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Exogenous Irritant-Induced Airway Hyperreactivity and Inhibition of Soluble Guanylyl Cyclase

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The majority of nitric oxide (NO) effects in the respiratory system are caused by stimulation of soluble guanylyl cyclase (sGC) with subsequent increase of cyclic guanosine monophosphate (cGMP) production. The importance of this mechanism of NO action in airway hyperreactivity (AHR) pathogenesis is unknown. Therefore, the aim of our experiment was to examine the changes of airway reactivity enhanced by toluene vapor exposure in the presence or inhibition of sGC activity in guinea pigs. Animals were treated with a nonspecific sGC inhibitor, methylene blue, in a dose of 50 or 100 mg/kg body weight, administered by intraperitoneal injection 30 min before or after exposure to toluene vapors. The toluene exposure lasted 2 hr in each of 3 consecutive days under in vivo conditions. Thereafter, the tracheal and lung tissue smooth muscle response to cumulative doses of mediators (histamine or acetylcholine) was recorded under in vitro conditions. The exposure to toluene vapors significantly increased the airway reactivity to both mediators in comparison with the healthy animal group. The administration of methylene blue decreased the amplitude of airway smooth muscle contraction in toluene-induced hyperreactivity. The decreases were dependent on the inhibitor doses, on a regimen of administration (before or after toluene inhalation), the level of the respiratory system (trachea, lung), and the bronchoconstrictor mediators. Our results suggest that the interaction between NO and sGC may be important for airway reactivity changes, but other mechanisms of NO action are important in AHR pathogenesis, too.

Keywords: airway reactivity; nitric oxide; soluble guanylyl cyclase; cGMP

Airway hyperreactivity (AHR) is a grave symptom connected with asthma and other predominantly chronic respiratory diseases. The hyperreactivity is characterized as a tendency of the tracheobronchial smooth muscle to contract more intensively in response to various stimuli. Many different cells (e.g., epithelial, endothelial, vascular and bronchial smooth muscle cells) and mediators (e.g., leukotrienes, prostaglandins, histamine, neurokinins and nitric oxide [NO]) participate in the pathogenesis of hyperreactivity, but it is not known whether any of these mediators are pathogenic. This study examines the role of NO in AHR pathogenesis.

NO has been recognized during the past two decades as a versatile player in the regulation of many physiological and pathophysiological processes in the respiratory system. It is the main neurotransmitter of the inhibitory nonadrenergic noncholinergic system that participates in the regulation of the airway smooth muscle tonus, ciliary motility, mucous secretion, plasma exudation, and more. NO mediates these effects through various mechanisms (Murad, 1999). Biological signaling by NO can be divided into direct and indirect actions. The direct action of NO is connected with low NO concentrations, as generated by the constitutive NO synthases (NOS)—neuronal and endothelial. Here, NO interacts directly with positively charged metal ions in proteins, for example

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the iron atom in hemoglobin, myoglobin, peroxidases, and soluble guanylyl cyclase (sGC; Kagan & Laskin, 2001). The sGC is a major receptor for NO and mediates a wide range of physiological effects via elevation of intracellular cyclic guanosine monophosphate (cGMP) levels (Bellamy, Wood, & Garthwait, 2002). sGC exists as heterodimers of α and β subunits. It is known that NO activates sGC via interaction with its prosthetic heme group (Nakane, 2003). According to the conventional scheme, NO binds to a prosthetic heme group associated with the β subunit, after which the bond between the heme and a nearby histidine residue breaks, causing a conformational change that propagates to the catalytic domain, greatly speeding the conversion of GTP into cGMP (Denniger & Marletta, 1999; Ignarro, 1991; Koesling, Russwurm, Mergia, Mullershausen, & Friebe, 2004; Roy & Garthweith, 2006). cGMP induces smooth muscle relaxation by multiple mechanisms: (a) it inhibits the calcium entry into cells and decreases intracellular calcium concentrations; (b) it activates potassium (K^+) channels which leads to hyperpolarization and relaxation; and (c) it stimulates a cGMP-dependent protein kinase that activates myosin light-chain phosphatase, the enzyme that dephosphorylates myosin light chains which leads to smooth muscle relaxation (Klabunde, Tse, & Weiss, 1998). These mechanisms are characterized as cGMP dependent (Friebe & Koesling, 2003; Ricciardolo, Sterk, Gaston, & Folkerts, 2004). The reactions of NO/cGMP signaling cascade are also involved in the regulation of smooth muscle tonus in the respiratory system.

