EXPERIMENTAL STUDY

Inhibition of guanylyl cyclase in the airways hyperreactivity

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Abstract

Background: **The majority of nitric oxide (NO) effects in the respiratory system are mediated via the stimulation of soluble guanylyl cyclase with subsequent generation of the second messenger – cyclic guanosine monophosphate (cGMP).**
Objectives: **We were interested in the effect of non-selective soluble guanylyl cyclase inhibitor – methylene blue on the exogenous irritant-induced bronchial hyperreactivity.**
Methods: **Male guinea pigs were used in the experiment. The animals received non-selective soluble guanylyl cyclase inhibitor – methylene blue in a dose of 50 or 100 mg/kg b.w. 30 minutes before inhalation of the exogenous irritant – toluene vapours. The toluene exposition lasted three consecutive days during two hours in in vivo conditions. The monitoring of tracheal and lung tissue strips reactivity changes was carried out in in vitro conditions. The brochoconstrictor mediators histamine and acetylcholine in the cumulative doses (10^{-8}–10^{-3} mol/l) were used in the experiment.**
Results: **The methylene blue pretreatment induced the decrease of tracheal and lung tissue smooth muscle contraction amplitude increased by exogenous irritant – toluene. We recorded different smooth muscle response depending on the doses of inhibitor. Methylene blue in a dose of 50 mg/kg b.w. affected mainly tracheal smooth muscle, in a dose of 100 mg/kg b.w. mainly the lung tissue.**
Conclusion: **The interaction between nitric oxide and soluble guanylyl cyclase can be important for bronchial reactivity changes. The changes depended on the dose of inhibitor and on the type of respiratory system tissue (trachea, lung). We can summarise that changes of the airways reactivity are not only evoked by NO/cGMP pathway but probably by any other mechanisms *(Fig. 5, Ref. 26)*.**
Key words: nitric oxide, soluble guanylyl cyclase, airways reactivity.

Nitric oxide (NO) is the main neurotransmitter of inhibitory non-adrenergic non-cholinergic system (NANC). It is very important in the regulation of the airway smooth muscle tone.

Nitric oxide mediates bronchodilation through various mechanisms (1). The mechanisms of nitric oxide action are divided into two groups – cyclic guanosine monophosphate (cGMP)-dependent or cGMP-independent. cGMP-independent mechanism is mediated by ion channels (Ca^{2+}-dependent K^+ channels, K_{ATP} channels etc.) or by free radicals production and release or by means of NO direct effect that represents NO diffusion from the site of generation to the target tissue (2). The majority of NO effects are cGMP-dependent.

cGMP is the second messenger produced through activation of guanylyl cyclase (3, 4). Two major classes of guanylyl cyclase are known: particulate (pGC) and soluble (sGC). pGC is a membrane bound receptor for natriuretic peptides. This enzyme has various isoforms. Particulate GC-A acts as the receptor for the atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). Particulate GC-B displays the highest affinity for the natriuretic peptide of the C-type (CNP). Further GC isoforms GC-C, GC-D, GC-E, GC-F have more specific localization and activity (5, 6).

Soluble guanylyl cyclase, a haeme-containing enzyme plays a pivotal role in the transduction of inter- and intracellular signals conveyed by the signal molecule NO. It is known that NO activates sGC via interaction with their haeme prostethic group (7). Thus activated enzyme catalyzes the conversion of guanosine

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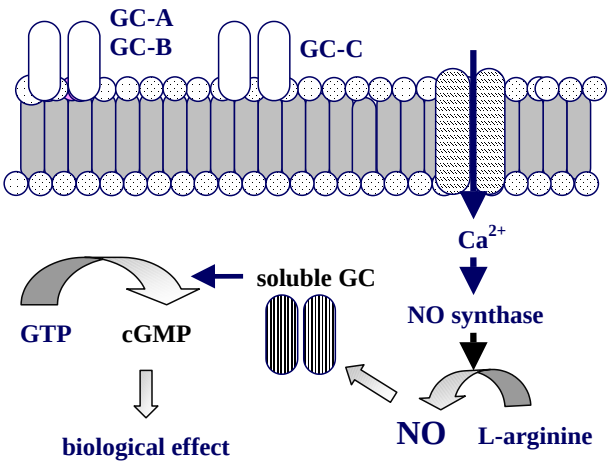

