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Relaxing effect of a new ruthenium complex nitric oxide donor on airway smooth muscle of an experimental model of asthma in rats

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ABSTRACT

NO is a potent bronchodilator and NO-donor compounds have demonstrated clinical significance for obstructive airway diseases. This study evaluated the relaxation mechanisms of two NO donors, a ruthenium compound (TERPY), and sodium nitroprusside (SNP), in rat tracheas with ovalbumin-induced asthma (OVA group) and in another control group. The effect of TERPY and SNP was evaluated in tracheal rings in an isolated organ chamber. The contribution of K⁺ channels, sGC/cGMP pathway, phosphodiesterases, and extra and intracellular Ca²⁺ sources were analyzed. The TERPY and SNP induced tracheal smooth muscle relaxation in both groups. However, the maximum effect induced by TERPY was higher than that of SNP in both control (110.2±3.2% vs 68.3±3.1%, p<0.001) and OVA groups (106.1±1.5% vs 49.9±2.7%, p<0.001). In the control group, TERPY relaxation was induced by the activation of K⁺ channels and reduction of the calcium influx, while in the OVA group, these same effects were also brought about by TERPY, but with participation of sGC/cGMP pathway. In both groups, SNP-induced relaxation occurred through the activation of K⁺ channels, sGC/cGMP pathway and reduction of calcium influx. However, the activation of sGC pathway and reticular Ca²⁺-ATPase seemed to be reduced in the OVA group. Furthermore, TERPY is capable of reversing the contraction of carbachol in asthmatic bronchioles. Finally, TERPY and SNP relaxation mechanisms were modified by asthma. SNP presented less relaxation than TERPY, which induced full relaxation with greater participation of K⁺ and Ca²⁺ fluxes through the membrane, thereby making TERPY a promising drug for reversing the narrowing of airways.

Keywords: airway smooth muscle; asthma; K⁺ channels; nitric oxide; ruthenium complex

INTRODUCTION

Asthma, a chronic inflammatory disease associated with hypersensitivity in the airways, leads to wheezing, dyspnoea, and coughing, particularly at night or in the early morning. The prevalence of asthma varies between 1% and 16% of the world's population and its clinical treatment is based on the use of bronchodilators and antiinflammatory drugs.¹ However, some patients remain refractory to these drug groups.^{2,3}

New alternatives have been made possible through pharmacological studies in animal models of airway inflammation. Sensitization with ovalbumin (OVA) is the classic model for inducing experimental asthma in rodents. OVA-sensitization leads to inflammation, and recruits cells, such as

eosinophils, mast cells, lymphocytes, and neutrophils, and is hyper-responsive to constrictor agonists, which is similar to what has been described in patients with asthma.^{1,4}

Nitric oxide (NO) is a key factor in the regulation of various physiological and pathological processes including airway smooth muscles relaxation, controlling the frequency of ciliary beating, helping in the transportation of electrolytes through the epithelium, and participating in the innate immune response, all of which make it an effective alternative for the treatment of inflammatory respiratory diseases such as asthma.⁵ For many years NO-donors have been recognized as inducers of bronchial relaxation.⁶

NO is very unstable in physiological conditions and can react almost instantly to molecules with unpaired electrons.⁷ An alternative is the use of NO-donor molecules, such as sodium nitroprusside (SNP). However, there are serious limitations to the use of this drug because of the liberation of cyanide as a product of biotransformation. Moreover, the administration of SNP can lead to a quick and severe drop in blood pressure and reflex tachycardia.⁸ In addition to these side effects, it only exerts a mild effect in relaxing airway smooth muscle.^{9,10}

An alternative to SNP could be ruthenium compounds, which have shown biological activities similar to those of NO, such as vascular and tracheal smooth muscle relaxation, a lowering of blood pressure, an inhibition of platelet aggregation, and an increase in intracellular cGMP levels.^{11,12}

A compound derived from ruthenium $[\text{Ru}(\text{terpy})(\text{bdq})\text{NO}^+]^{3+}$, (TERPY), [terpy = terpyridine and bdq = 1,2-benzoquinone-diimine] (Fig. 1)¹³, is a promising NO-donor compound whose action has been described as vasodilatory in both the resistance and conductance arteries of normotensive and hypertensive rats^{11,14,15} and as bronchodilatory in healthy rats.¹⁰ Significantly, it does not affect the vasculature of the central nervous system, which would leave it free of central side effects, such as headaches, which are common to other NO-donors.¹⁶ However, no research has yet evaluated the effects of TERPY in inducing relaxation in the airways of asthmatic rats. With that in mind, this study set out to test and characterize the action mechanisms of TERPY in the tracheas of rats with experimentally-induced asthma and compare them to the relaxation induced by the classic NO-donor SNP.