Indirect NO signal mechanisms are cGMP independent. They are extremely complex and mediated through ion channels (NO activated Ca^{2+}-dependent K^+ channels, K_{ATP} channels, etc.) through a generation of reactive nitrogen oxide species that chemically modify enzymes, signaling molecules such as kinases and transcription factors (Perkins, Pabelick, Warner, & Jones, 1998). However, the majority of NO effects are cGMP dependent.

The position of NO in the airways and the importance of the NO/cGMP signaling cascade bring many questions about the role of this pathway in the symptoms of respiratory diseases. Some researchers propose that NO/cGMP signaling is involved in hyperreactivity pathogenesis through the following reactions: NO-sensitive sGC catalyzes the formation of cGMP, which subsequently activates the cGMP-dependent protein kinase G and protein kinase A, which ultimately decreases both cytosolic Ca^{2+} concentration and the phosphorylation of many intracellular proteins (Mokrý, 2006; Somlyo & Somlyo, 1994). The final effect is airway smooth muscle relaxation that is terminated by the action of the cyclic nucleotide–hydrolyzing phosphodiesterases (Hamad, Clayton, Islam, & Knox, 2003). Failure in this signaling cascade can lead to the development of AHR.

The accessible information about this problem is controversial at present. Papapetropoulos, Simoes, Xanthou, Roussos, & Gratziou (2005) suggest that inhibition of sGC in animals sensitized and challenged with ovalbumin can result in AHR. On the other side, Osanai et al. (2000) demonstrated that pretreatment with methylene blue enhanced prostacyclin production with subsequent inhibition of AHR. If relaxation of airway smooth muscle is cGMP dependent, we hypothesize that the inhibition of sGC by a nonspecific sGC inhibitor, methylene blue, should modify the AHR if NO is involved. In our previous studies we showed that exposure to an exogenous irritant, toluene vapor, evoked AHR (Antošová Turčan, Strapková, & Nosáľová, 2005; Strapková, Nosáľová, & Hanáček, 1996). Therefore, the purpose of this study was to test the hypothesis that the NO/cGMP signaling cascade plays a role in AHR. Different authors point out changes in different tissues when the experimental agents are administered before and after the development of AHR (Leigh, Ellie, Wattie, Donaldson, & Inman, 2004; Schuiling, Meurs, Zuidhof, Venea, & Zaagsma, 1998). Therefore, we administered methylene blue in different doses and different therapeutic regimens, before or after toluene vapor exposure, to determine the development of AHR in the presence or inhibition of the NO/cGMP pathway.

Methods

Animals

Pathogen-free male TRIK guinea pigs (280–350 g) were used in the present study. The animals came from an accredited breeder (Department of Toxicology and Breeding of Experimental Animals, Department of Experimental Pharmacology, Slovak Academy of Sciences, Good Water, Slovak Republic) and after delivery were housed individually in climate-controlled animal cages. The guinea pigs were weighed daily and had ad libitum access to food and water.

Table 1. The Scheme of Treatment Procedures ($n = 8$ for each group)

First Therapeutic Regimen—Pretreatment Before Toluene Exposure	First Group	Second Group	Third Group (Control)
Agent	Methylene blue	Methylene blue	Aqua pro injection
Dose	50 mg/kg b.w. i.p.	100 mg/kg b.w. i.p.	1 ml/kg b.w. i.p.
Time of application	Each day 30 min before toluene exposure	Each day 30 min before toluene exposure	Each day 30 min before toluene exposure
Toluene exposure	2 hr each day	2 hr each day	2 hr each day
Second Therapeutic Regimen—Pretreatment After Toluene Exposure	**Fourth Group**	**Fifth Group**	**Sixth Group (Control)**
Agent	Methylene blue	Methylene blue	Aqua pro injection
Dose	50 mg/kg b.w. i.p.	100 mg/kg b.w. i.p.	1 ml/kg b.w. i.p.
Time of application	Each day 30 min after toluene exposure	Each day 30 min after toluene exposure	Each day 30 min after toluene exposure
Toluene exposure	2 hr each day	2 hr each day	2 hr each day

NOTES: Treatments occurred on each of 3 consecutive days; b.w. = body weight; i.p. = intraperitoneal.

