Effect of Nitric Oxide Synthases Inhibitors on Exogenous Irritant-Induced Bronchial Hyper-Reactivity in Guinea Pigs

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Abstract. Nitric oxide (NO) is an important endogenous mediator involved in many biological functions in both physiological and pathological conditions. Many of studies suggest that high level of NO may play a role in the pathogenesis of various diseases including respiratory diseases with bronchial hyper-reactivity (BHR). The aim of our study was to examine the relationship between NO production and BHR. The reactivity of tracheal and lung tissue smooth muscle to histamine and acetylcholine was measured *in vitro* in male guinea pigs pre-treated with NO synthase (NOS) inhibitors. The drugs were administered *in vivo* during either 3 or 17 days. Furthermore, the animals were exposed *in vivo* to the toluene vapours after administration of agents. NOS inhibitors showed mainly beneficial effect in the presented study. They decreased the hyper-reactivity of the tracheal and lung tissue smooth muscle 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 one. A more significant effect was recorded after the pre-treatment with non-selective inhibitor L-NAME. The results showed possible participation of constitutive forms of NOS in the BHR.

Key words: Nitric oxide — NOS — Airway hyper-reactivity — L-NAME — Aminoguanidine

Introduction

Nitric oxide (NO) plays an important role in the regulation of physiological pulmonary function as well as in the respiratory diseases associated with hyper-reactivity. The changes of airway smooth muscle tone and the response to different mediators is one of the manifestations of an abnormal production and activity of NO in the airways. In previous experiments, by other authors was demonstrated that exogenous NO administration, administration of NO releasing substances as

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well as substances influencing the activity of NO synthases (NOS) might contribute to the modification of the processes regulated by NO (Barnes and Belvisi 1993).

NO synthesis is catalysed by a family of NOS that are localised in the cells of smooth muscle, epithelium, nerves, endothelium, vessels and inflammatory tissue. The activity of constitutive isoforms (neuronal – nNOS and endothelial – eNOS) depends on the intracellular calcium level. These isoforms produce picomolar amounts of NO that are involved in various physiological regulatory mechanisms – neuronal transmission, neuro-endocrine activity, blood pressure, congestion, adhesion and aggregation of trombocytes, the tone of the airways smooth muscle, mucociliary transport, vessels permeability etc.

Inducible calcium-independent form of NOS enzyme (iNOS) is responsible for NO production in the nanomolar amounts. It is activated by various defence reactions of the organism such as inflammation. NO manifests antimicrobial, antitumor and cytotoxic effects associated with reactive free radicals production in these higher concentrations. Large amounts of NO produced by iNOS isoform contribute to the development of pulmonary pathological processes (Singh and Evans 1997; Ricciardolo 2003).

These special characteristics of NOS provoked us to use NOS inhibitors under our experimental conditions. In our previous experiments we have demonstrated an increase in airway smooth muscles reactivity in guinea pigs after the toluene exposure (Strapková et al. 1995, 1996; Strapková and Nosáľová 2001). 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 pathogenetic mechanisms of the airway reactivity changes. The aim of our study was to evaluate the influence of the substances inhibiting NO synthesis on the reactivity of tracheal and lung tissue smooth muscle changed by toluene vapours exposure in guinea pigs. Aminoguanidine (selective inhibitor of iNOS) or N^{ω}-nitro-L-arginine methylester (L-NAME; non-selective inhibitor of NOS that inhibits all isoforms of NOS) were chosen to change the NO levels before the irritant exposure.