Fig. 1. The illustration of the NO effect mediated by cGMP. GC — guanylyl cyclase, GC-A, GC-B, GC-C — the isoforms of particulate guanylyl cyclase, NO — nitric oxide, GTP — guanosine triphosphate, cGMP — cyclic guanosine monophosphate.

triphosphate (GTP) to cGMP (Fig. 1). cGMP activates subsequently cGMP-dependent proteinkinase G (PKG) and proteinkinase A (PKA) which ultimately decreases both cytosolic Ca^{2+} concentration and the phosphorylation of many intracellular proteins (8). The final effect is the relaxation of airway smooth muscle. Soluble guanylyl cyclase has been localized in the bronchial and vascular smooth muscle in the lungs of various species (6).

The discovery of the role of NO in the regulation of the respiratory system functions and the importance of the NO-GC-cGMP pathway raises many other questions on the participation of NO-GC-cGMP pathway in some symptoms of respiratory diseases. The bronchial hyperreactivity (BHR) is one of important hallmarks in various respiratory diseases including asthma and chronic obstructive pulmonary disease. There are some possible mechanisms of BHR origin at present time. It is supposed that one of the factors contributing to BHR is the change of NO and cGMP level in the respiratory system. There is not enough information on this question in the literature. Therefore the aim of our study was to participate in investigation of the NO/cGMP pathway involvement in the exogenous irritant-induced bronchial hyperreactivity by using the non-specific soluble guanylyl cyclase inhibitor methylene blue.

Material and methods

Animals and drugs

Pathogens free male Trick guinea pigs (280–350 g) were divided in to three groups that were used in the presented 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 nonselective inhibitor of soluble guanylyl cyclase – methylene blue (subst. Sigma) in a dose of 50 mg/kg b.w. intraperitoneally. The second group (n=8) received a nonselective inhibitor of soluble guanylyl cyclase – methylene blue in a dose of 100 mg/kg b.w. intraperitoneally. The third group (n=8) – the control group received aqua pro injectione. The inhibitor was administered once a day 30 minutes before inhalation of the exogenous irritant – toluene for 3 days. The Ethics Committee of Jessenius Faculty of Medicine has approved the study protocol.

Toluene exposure

The method of in vivo exposition to the toluene vapours described by Strapková et al (9) was used in this experiment. The animals were spontaneously breathing toluene vapours in a special exposure chamber made of plexiglass. The chamber consists of compressor, flowmeter, vaporizer and exposure cage. The device was situated in the fume-cupboard at 22 °C. Toluene vapours were supplied into the cage with constant flow of 4 liter/min. The average concentration of toluene was 6 mg/l (1600 ppm). The duration of exposure was two hours each of the three consecutive days (9).

Airways responsiveness

Animals were killed 24 hours after last toluene exposure and trachea and lung were removed. The trachea and lung tissue thin strips were 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). The Krebs-Henseleit solution was maintained at 36±0.5 °C and continuously gassed with mixture of 95 % O_2 and 5 % CO_2 pH 7.5±0.1. The upper part of the tissue strip was connected to a force transducer and an amplifier and tension recordings were performed on PC software (RES, Martin, Slovak Republic). The strips were set to the initial resting tension of 4 g (30 minutes) and thereafter were readjusted to a baseline of 2 g (30 minutes – adaptive phase). Krebs-Henseleit solution was changed every 10 minutes. The strips were contracted by cumulative doses of histamine and acetylcholine (10^{-8}–10^{-3} mol/l). Statistical analysis was performed using the Student's t-test. P values of less than 0.05 were considered significant. All results are expressed as mean±S.E.M.