Study Design

First, we demonstrated that toluene exposure evoked the AHR in our conditions. Two groups of guinea pigs were used for this demonstration. The control group represents healthy animals spontaneously breathing room air ($n = 8$). The animals exposed to and exogenous irritant, toluene vapors, represented the experimental group ($n = 8$). The toluene exposure was performed for 2 hr every day for 3 consecutive days. We surveyed the changes in airway reactivity to histamine and acetylcholine (substances from Sigma Aldrich) in cumulative concentration (10^{-8}–10^{-3} mol/l) in vitro 24 hr after the last toluene exposure by organ chamber methods.

We divided the other animals into 6 groups, 4 experimental and 2 control groups. All experimental animals received the nonselective inhibitor of sGC methylene blue (Sigma Aldrich), dissolved in Aqua pro injectione (Biotika a.s.) by intraperitoneal (i.p.) injection in one of two dosages (50 or 100 mg/kg body weight [b.w.]) and in different therapeutic regimens (before or after toluene exposure). Both control groups received Aqua pro i.p. injections (1 ml/kg b.w.) in the same therapeutic regimens. Table 1 shows the treatment scheme.

Pretreatment with methylene blue and toluene exposure was performed for 3 days. The animals were killed on the 4th day by decapitation with a small guillotine. The trachea and lung tissue were removed and the changes of airway reactivity to histamine and acetylcholine in cumulative concentration (10^{-8}–10^{-3} mol/l) were recorded in vitro conditions by organ chamber methods.

The local Ethical Committee of Jessenius Faculty of Medicine approved the study protocol. Animal care, toluene inhalation procedures, and euthanasia with the small guillotine were performed in accordance with internationally accepted recommendations: the World Medical Association Declaration of Helsinki, European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (directive 86/609/EHS, 1986), and a relevant statute in the Sloval Republic (law no. 289/2003, Statute Book of the Slovak Republic).

Exposure to an Exogenous Irritant

We used the method described Strapková et al. (1996) in our experiments. The guinea pigs were exposed to toluene vapors (6 mg/l–1,600 ppm) in a special exposure chamber made of Plexiglas. The chamber consists of a compressor, flowmeter, vaporizer, and exposure cage. The animals were spontaneously breathing toluene vapors that were supplied into the cage with a constant flow of 4 l/min. The duration of exposure was 2 hr every day for 3 consecutive days.

Measurement of Airway Reactivity

Airway responsiveness was measured in vitro conditions in response to cumulative doses (10^{-8}–10^{-3} mol/l) of histamine and acetylcholine. The trachea

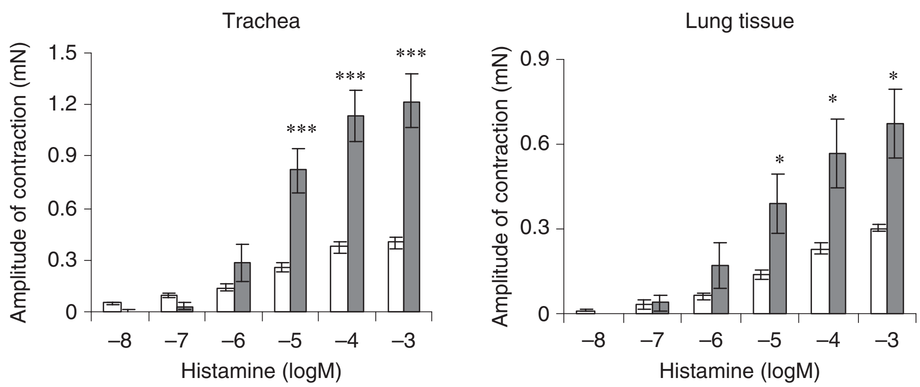

Figure 1. Effect of 3-day toluene exposure on tracheal and lung tissue smooth muscle reactivity (■) compared to a control group of air-breathing animals (□). The columns represent average values of the contraction amplitude (mean ± *SEM*). The x-axis indicates the concentration of the histamine in log M; and the y-axis indicates the amplitude of the contraction in mN. $^{*}p < .05$, $^{***}p < .001$.