Materials and Methods

Animals and agents

Pathogens-free male Trik strain guinea pigs (250–350 g) were used in the study. The animals were housed in individual cages in climate-controlled animal quarters and received water and food *ad libitum*. The first group ($n = 8$) received a selective inhibitor of iNOS – aminoguanidine (Sigma) at a dose of 50 mg/kg b.w. intraperitoneally 30 min before toluene exposure during 3 consecutive days. The second group ($n = 8$) received aminoguanidine at equal dose during 17 days. During last 3 days, the animals received aminoguanidine 30 min before toluene exposure, too. The third ($n = 8$) and fourth group ($n = 8$) received a non-selective inhibitor of NOS – L-NAME (Sigma) at a dose of 40 mg/kg b.w. intraperitoneally in the same time regime. The fifth ($n = 8$) and sixth group ($n = 8$) received a combination

of both inhibitors during 3 or 17 days. The inhibitors were dissolved in Aqua pro injectione and administered once a day. Animals in the seventh ($n = 8$) and eighth group ($n = 8$) were considered to be the control group and received Aqua pro injectione during 3 or 17 days. The administration of agents during 3 days was considered to be a short-term administration, during 17 days – long-term administration. The Ethical Committee of Jessenius Faculty of Medicine approved the study protocol.

Toluene exposure

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

Airway responsiveness

Animals were killed 24 h after last toluene exposure. The strips from trachea and lung tissue were prepared and placed into organ bath with Krebs—Henseleit solution (in mol/l: 110.0 NaCl, 4.8 KCl, 2.35 $CaCl_2$, 1.20 $MgSO_4$, 1.20 $KHPO_4$, 25.0 $NaHCO_3$ 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℃. 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 recording were made by 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). 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 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

The effect of toluene exposure on the tracheal and the lung tissue smooth muscle responsiveness to histamine

The tracheal smooth muscle responsiveness to histamine is compared in animals that inhaled air (white columns) and animals that inhaled toluene vapours 2 h in each of three consecutive days (black columns) in the Fig. 1, part a. The responsiveness was significantly increased in the histamine concentration 10^{-5}–10^{-3} mol/l.

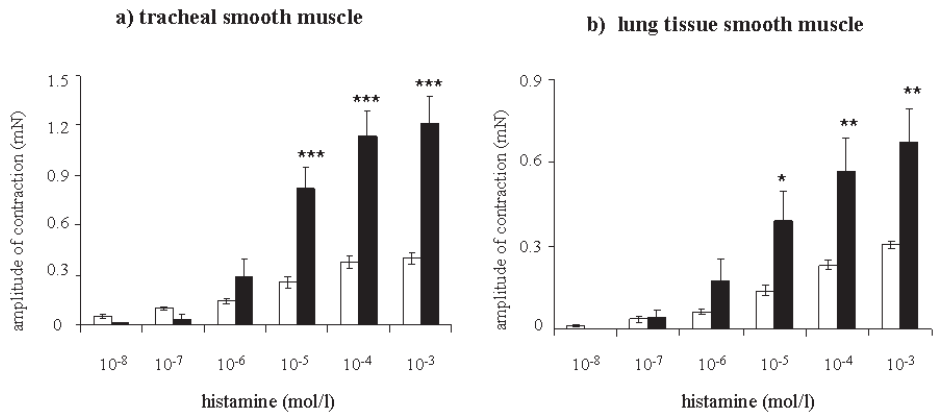

Figure 1. The effect of toluene exposure on tracheal (a) and lung tissue smooth muscle (b) responsiveness to histamine. □ animals inhalated air; ■ animals inhalated toluene vapours; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The increase in tracheal smooth muscle responsiveness is more prominent in lung tissue smooth muscle responsiveness (Fig. 1, part b) to all inscribed concentration of histamine.

The effect of toluene exposure on the tracheal and the lung tissue smooth muscle responsiveness to acetylcholine was similar, therefore, the figures are not shown.

The effect of 3- and 17-day administration of NOS inhibitors on the tracheal smooth muscle responsiveness to histamine

We compared the changes of the airways reactivity evoked by toluene exposure after 3- and 17-day administration of NOS inhibitors with the control animals exposed to toluene with Aqua pro injectione pre-treatment. We noticed the decrease in tracheal smooth muscle response to histamine in all three groups of animals that received NOS inhibitor during 3 days (Fig. 2, part a). However, a more prominent decrease was observed after administration of the inhibitors combination (L-NAME + aminoguanidine) than that observed after a dose of single inhibitors. The amplitude of contraction was significantly reduced at histamine concentration of 10^{-5}–10^{-3} mol/l. We observed similar but statistically less significant effect after administration of L-NAME. The aminoguanidine did not show any statistically significant effect under these conditions. After 17-day administration of inhibitors (Fig. 2, part b) we did not observe any statistically significant changes of tracheal smooth muscle reactivity to histamine, only the decreased reactivity at the concentration of 10^{-3} mol/l of histamine was found after administration of the non-selective inhibitor L-NAME.

