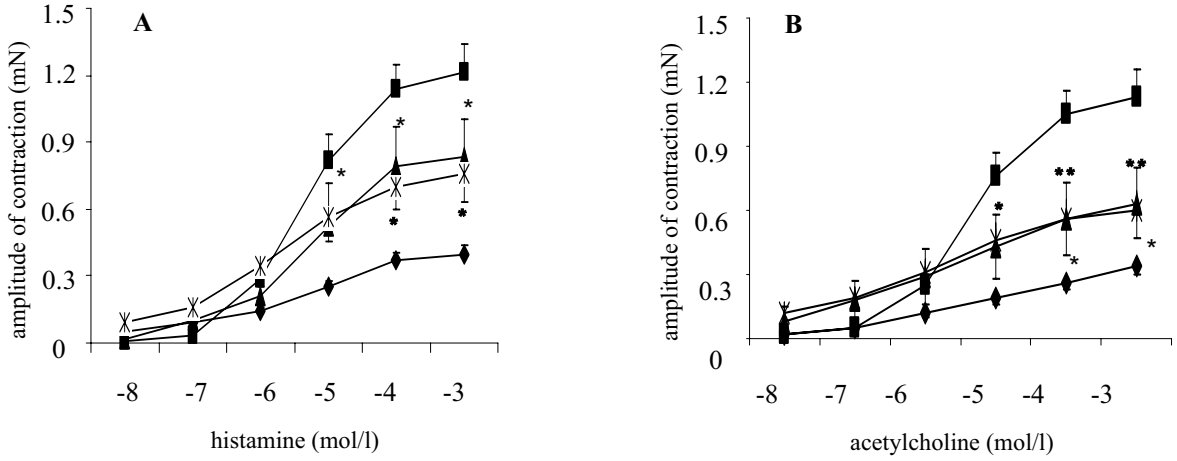

Figure 1. Response of tracheal smooth muscle to histamine (A) or acetylcholine (B) in the healthy animal (◆) with healthy animals received L-arginine in a dose of 300 mg/kg i.p. in the 3 consecutive days (▲) and animal with toluene-induced airway hyperreactivity (■) with animal received L-arginine in a dose of 300 mg/kg i.p. administered 30 min before the provocation of the airways hyperreactivity by toluene exposure in the 3 consecutive days (✕). The lines represent average values of the contraction amplitude with mean mistake average ± S.E.M. * $p < 0.05$, ** $p < 0.01$.

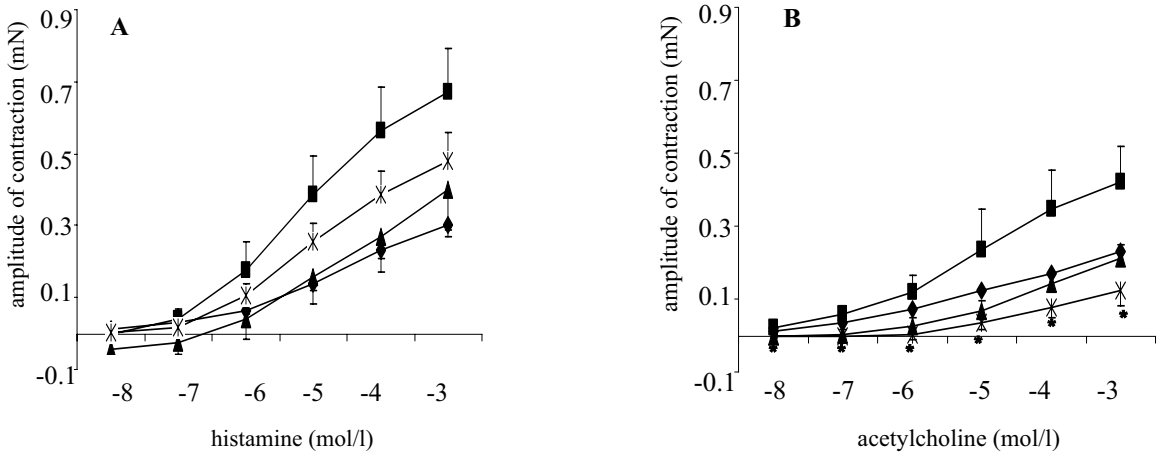

Figure 2. Response of lung tissue smooth muscle to histamine (A) or acetylcholine (B) in the healthy animal (◆) with healthy animals received L-arginine in a dose of 300 mg/kg i.p. in the 3 consecutive days (▲) and animal with toluene-induced airway hyperreactivity (■) with animal received L-arginine in a dose of 300 mg/kg i.p. administered 30 min before the provocation of the airways hyperreactivity by toluene exposure in the 3 consecutive days (✕). * $p < 0.05$.