Results

In our experiment we compared the changes of the bronchial reactivity evoked by toluene exposure after 3 days administration of soluble guanylyl cyclase inhibitor – methylene blue in two doses (50 or 100 mg/kg b.w.) with the control group exposed to the toluene vapours following aqua pro injectione pretreatment.

Guinea pig trachea

We observed a decrease of tracheal smooth muscle response to histamine and acetylcholine in both groups of animals that received methylene blue in two different doses. However, more marked decrease was observed after administration of the in-

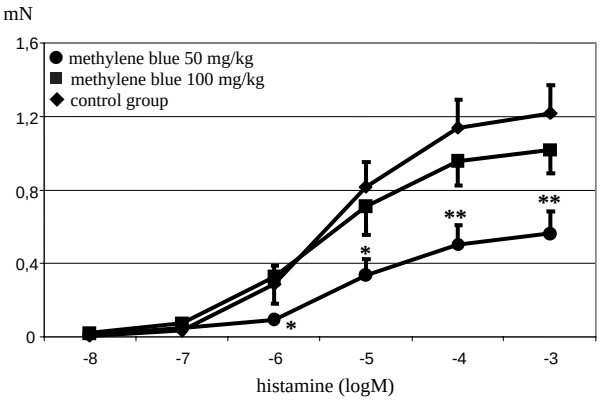

Fig. 2. The changes of tracheal smooth muscle reactivity to histamine. The effect of the 3-days pretreatment with methylene blue in a dose of 50 mg/kg b.w. (●), methylene blue in a dose of 100 mg/kg b.w. (■) compared with toluene group (◆). The curve represents average values of the contraction amplitude with mean error of average±S.E.M. Axis x — the concentration of histamine in log M, axis y — the amplitude of the contraction in mN. Significance p<0.05 — one asterisk, p<0.01 — two asterisks.

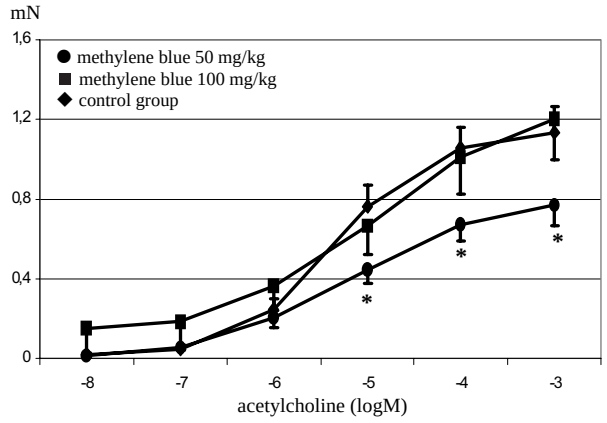

Fig. 3. The changes of tracheal smooth muscle reactivity to acetylcholine. The effect of the 3-days pretreatment with methylene blue in a dose of 50 mg/kg b.w. (●), methylene blue in a dose of 100 mg/kg b.w. (■) compared with toluene group (◆). The curve represent average values of the contraction amplitude with mean mistake of average±S.E.M. Axis x — the concentration of the acetylcholine in log M, axis y — the amplitude of the contraction in mN. Significance p<0.05 — one star.