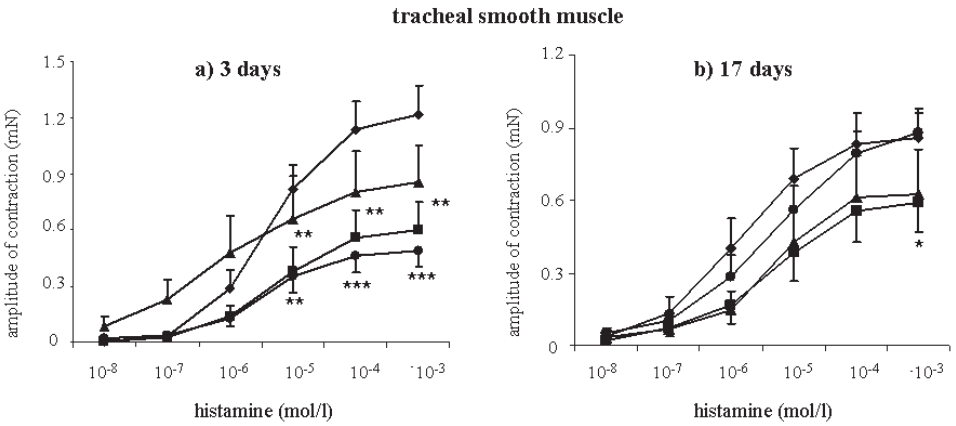

Figure 2. The effect of the 3-day (a) and 17-day (b) pre-treatment with L-NAME (■), aminoguanidine (▲) and combination of L-NAME and aminoguanidine (●) on the tracheal smooth muscle reactivity to histamine after exposure to the toluene compared with toluene group (◆). The columns represent average values of the contraction amplitude with mean mistake of average ± S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The effect of 3- and 17-day administration of NOS inhibitors on lung tissue responsiveness to histamine

The smooth muscle of lung tissue showed significant and almost identical decrease in the amplitude of contraction to histamine at the concentration 10^{-4}–10^{-3} mol/l after 3-day administration of L-NAME and aminoguanidine. We were unable to decrease the lung tissue reactivity to histamine by the pre-treatment with the combination of inhibitors (Fig. 3, part a). A significant effect on lung tissue responsiveness to histamine in long-term pre-treatment was found only after administration of L-NAME (Fig. 3, part b). This inhibitor significantly decreased the amplitudes of the contraction at the concentration of 10^{-6}–10^{-3} mol/l of histamine. We did not observe any significant decrease in lung tissue responsiveness in other groups of pre-treated animals.

The effect of 3- and 17-day administration of NOS inhibitors on tracheal responsiveness to acetylcholine

The significant decrease in tracheal smooth muscle reactivity to acetylcholine was evoked by short-term administration of single inhibitors as well as by their combination. The changes were similar except L-NAME administration where the statistical significance of changes was higher (Fig. 4, part a). After long-term administration of L-NAME and aminoguanidine, no significant changes were observed in the tracheal smooth muscle reactivity to acetylcholine (Fig. 4, part b). The contraction curves were almost identical after administration of inhibitors comparing to the control