tissue smooth muscle reactivity in all concentrations of acetylcholine (10^{-8} – 10^{-3} mol/l, $p < 0.05$) in animals with irritant–induced hyperreactivity with L-arginine pre-treatment. The group without airways hyperreactivity that received L-arginine in short regimen did not show statistically significant reactivity changes in the comparison with healthy group without L-arginine pre-treatment (Fig. 2B).

Fig. 3 shows and compares the effect of the long-term pre-treatment (17 days) with L-arginine in a dose of 300 mg/kg i.p. in healthy animals and animals with toluene-induced hyperreactivity. We did not evoke statistically significant changes in the reactivity of tracheal and lung tissue smooth muscle to acetylcholine in the group of animals with hyperreactivity that received this precursor. The amplitude of contraction of tracheal smooth muscle was increased in the group of animals without airways hyperactivity that received L-arginine. The response of lung tissue was less expressed (B) than the overall tracheal smooth muscle response (A).

In Fig. 4, the effect of *in vitro* administered L-arginine was showed. The amplitude of tracheal smooth muscle contraction was statistically significantly decreased in concentration of acetylcholine 10^{-4} – 10^{-3} mol/l ($p < 0.05$) if L-arginine was added directly into the organ bath in concentration of 10^{-4} mol/l to the strip from animals with airway hyper-

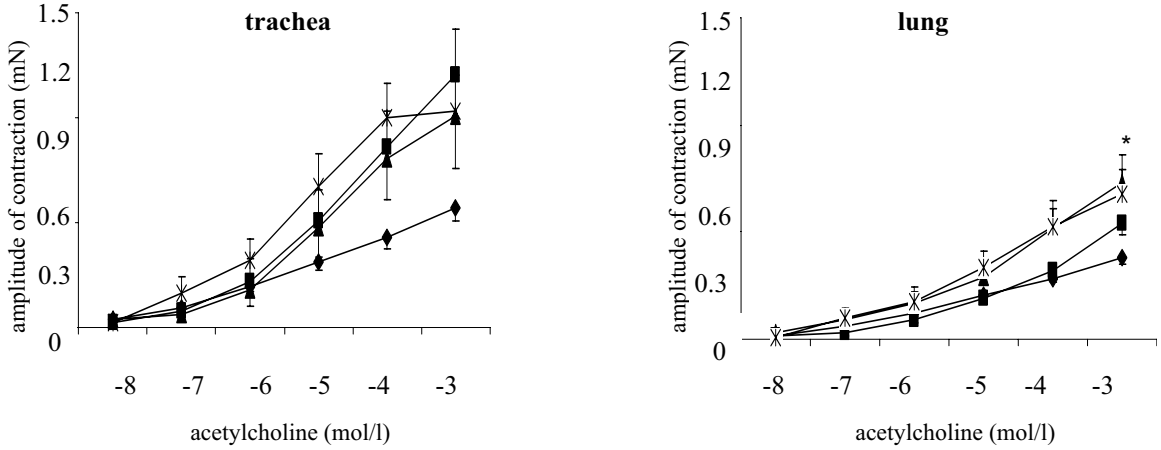

Figure 3. Effect of 17 days pre-treatment with L-arginine in a dose of 300 mg/kg i.p. on the tracheal and lung tissue smooth muscle reactivity to acetylcholine in healthy animals (◆), healthy animals with L-arginine pre-treatment (▲), animals with toluene-induced airway hyperreactivity (■) and animals received L-arginine in a dose of 300 mg/kg i.p. administered 30 min before the provocation of the airways hyperreactivity by toluene exposure in the 3 consecutive days (✕). * $p < 0.05$.