RESULTS

Experimental asthma model

The histological slices in Fig. 2 show cylindrical pseudostratified ciliated epithelium, fibrous connective tissue and smooth muscles attached to hyaline cartilage. Figs. 2A-D present the control group trachea while Figs. 2E-2H represent the OVA group trachea. Figs. 2E and 2F show intense mononuclear inflammatory infiltrate, and subepithelial permeated by small vessels (see arrow) when compared to Figs. 2A and 2B. Masson's trichrome showed smooth muscles, stained in red, in both groups (Figs. 2C and 2G – see arrow) characterized by elongated cells and flattened nuclei. Figs. 2D and 2H show mast cells dyed in purple (see arrow). The control group showed 14.7 ± 4.3 mast/ μm^2 and the OVA group showed 101.0 ± 12.0 mast/ μm^2 ($p < 0.05$). Moreover, the contraction evoked by carbachol ($0.5 \mu\text{mol/L}$) in the tracheal rings of the OVA group ($2.3 \pm 0.3\text{g}$, $n=15$) showed an increase of about 44% ($p < 0.01$) when compared to the control group ($1.6 \pm 0.17\text{g}$, $n=18$) (data not shown in Figure).

Relaxant activity of SNP and TERPY in rat tracheal smooth muscle

TERPY and SNP provoked relaxing effects in the tracheas of both control and OVA rats. Both NO-donors (10 nmol/L to $100 \mu\text{mol/L}$) cumulatively added to the bath solution during the sustained contraction induced by carbachol (EC_{50} , $0.5 \mu\text{mol/L}$) caused concentration-dependent relaxation (Fig. 3).

TERPY was more effective than SNP in both groups. It produced no significant difference in E_{max} between the control group ($110.2 \pm 3.2\%$, $n=9$) and the OVA group ($106.1 \pm 1.5\%$, $n=9$). The maximum effect of SNP on the control group was $68.3 \pm 3.1\%$ ($n=9$) while in the OVA group it was $49.9 \pm 2.7\%$ ($n=6$), which shows about 27% impairment ($p < 0.001$) in the relaxation of OVA rats (Fig. 3).

The pD_2 of SNP in the control group was 6.1 ± 0.07 ($n=9$) and 6.2 ± 0.06 ($n=6$) in the OVA group. For TERPY, the pD_2 was 5.1 ± 0.06 , ($n=9$) for the control group and 5.1 ± 0.09 , ($n=9$) for the OVA group, which shows that the inflammatory states in the tracheas did not alter the potency of SNP or TERPY (Fig. 3).

Participation of soluble guanylyl cyclase (sGC) and K⁺ channels in tracheal relaxation

The presence of the sGC inhibitor (ODQ) altered the maximum effect and potency of the SNP in the control group (Fig. 4A, Table 1). The $E_{\max}$ and pD_2 values of the SNP were significantly reduced (49% and 15%, respectively, $p<0.001$) after ODQ treatment in the control group. In the OVA group, the ODQ treatment only affected the potency of SNP (Fig. 3A, Table 1). The relaxation stimulated by TERPY was not affected by the ODQ treatment in the control group. In the OVA group, on the other hand, only the potency of TERPY was affected, and underwent a reduction from 5.0 ± 0.09 to 4.3 ± 0.07 ($p<0.001$) after sGC inhibition (Fig. 3B, Table 1).

The K⁺ channel blocker (TEA) increased the $E_{\max}$ of SNP in the control and OVA groups to about 19% and 63% ($p<0.05$ and $p<0.001$), respectively (Fig. 4C and Table 1). The inhibition provoked by TEA reduced the $E_{\max}$ of TERPY to about 56% and 87% in the control and OVA groups, respectively (Fig. 4D and Table 1). TEA did not alter the pD_2 of either NO-donor.