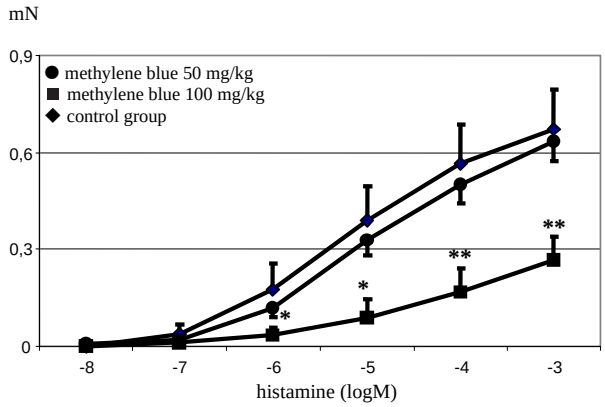

Fig. 4. The changes of lung tissue smooth muscle reactivity to histamine. The effect of the 3-days pretreatment with methylene blue in a dose of 50 mg/kg b.w. (●), methylene blue in a dose of 100 mg/kg b.w. (■) compared with toluene group (◆). The curve represents average values of the contraction amplitude with mean error of average±S.E.M. Axis x — the concentration of histamine in log M, axis y — the amplitude of contraction in mN. Significance p<0.05 — one asterisk, p<0.01 — two asterisks.

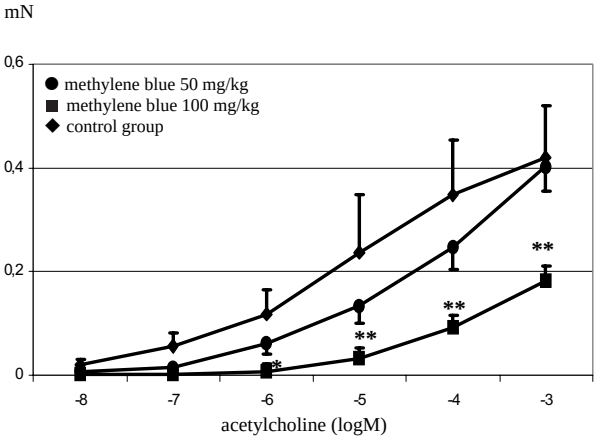

Fig. 5. The changes of lung tissue smooth muscle reactivity to acteylcholine. The effect of the 3-days pretreatment with methylene blue in a dose of 50 mg/kg b.w. (●), methylene blue in a dose of 100 mg/kg b.w. (■) compared with toluene group (◆). The curve represents average values of the contraction amplitude with mean error of average±S.E.M. Axis x — the concentration of acetylcholine in log M, axis y — the amplitude of contraction in mN. Significance p<0.05 — one asterisk, p<0.01 — two asterisks.

hibitor in a dose of 50 mg/kg b.w. intraperitoneally. The amplitude of contraction was significantly reduced at histamine concentration of 10^{-6}–10^{-3} mol/l (Fig. 2) and acetylcholine 10^{-5}–10^{-3} mol/l (Fig. 3). We did not observe any statistically significant changes of tracheal smooth muscle reactivity to histamine or acetylcholine after administration of methylene blue in a dose of 100 mg/kg b.w.

Guinea pig lung tissue

The smooth muscle of lung tissue showed significant decrease of the amplitude of contraction to histamine (Fig. 4) and to acetylcholine (Fig. 5) at the concentration 10^{-6}–10^{-3} mol/l after administration of methylene blue in a dose of 100 mg/kg b.w. intraperitoneally. The contraction amplitude was almost identical with control group amplitude after administration of the inhibitor in a dose of 50 mg/kg b.w. for histamine and it was not statistically significant for acetylcholine.

Discussion

The influence of NO-GC-cGMP relationship can be one of the factors determining changes of the airways reactivity. Our

current understanding of the mechanisms by which NO leads to changes of the smooth muscle tonus results mostly from studies with vascular smooth muscle. Numerous studies with vascular smooth muscle support the hypothesis that stimulation of soluble guanylyl cyclase is the initial event in NO-induced relaxation. However, the role of soluble guanylyl cyclase in response to NO and other vasodilators in the airways is controversial (10). We used the inhibition of soluble guanylyl cyclase to clarify participation of NO-GC-cGMP relationship in the airways reactivity changes evoked by exogenous irritant.