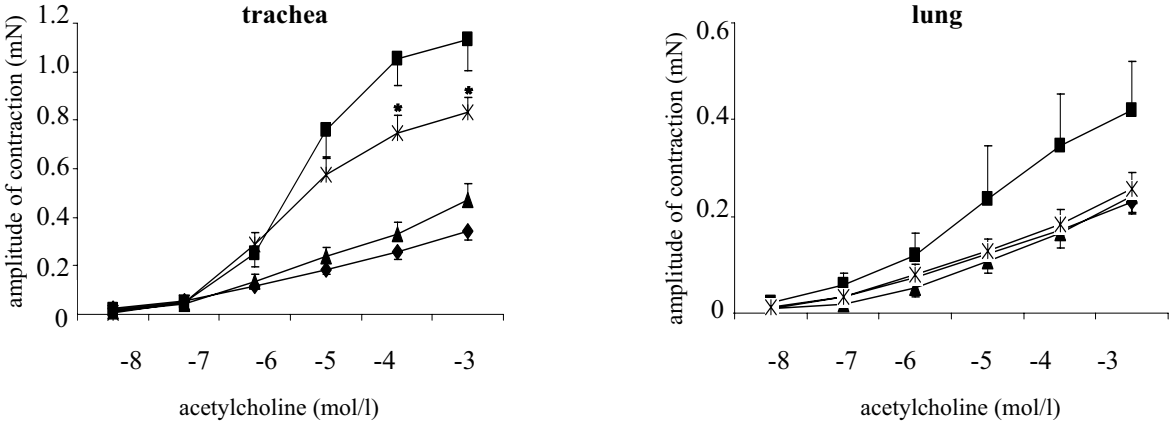

Figure 4. Effect of *in vitro* incubation of tracheal and lung tissue strips with L-arginine in the concentration of 10^{-4} mol/l and response of preparations to acetylcholine from healthy animals (◆), healthy animals with L-arginine pre-treatment (▲), animals with toluene-induced airway hyperreactivity (■) and animals received L-arginine in a dose of 300 mg/kg i.p. administered 30 min before the provocation of the airways hyperreactivity by toluene exposure in the 3 consecutive days (✕). * $p < 0.05$.

reactivity in the comparison with group of animals with airway hyperreactivity without L-arginine pre-treatment. We did not record statistically significant changes of tracheal smooth muscle response in the group of animals without airways hyperactivity. The incubation of the lung tissue strip from animals with irritant-induced hyperreactivity with L-arginine in equal concentration evoked also a decrease of response to acetylcholine that is not statistically significant in comparison with the group of animals with hyperreactivity without L-arginine. We did not observe changes in the amplitude of contraction in the group of animals without airways hyperreactivity.

Discussion

The airway hyperreactivity is one of important hallmarks in various respiratory diseases including asthma and chronic obstructive pulmonary disease. At present there are some possible mechanisms of origin in the airway hyperreactivity; the interference in NO homeostasis with the deficiency of L-arginine can be one of the factors contributing to this symptom (Fraňová 2001; Strapková and Nosáľová 2001; Morris et al. 2004). Therefore, we aimed to investigate a participation of L-arginine in the exogenous irritant-induced bronchial hyperreactivity by using supplementation of this precursor. Toluene vapours were used as exogenous irritant for the provocation of the airway hyperreactivity because we demonstrated an increase in airway smooth muscles reactivity in guinea pigs after the toluene exposure in our previous experiments (Strapková et al. 1996). This increase can be associated with an enhanced free radicals production and pathological changes in the respiratory tract (Mattia et al. 1991). We suppose that NO participates in the pathomechanisms of the airway reactivity changes evoked by this substance. If we suppose that the deficiency of basic substrate for NO production in this model of hyperreactivity can arise then the supplementation of precursor can enhance NO production and may prevent its development.

Different mechanisms occur to cause a limitation of L-arginine. Firstly, toluene-induced hyperreactivity simulates the pulmonary oxidative stress that can be connected with free radicals production as well as with the increased demand of L-arginine as NO precursor. It is known that beneficial role of NO in optimal conditions is probably a result of its ability to scavenge free radicals and with the ability to reduce lipid peroxidation (Lin et al. 2005). Further, the deficiency of L-arginine can result in switching of NOS from NO production to superoxide formation and cellular injury or can cause the inhibition of cNOS. This situation activates inducible NOS isoform that begins the production of the detrimental amount of NO (Boer et al. 1999, 2001).