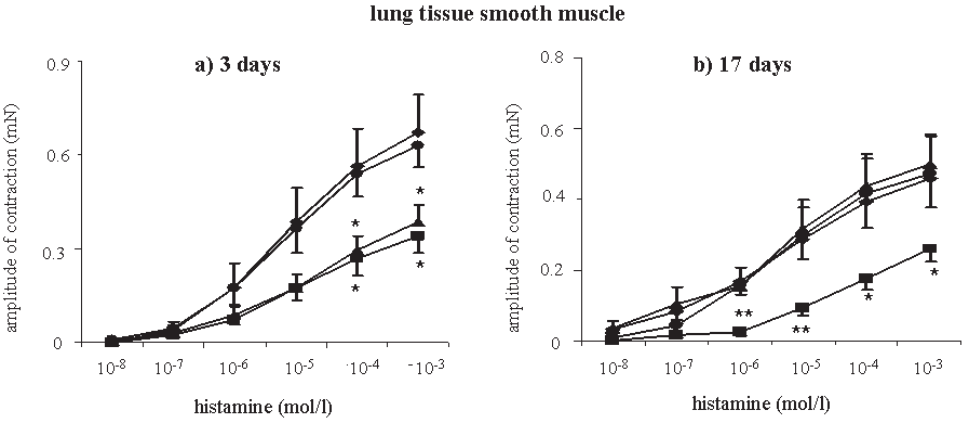

Figure 3. The effect of the 3-day (a) and 17-day (b) pre-treatment with L-NAME (■), aminoguanidine (▲) and combination of L-NAME and aminoguanidine (●) on the lung tissue smooth muscle reactivity to histamine after exposure to the toluene compared with toluene group (◆). * $p < 0.05$; ** $p < 0.01$.

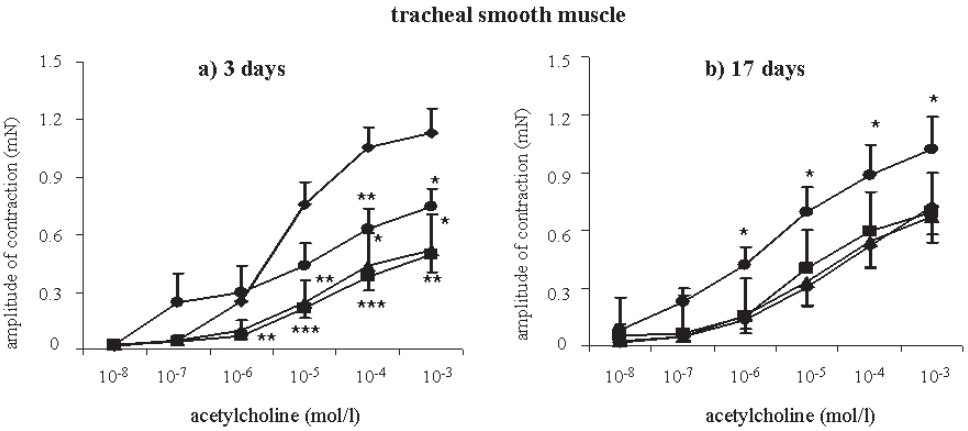

Figure 4. The effect of the 3-day (a) and 17-day (b) pre-treatment with L-NAME (■), aminoguanidine (▲) and combination of L-NAME and aminoguanidine (●) on the tracheal smooth muscle reactivity to acetylcholine after exposure to the toluene compared with toluene group (◆). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

group. However, significant increase was found in the tracheal smooth muscle reactivity to acetylcholine at the concentrations of 10^{-6}–10^{-3} mol/l after long-term pre-treatment with combination of both inhibitors.