and lungs were dissected and cleaned of connective tissue. Thin strips from the lower part of the trachea and diaphragmatic parts of the lungs were prepared and placed into organ chambers with Krebs-Henseleit solution composition: 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 (Microchem, Slovakia). The solution was continuously aerated with a mixture of 95% O_2 and 5% CO_2 at pH 7.5 ± 0.1 and temperature 36 ± 0.5°C. Each strip was connected with two hooks inserted on the ends of the strip. The upper part of the tissue strip was connected to a force transducer and an amplifier (RES s.r.o, Martin, Slovak Republic). Isometric tension was recorded on a computer with specialized software (RES s.r.o, Martin, Slovak Republic). The tissue strips were exposed initially to 4 g tension (30-min loading phase). Thereafter, the tension was readjusted to a baseline of 2 g (30-min adaptive phase). We recorded the changes of airway tissue reactivity to cumulative doses of both bronchoconstrictor mediators after 1 hr of tissue incubation. A cumulative concentration-response curve to 10^{-8}–10^{-3} mol/l histamine or acetylcholine was determined for each strip. The amplitude of contraction of the strips in response to increasing doses of histamine (10^{-8}–10^{-3} mol/l) or acetylcholine (10^{-8}–10^{-3} mol/l) was used as a parameter of reactivity in tracheal and lung tissue smooth muscle. The amplitude of contraction to every concentration of mediators was automatically evaluated by the analysis software.

Statistical Analysis

Statistical analysis was done using one-way ANOVA followed by Dunnett's test to compare the control group with each experimental group. The comparisons of baseline values between groups were analyzed by Student's two-sided *t*-tests. The influence of toluene inhalation (the group of "hyperreactive" guinea pigs) versus control air-breathing guinea pigs was evaluated by a one-sided *t*-test. All statistical analyses were done with Microsoft Excel and Microcal Origin 7.0 (OriginLab). All results are expressed as mean value ± SEM. Differences between the groups were considered statistically significant at $p < .05$.

Results

The Effect of Toluene Inhalation on Airway Reactivity

The repeated exposure of guinea pigs to toluene vapors evoked a significant increase in airway reactivity. The tracheal smooth muscle reactivity was significantly enhanced at concentrations of 10^{-5}–10^{-3} mol/l of histamine and 10^{-6}–10^{-3} mol/l of acetylcholine. The amplitude of lung tissue contraction was significantly increased at concentrations of 10^{-5}–10^{-3} mol/l of histamine and 10^{-4}–10^{-3} mol/l of acetylcholine. The changes in airway reactivity to histamine are shown in Figure 1.

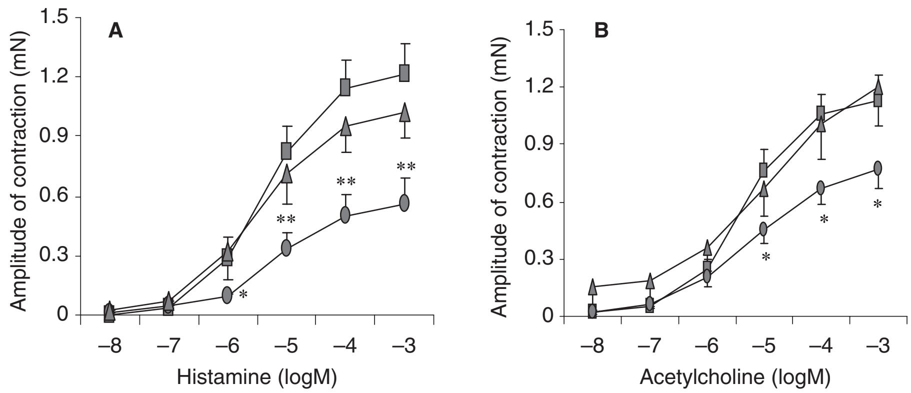

Figure 2. Effect of pretreatment with methylene blue in a dose of 50 mg/kg b.w. (●) and 100 mg/kg b.w. (▲) administered before exogenous irritant exposure on tracheal smooth muscle reactivity to histamine (A) and to acetylcholine (B) compared to the control group (■). Values are mean ± *SEM*. $^{*}p < .05$, $^{**}p < .01$. b.w. = body weight.