Effects of the PDE and SERCA inhibitors on the relaxation induced by TERPY and SNP

The non-selective PDE inhibitor (IBMX) increased the $E_{\max}$ of SNP in the control and OVA groups to about 24% and 104%, respectively (Fig. 5A, Table 1). It also increased the pD_2 values of the SNP from 6.2 ± 0.06 to 6.6 ± 0.07 in the OVA group. For TERPY, IBMX did not affect $E_{\max}$. However, the pD_2 was significantly increased after IBMX treatment in both the control ($p<0.01$) and OVA groups ($p<0.001$) (Fig. 5B, Table 1).

The SERCA inhibitor (CPA) altered the $E_{\max}$ and pD_2 of SNP in the control group. The $E_{\max}$ of SNP was reduced by about 43%, while the pD_2 was reduced from 6.1 ± 0.07 to 5.5 ± 0.09 ($p<0.01$) (Fig. 5C, Table 1). CPA did not alter either the $E_{\max}$ or pD_2 of SNP in the OVA group. On the other hand, CPA reduced the pD_2 of TERPY from 5.0 ± 0.09 to 3.7 ± 0.24 in the OVA group and did not have any significant effect on either the $E_{\max}$ or pD_2 in the control group (Fig. 5D, Table 1).

Effect of TERPY and SNP on extracellular Ca^{2+} -induced contractions

Cumulative additions of CaCl_2 to the bath solution produced concentration-dependent tracheal smooth muscle contractions in the presence of carbachol (Fig. 6).

In the control group, the Ca^{2+} influx led to contractions of $118.0 \pm 6.0\%$ ($n=5$) in control preparations. Treatment with SNP (100 $\mu\text{mol/L}$ for 20 min) caused a reduction of approximately 35% in the maximum contraction ($77.0 \pm 9.6\%$, $n=5$, $p<0.001$) while treatment with TERPY (100 $\mu\text{mol/L}$ for 20 min) significantly reduced the contraction by approximately 77% compared with the control group ($27.0 \pm 4.1\%$, $n=4$, $p<0.001$).

In the OVA group, the Ca^{2+} influx led to a contraction of $123.7 \pm 76\%$ ($n=5$) in control preparations. Treatment with SNP (100 $\mu\text{mol/L}$ for 20 min) caused a reduction of approximately 32% in the maximum contraction (84.1 ± 7.4 , $n=5$, $p<0.001$) while treatment with TERPY (100 $\mu\text{mol/L}$ for 20 min) completely eliminated the contraction stimulated by Ca^{2+} influx ($-1.6 \pm 2.1\%$, $n=5$, $p<0.001$).

Bronchiolar relaxation induced by TERPY

Fig. 7A shows the bronchoalveolar slice of the asthmatic animal with an initial area of $14,456.36 \mu\text{m}^2$ before adding carbachol. Fig. 7B shows the contraction 180s after stimulation by carbachol (0.5 $\mu\text{mol/L}$) with an area of $11,603.65 \mu\text{m}^2$ while Fig. 7C represents the relaxation induced by TERPY (100 $\mu\text{mol/L}$) 360s after the carbachol-induced contraction (final area of $13,196.14 \mu\text{m}^2$). The average contraction induced by carbachol in the bronchoalveolar slices was $17.6 \pm 4.9\%$ and the relaxation provoked by TERPY was $28.3 \pm 13.2\%$ ($n=5$, $p<0.01$) (Fig. 7D).

DISCUSSION

This study contributes to the knowledge of the effects of NO on the airway smooth muscle and on the use of a promising new NO-donor compound (TERPY) in the isolated airways of asthmatic rats. Both NO-donors studied caused concentration-dependent relaxation in both the asthmatic and healthy rat tracheas. TERPY was more effective than SNP and presented similar relaxation levels in both groups of animals, leading to full relaxation. Thus, we can state that the asthma condition did not alter the pharmacological parameters of relaxation induced by TERPY. Our results with SNP corroborate previous studies in which this drug

only induced a half-relaxation in airways.^{9,10} On the other hand, the asthma condition inhibited further relaxation, which indicated that the inflammatory process could reduce the relaxing effect of SNP. Our experiments have shown that the new NO-donor TERPY induces 100% relaxation in rat trachea, irrespective of the presence of asthma, and this could be a major advantage in relation to other NO-related drugs.