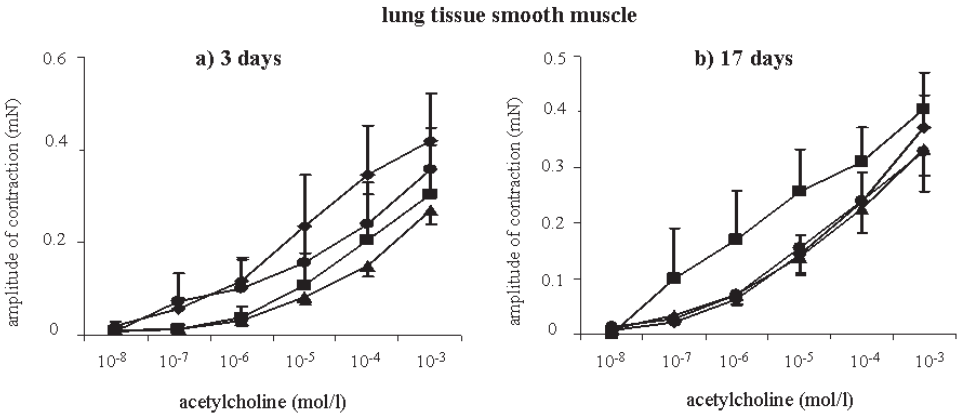

Figure 5. The effect of the 3-day (a) and 17-day (b) pre-treatment with L-NAME (■), aminoguanidine (▲) and combination of L-NAME and aminoguanidine (●) on the lung tissue smooth muscle reactivity to acetylcholine after exposure to the toluene compared with toluene group (◆).

The effect of 3- and 17-day administration of NOS inhibitors on lung tissue responsiveness to acetylcholine

We did not observe any significant changes in the lung tissue smooth muscle reactivity to acetylcholine after short-term administration of NOS inhibitors (Fig. 5, part a). The lung tissue smooth muscle responses to acetylcholine were similar after long-term pre-treatment with NOS inhibitors (Fig. 5, part b) in comparison to the changes caused by 3-day administration of NOS inhibitors. The contraction amplitude was almost identical when comparing to the control group and it was not statistically significant.

Discussion

In presented study we showed that NOS inhibitors (aminoguanidine, L-NAME or their combination) had mainly beneficial effect on the airways response. The administration of inhibitors ameliorated toluene-induced hyper-reactivity in guinea pigs. The NOS inhibitors decreased the reactivity of tracheal and lung tissue smooth muscle strips in case of both bronchoconstrictor mediators – histamine and acetylcholine. Some differences in the response of tissue at different airway levels could be associated with different isoforms of NOS in upper and lower airway. These various effects of NOS inhibitors were dependent on the type of inhibitors as well as on the duration of their administration. The short-term administration of both inhibitors was more effective comparing to the long-term one. We suppose that this observation can result either from adaptation of the mechanisms controlling

the airway-reactivity or it is associated with desensitization of the guanylyl cyclase. Furthermore, we suppose that NO may participate in the development of hyper-reactivity evoked by toluene vapours exposure.

It is known that high levels of NO produced mainly by iNOS are responsible for the development of many respiratory diseases and their symptoms including bronchial hyper-reactivity (BHR). Some of the experimental studies showed both beneficial (modulating the airway smooth muscle tone) and detrimental effect (deepening the airway inflammation) of iNOS-derived NO on the BHR. Schuiling et al. (1998) found dual effect by using of the selective iNOS inhibitor aminoguanidine in ovalbumin-induced airways hyper-reactivity. The administration of aminoguanidine at small doses increased the airways reactivity to histamine in an acute state. Preventive treatment with high doses of this agent stopped the airways hyper-reactivity (Eynott et al. 2002). Schuiling's study indicates that NO derived mainly by iNOS is involved in the reversal of the hyper-reactivity. However, the inhalation of aminoguanidine after the late asthmatic reaction to allergen challenge enhanced BHR in ovalbumine-sensitized guinea pigs but the inhalation of aminoguanidine before development of the late asthmatic reaction reduced BHR and decreased the number of inflammatory cells (Schuiling et al. 1998; Silkoff et al. 2000).

The effect of iNOS inhibitor aminoguanidine was predominantly beneficial also in our short-term experiment. At a dose of 50 mg/kg (intraperitoneally) 30 min before the toluene exposure, it decreased the airways responses to histamine and acetylcholine. We suggest that the effect of iNOS-derived NO can be deleterious to BHR. We assume that blockade of NO synthesis by iNOS reduces the generation and the activity of free radicals on the tracheal smooth muscle that results in decelerating of lung and epithelial damage.