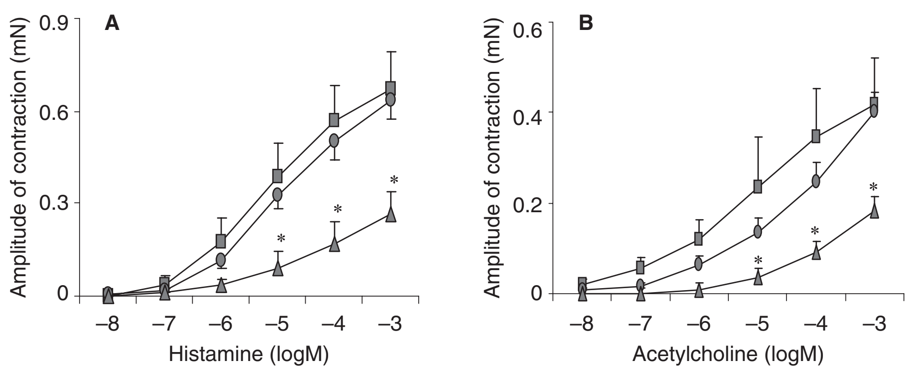

Figure 3. Effect of pretreatment with methylene blue in a dose of 50 mg/kg b.w. (●) and 100 mg/kg b.w. (▲) administered before exogenous irritant exposure on lung tissue smooth muscle reactivity to histamine (A) and acetylcholine (B) compared to the control group (■). Values are mean ± *SEM*. $^{*}p < .05$. b.w. = body weight.

The Effect of Pretreatment With Methylene Blue Administered Before Exogenous Irritant Exposure on Airway Reactivity

We observed a decrease in tracheal smooth muscle response at concentrations of 10^{-6}–10^{-3} mol/l of histamine (Figure 2a) and 10^{-5}–10^{-3} mol/l of acetylcholine (Figure 2b) in animals that received methylene blue in a dose of 50 mg/kg b.w. i.p. 30 min before irritant exposure in comparison to the control group. Methylene blue applied before toluene exposure in the higher dose of 100 mg/kg b.w. did not evoke any statistically significant changes in tracheal smooth muscle reactivity to either of the mediators.

Pretreatment with 50 mg/kg b.w., of the sGC inhibitor, before irritant exposure did not evoke any statistically significant changes in lung tissue response to histamine and acetylcholine in comparison with the control group (Figure 3). The higher dose of methylene blue (100 mg/kg b.w.) significantly decreased the lung tissue reactivity to both the mediators at a concentration of 10^{-5}–10^{-3} mol/l.

The Effect of Pretreatment With Methylene Blue Administered After Exogenous Irritant Exposure on Airway Reactivity

The administration of the sGC inhibitor methylene blue at 50 mg/kg b.w. i.p., 30 min after toluene exposure did not evoke significant changes in tracheal smooth muscle response to histamine and acetylcholine. Increasing the dose of methylene blue to 100 mg/kg b.w. induced a more expressive decrease of

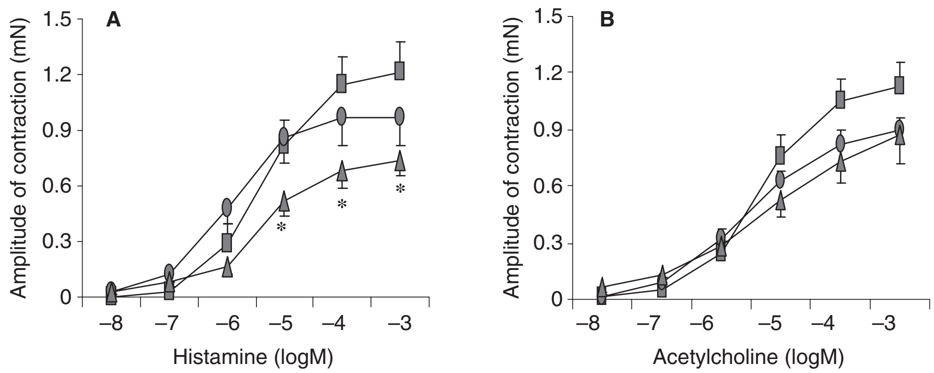

Figure 4. Effect of pretreatment with methylene blue in a dose of 50 mg/kg b.w. (●) and 100 mg/kg b.w. (▲), administered after exogenous irritant exposure on tracheal smooth muscle reactivity to histamine (A) and acetylcholine (B) compared to the control group (■). Values are mean ± *SEM*. $^*p < .05$. b.w. = body weight.