Secondly, every cell needs to transport L-arginine through plasma membrane. Specialized carrier proteins are necessary to provide an adequate import and export. In human airway cells there are at least four distinct cationic amino acid transporter systems for amino acids transport (Closs et al. 2004; Rotoli et al. 2005). Observations of Boer et al. (1999) as well as other investigators have indicated that reduced availability of substrate may be caused by reduced cellular uptake of L-arginine by these transporters that is induced by inflammatory changes in the airways.

The enhanced competition between NOS and arginase for substrate and thereby the negative co-regulation of the function of each of them could be another mechanism involved in the deficiency of L-arginine. Thus, the modulation of L-arginine levels may affect L-arginine metabolism by both NOS and arginase pathway. 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 (Meuers et al. 2003; King et al. 2004; Morris et al. 2004).

It is interesting reaction of the tracheal smooth muscle to the L-arginine pre-treatment in the group of healthy animals without the airways hyperreactivity. We can see the increase in the amplitude contraction in healthy animals received L-arginine in the short- or long-term regimen. The cause of this phenomenon can be only hypothesized. One of explanation is that an increase in the L-arginine concentration may augment NO formation through the increase in iNOS expression (Grasemann et al. 2005). Another explanation is connected with different situations in the radical production and NO's role, prostaglandins or cytokines production in the healthy and in the diseased tissue (Boer et al. 1998).

In the present study we demonstrated that the administration of the NO precursor L-arginine had overall reduced the responsiveness of the airway smooth muscle to acetylcholine and to histamine in animals with exogenous irritant-induced airway hyperreactivity. The effect was more expressed in the tracheal smooth muscle after shorter pre-treatment with L-arginine and after incubation of strips with this substrate. We suppose that supplementation of L-arginine in a shorter regime normalizes NO production and its activity under the conditions of airway hyperreactivity. The beneficial effect of strip incubation with L-arginine may be caused by better reach of the important elements that regulate bronchomotor tone (e.g. cNOS in smooth muscle or nerve fibres).

The favourable effects of long-term pre-treatment with L-arginine we did not recorded – there is information in the literature about the effects of oral L-arginine on airway hyperresponsiveness to histamine in asthma patients where oral L-arginine does not influence airway hyperresponsiveness, although the dose-response slope was slightly reduced and the level of exhaled NO was increased (de Gouw et al. 1999). Takano et al. (1998) achieved similar results when oral

administration of L-arginine potentiated the airway inflammation. We suppose that the long-term administration of L-arginine in our conditions may provide the NO excess that will inhibit beneficial effects on the bronchial hyperreactivity *via* its detrimental actions. The excess of substrate probably inhibits cNOS and the result of this action is the amplification of the bronchoconstriction. L-arginine may increase NO formation by inducible NOS or the production of other radical species that may be associated with the increase in the airway smooth muscle response to the bronchoconstric mediators.

We recorded the difference in the L-arginine action at different levels of the airway. 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. 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 (Prado et al. 2005). It is probably a reason for the finding that NO prevents more the contraction of the large airways than small ones (Ward et al. 1995; Dewachter et al. 1997). It is necessary to take into consideration the participation of vessels in case of lung tissue (Mokrý 2005). 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 (Nelin et al. 2002; Baydoun and Mann 2003).

We observed 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 be due to these responses (Matsumoto et al. 1997).

Our experiments confirm that L-arginine/NO pathway may be one of factors influencing the airway reactivity. We suppose that L-arginine level in our model of the bronchial hyperreactivity is decreased that can be the reason of detrimental influence on the inhibitory nonadrenergic noncholinergic neurotransmission and the origin of the airway hyperreactivity. Supplementation of the precursor – L-arginine may modify this state. The protective influence of exogenous L-arginine on irritant-induced bronchial hyperreactivity discovered in our paper shows the importance of the optimal level of L-arginine for the control of bronchomotor tone on one hand and the possibility of the use of therapeutic activities that increase the bioavailability of this precursor on the other hand.

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