However, the effect of aminoguanidine was predominantly detrimental during the long-term administration. The contractile responses of tracheal and lung tissue smooth muscle to acetylcholine and histamine were predominantly increased or identical comparing to control group. We suppose that the effect of aminoguanidine was probably affected by desensitization of iNOS after long-term administration of this iNOS inhibitor.

There were described different effects of the inhibitor of constitutive NOS isoforms L-NAME in various experimental studies, too. L-NAME inhibits all types of NOS with subsequent inhibition of the NO release by various cells including the airway smooth muscle cells. The administration of this non-selective inhibitor induced an increase in the baseline tissue resistance that was inhibited by NO donors or by inhaled NO (Khassawnwh et al. 2002). Mehta et al. (1997) have described an increase in the contractile responses to histamine after inhibition of NO production with L-NAME in guinea pigs. They reported that L-NAME enhanced bronchial reactivity to histamine in control group but not in antigen-challenged guinea pigs. This can determine a defect in NO bronchodilating activity in challenged animals. This defect probably did not arise from decreased NO production because exhaled concentration was the same in control and challenged animals (Silkoff et al. 2000). This suggests that the effect of L-NAME depends on doses or on different mech-

anisms of action than NOS inhibition. Furthermore, it was speculated that low doses of L-NAME were not able to penetrate through the epithelium to the airway sensory nerves. High doses of L-NAME were able to penetrate into the airway parenchyma and could influence the NOS (Taylor et al. 1998; Nevin and Broadley 2002).

The effects of L-NAME were mainly protective on BHR to histamine and acetylcholine during short- and long-term administration. A more expressive modulation of the reactivity by inhibition of constitutive NOS suggests that these isoforms can be more important in BHR under our conditions. Our results showed that the effect of L-NAME on tracheal smooth muscle reactivity to histamine and acetylcholine was beneficial predominantly during a short-term administration; in the lung tissue this effect was protective only to histamine but during short- and long-term administration. These findings can result from various factors. Was the dose of 40 mg/kg applied intraperitoneally too low or did L-NAME have any effect on other NOS isoforms? From this point of view, the position of vasoactive intestinale peptide (VIP) in these conditions is also very interesting. We suppose that NO alone is active predominantly in the trachea and NO together with VIP mediates protective effects in the lung tissue. However, it is necessary to look for others answers to these questions in the future.

The combination of NOS inhibitors had different effects that were dependent on the duration of the pre-treatment. A protective effect (the decrease of the airways reactivity) was registered during short-term administration while a detrimental effect (enhanced reactivity mainly to acetylcholine) was observed during long-term administration. These findings are in concordance with previous experimental studies. Aizawa (1999) showed that the pre-treatment with aminoguanidine plus L-NAME had no effects on airway hyper-responsiveness immediately after ozone exposure but led to the significant inhibition of the airways reactivity 5 h after ozone exposure. The toluene exposure has similar effects as ozone. Both exogenous irritants can enhance bronchial reactivity by modulation of NOS activity, because they lead to the damage of the airway epithelium where all isoforms of NOS are located.

There are also other questions: are the NOS inhibitors able to evoke the airways hyper-reactivity or do they have a protective effect? Which isoforms are more important in this process? In our model of the BHR we observed more expressive participation of constitutive isoforms. However, various experimental conditions must be taken into account in provocation of hyper-reactivity (environmental irritants, allergen-induced BHR) as well as type of animals, doses of used inhibitors, the way of evaluating the reactivity (*in vitro* or *in vivo*) (Bánovčin et al. 1996). Furthermore, different NO effects can be expected in acute and serious clinical states where mainly the deleterious NO effect mainly it is manifested. On the other hand, NO under physiological conditions has mainly beneficial effect (van der Vliet et al. 2000; Samb et al. 2001; Himashree et al. 2003). Further studies will be needed for better understanding of these processes and to confirm our hypothesis. Therefore it is necessary to continue in the research of the NO role in the respiratory system

(Dweik 2001). This research may bring some of new therapeutic approaches in the treatment of many respiratory diseases with BHR.

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