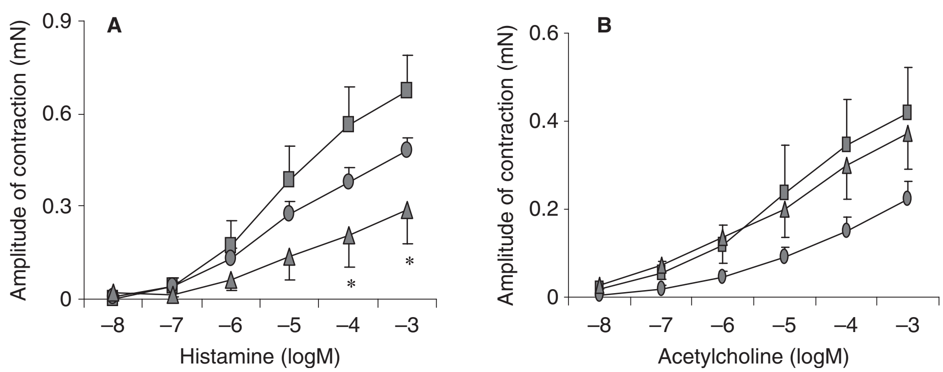

Figure 5. Effect of pretreatment with methylene blue in a dose of 50 mg/kg b.w. (●) and 100 mg/kg b.w. (▲), administered after exogenous irritant exposure on lung tissue smooth muscle reactivity to histamine (A) and acetylcholine (B) compared to the control group (■). Values are mean ± *SEM*. $^*p < .05$. b.w. = body weight.

tracheal smooth muscle response only to histamine. The response was statistically significant at a concentration of 10^{-5}–10^{-3} mol/l (Figure 4).

The response of lung tissue was similar to that of tracheal smooth muscle. The smaller dose of methylene blue applied 30 min after toluene exposure did not cause statistically significant changes in lung tissue reactivity to either bronchoconstrictor mediator (Figure 5). We recorded statistically significant changes after pretreatment with methylene blue in the higher dose to histamine at a concentration of 10^{-4}–10^{-3}mol/l only.

Discussion

NO generated by NOS diffuses out of the generator cell to target cells. Higher NO amounts can produce three chemical reactions: rapid NO reaction with hemoglobin, oxidation by superoxide, and activation of sGC with stimulation of cGMP synthesis. cGMP production in the airways decreases smooth muscle tone. We proposed that the inhibition of sGC would evoke an increase of airway smooth muscle response. Our findings support the results of other papers. For example, Sadeghi-Hashjin et al. (1996) showed that inactivation of NO or guanylyl cyclase by a selective inhibitor increased airway reactivity. This suggests that drugs that enhance cGMP levels in the airway smooth muscle may be useful in the treatment of airway obstruction and enhanced reactivity. A similar effect was described by Papapetropoulos et al. (2005), who showed that expression of sGC is reduced in a model of allergic asthma and that sGC inhibition results in AHR. The inhibition of sGC by methylene blue modified the AHR in guinea pigs in our experimental conditions but with an opposite effect. We recorded a significant decrease of tracheal and lung tissue reactivity to histamine and acetylcholine in pretreated animals compared with control groups. The effect of methylene blue was dependent on dose, time of administration (before or after irritant exposure), the bronchoconstrictor mediator used (histamine or acetylcholine), and the airway level (trachea, lung tissue).