The sources of cGMP include both soluble (sGC) and particulate (pGC) forms of guanylyl cyclase. The smooth muscle of airways expresses both sGC and pGC. The inhibition of soluble guanylyl cyclase with methylene blue reduced the toluene vapour-induced airways bronchial hyperreactivity in the guinea pigs in our experimental conditions. We observed a decrease of tracheal and lung tissue smooth muscle response to histamine and acetylcholine in both groups of animals that received methylene blue in two different doses. Surprisingly, different response of tracheal and lung tissue smooth muscle in depending on the dose of methylene blue was recorded. The tracheal smooth muscle showed more significant response in animals pretreated with 50 mg/kg b.w. while lung tissue smooth muscle responds to dose of 100 mg/kg b.w. only. These results may be caused by the following:

The possibility of the different penetration of methylene blue in the dissimilar tissue types and distinct sensitivity of the smooth muscles to cGMP must be taken in to consideration (11, 12). We may speculate that methylene blue possibly influences the redistribution of blood flow among different organs. The effects of methylene blue on regional blood flow may be different (13). We used the intraperitoneal administration that has no specific influence in the respiratory system but other ways of administering were not possible to use in our conditions. Methylene blue is markedly coloured agent and its use by inhalation or in in vitro conditions is very inconvenient. On the other side, direct intravenous application to right ventricle and subsequent distribution to the lung circulation in in situ conditions is possible.

We suppose that there may be species differences in the mechanism of NO/cGMP pathway on the airway smooth muscle function (14). For example, the relaxation elicited by the NO donor in canine trachea is inhibited by methylene blue, whereas relaxations in porcine airways are not (15). Various studies in guinea pigs airways have suggested also a dichotomy between relaxation to NO and accumulation of cGMP. Studies in human airways found that although methylene blue blocked the NO-induced cGMP elevations it had no effect on relaxation (16, 17).

A significant limitation in the majority of studies is the lack of powerful pharmacological tools that could be used for study of the soluble guanylyl cyclase (12). We rely on methylene blue to inhibit soluble guanylyl cyclase but this compound is a weak non-specific inhibitor of soluble guanylyl cyclase. Moreover, it manifests other activities including the inhibition of iron-containing enzymes as NO synthase (NOS) and cyclooxygenase (COX) that play an important role in the controlling of the airways smooth muscle function (18).

There is an evidence for a biological interaction of NO with prostanoid that can take place at different levels. In the smooth muscle there also exists a synergy between the second messengers (cAMP and cGMP) of NO and prostanoid mediators. It can be supposed that the absence of NO which decreases cGMP levels induced a reduction of the PGI_2-dilating effects which is cAMP dependent (19, 20). The finding of other studies (21) showed that prostacyclin production is enhanced when NO production is suppressed. Osanai et al (22) investigated a role of cGMP in prostacyclin production by use of methylene blue. The results demonstrated that pretreatment with methylene blue enhanced prostacyclin production. This compensatory mechanism is likely to be present in our experiments, too. It is possible that the increase of prostacyclin production can provoke the decrease of amplitude contraction in the airways smooth muscle. There is information in the literature that methylene blue inhibits the generation of reactive oxygen species (ROS), particularly superoxides by competing with the molecular oxygen for the transfer of electrons by xanthine oxidase. This shunting of electrons to methylene blue thus attenuates the generation of reactive oxygen species (23) that are produced par example by toluene exposure (9). In addition, NO itself exists also in an oxidized or reduced form and all three forms can react with other radicals and molecules producing variety of reactive species (24).

In conclusion, methylene blue may interfere at these all levels (NOS-COX-ROS) that results in the airways reactivity changes presented in our study. The localization of cyclooxygenases, nitric oxide synthases and antioxidant mechanisms differ in the upper and lower airways that may result in different responses of these areas. It may be due to the different sensitivity of enzymes and mechanisms to cGMP dependent on the dose of methylene blue or to lower activity of sGC/cyclic GMP in trachea as compared to lung tissue (11, 25). It is necessary to take into consideration the participation of vessels in case of lung tissue (26). On the basis of the results of our experiments we can suggest that cGMP as well as other pathways are involved in the airways reactivity changes observed in our study.

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