Study of sGC-mediated processes has been difficult because of the lack of drugs that can selectively modulate this specific enzyme activity (Hobbs, 1997). We used the most accessible nonselective sGC inhibitor, methylene blue, in our studies. This substance has various effects that may account for its protective effect in hyperreactive conditions. First, methylene blue affects not only sGC but other enzymatic systems containing ferrous iron (e.g., NOS cyclooxygenase [COX]) that may also play a role in airway smooth muscle function. Second, methylene blue also inhibits superoxide production by competing with molecular oxygen for the transfer of electrons by xantine oxidase (Salaris, Babbs, & Voorheesiiw, 1991). Both mechanisms markedly interfere with the control of airway smooth muscle tone. Thus, we propose that the decrease of hyperreactivity in our study could be caused by a combination of these effects and could be related to the different enzyme systems (sGC, NOS, COX), free radical production, antioxidant mechanisms, or tissue sensitivity (Antošová et al., 2005; Weingartner et al., 1999). Evgenov, Evgenov, Mollnes, & Bjertnaes (2002) demonstrated that methylene blue inhibits the endotoxin-induced systemic release of COX products in vivo. The exact mechanisms of this effect remain unclear. The reduced systemic release of prostanoids could result from interference with receptor coupling mechanisms that control the release of arachidonic acid from membrane phospholipids, from a change in the amount and/or activity of COX and terminal prostaglandin synthesis through oxidation of the ferrous heme moiety of the enzymes, or from decreased extracellular secretion. The finding that methylene blue impairs the release of prostanoids in vivo refutes the contention that this dye acts only as a blocker of sGC and NOS (Evgenov et al., 2002). Alternatively, the study of Osanai et al. (2000) demonstrated that pretreatment with methylene blue enhanced prostacyclin production. This compensatory mechanism is likely to be present in our experiments also. The increase of prostacyclin production may provoke the decrease in contraction amplitude in the airway smooth muscle.

Toluene exposure represents a specific kind of oxidative stress (Mattia, Lebel, & Bondy, 1991) that can cause lipid peroxidation with changes in the airway tissues (Strapková et al., 1996). Thus methylene blue can modify this situation. But it is very difficult to define the exact mechanism by which methylene blue may evoke the airway reactivity changes seen in our study. We propose that methylene blue may interfere at all previously discussed levels—NOS-COX-ROS (reactive oxygen species).

A smaller dose of methylene blue decreased tracheal smooth muscle reactivity only when it was administered before toluene exposure. The higher dose reduced predominantly lung tissue hyperreactivity, independent of the time of application. We observed an expressive effect with histamine in both therapeutic regimens. Our results showed more expressive airway reactivity changes overall if methylene blue was applied before toluene exposure. Tracheal smooth muscle may be characterized as more sensitive because the decrease in reactivity occurred with the smaller dose of methylene blue. This result can be connected with the factors described above (a different localization of sGC, NOS, COX, free radical production, antioxidant mechanisms, or tissue sensitivity).

Variation in distribution of inhibitor, depending on the specific injection site and regional blood flow, may also influence the effects of methylene blue (Jones, Wong, Jankowski, Akao, & Warner, 1999; Weinbroum, Goldin, Kluger, & Szold, 2002). Other mechanisms of action as direct effects such as diffusion in the bronchial microcirculation or activation of NO-dependent channels with subsequent airway smooth muscle relaxation, may be occurring simultaneously.

More recent studies suggest that age plays a role. Belik, Hehne, Pan, and Behrends (2007) showed that sGC agonists significantly relaxed the newborn but not the adult bronchial muscle. Protein expression of the sGC α_1 and β_1 subunits was significantly lower in the adult compared with the newborn bronchial tissue. The investigators showed that NO-stimulated sGC activity was significantly greater in the newborn compared with the adult rat. We used adult guinea pigs in our experiments, and we can only hypothesize that the dominant effect of NO in these adult animals need not be cGMP dependent. The effect of methylene blue may also be species connected (Ellis, 1997).

This inhibitor antagonized relaxation of dog tracheal smooth muscle but relaxation of pig tracheal smooth muscle was uninfluenced (Stuart-Smith, Bynoe, Lindeman, & Hirshman, 1994).

In summary, our study demonstrates that the inhibition of guanylyl cyclase activity decreased the airway reactivity to exogenous irritant exposure. The decrease of airway reactivity was dependent on the regimen of methylene blue administration (before or after toluene exposure) and type of bronchoconstrictor mediator (histamine or acetylcholine). We observed different effects on different airway levels (trachea, lung tissue), depending on the dose of methylene blue used. The importance of this study lies in an explanation of NO mechanisms of action participating in the regulation of bronchial smooth muscle tone and in AHR pathogenesis. We suggest that NO/cGMP, as well as other pathways, is involved in airway reactivity changes evoked by exogenous irritants, but further experiments are warranted.

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