The histological findings on isolated tracheas showed that OVA triggered an inflammatory process associated with intense cell migration. Such findings were brought about by the increased reactivity of the tracheal smooth muscle in the presence of cholinergic stimulus, similar to that described in human asthmatic patients.^{1,2}

The NO activation of sGC induces the generation of cGMP, which leads to bronchodilations.⁹ However, in asthmatic patients, this bronchodilation is impaired even when large amounts of NO are used.¹⁷ In our study, the inhibition of sGC by ODQ in healthy trachea caused a significant reduction in SNP-induced relaxation. On the other hand, ODQ presented only a mild alteration in potency in asthmatic tracheas. This result suggests that asthma reduced the participation of the sGC/cGMP pathway in the SNP-induced relaxation. These data corroborate earlier findings, which have demonstrated that the sGC/cGMP pathway is impaired in asthmatic patients.^{18,19} Interestingly, the inhibition of sGC did not alter the pharmacological parameters of TERPY in healthy tracheas and only caused a reduction in potency in asthmatic tracheas. A possible explanation is that the relaxation induced by NO can also occur through cGMP-independent mechanisms and this seems to be the major route by which TERPY leads to relaxation in healthy tracheas causing, for example, activation of K⁺ channels.

The relaxing effect of TERPY after K⁺ channel blocking was largely reduced in both groups, which suggests that K⁺ channels participated in this effect. Moreover, the TEA almost completely eliminated the TERPY-induced relaxation in asthmatic tracheas, which shows the greater participation of K⁺ channels in this group. In contrast, these channels do not seem to be significant for SNP-induced relaxation. It is difficult to explain (and this study did not set out to do so) the unexpected effect of TEA on SNP-induced relaxation in both groups. The literature shows that TEA can exert secondary effects, such as the direct activation of gap junctions in various tissues including tracheas.^{20,21} These secondary effects of TEA could explain the unexpected increase in relaxation induced by SNP.

The results with IBMX reinforce the hypothesis that the sGC/cGMP pathway participates to a large extent in the relaxation induced by SNP in the tracheas of both groups. Since IBMX reduces degradation of the cGMP, an increase in the efficacy of SNP was to be expected, and was observed in both groups. However, IBMX provoked an increase only in

the potency of the TERPY in both groups. These results are in consonance with the data obtained after the sGC inhibition, using ODQ.

Intracellular Ca^{2+} concentration is responsible for determining tone in tracheal smooth muscles and several studies have shown that sarcoplasmic reticulum, which can be inhibited by cyclopiazonic acid (CPA), is involved in NO-smooth muscle relaxation.^{22,23} For SNP, we observed that the inhibition of SERCA reduced relaxation in healthy tracheas only, which shows that SNP can activate the SERCA. However, in the asthmatic group, the CPA treatment did not modify the SNP-induced relaxation, which suggests that the asthma condition could have desensitized the SERCA. In the physiological conditions, the SERCA activation is triggered by the sGC/cGMP pathway.²² Since the sGC/cGMP pathway could possibly be of secondary importance in the asthma condition, the SERCA inhibition did not change the relaxation induced by SNP. These results are also in agreement with the data obtained after the sGC inhibition, using ODQ. Unlike SNP, the treatment with CPA did not change the TERPY-induced relaxation in healthy tracheas, although it reduced the TERPY potency in asthmatic tracheas. This result along with the data obtained after sGC inhibition (using ODQ) suggest that the sGC/cGMP pathway could be a new mechanism by which TERPY leads to the relaxation of asthmatic tracheas. Apparently this pathway is not part of the relaxation in healthy trachea.

In airway smooth muscle, the extracellular Ca^{2+} influx is crucial to intracellular Ca^{2+} rise during the contraction phase and can be stimulated by a muscarinic agonist.^{23,24} Several different mechanisms for NO-induced inhibition of the Ca^{2+} influx through the plasma membrane have been proposed, including inhibition by cGMP-dependent mechanisms²⁵ or by membrane hyperpolarization due to direct or indirect cGMP-dependent activation of K^+ channels.²⁶ Our experiments also showed the importance of NO-induced extracellular Ca^{2+} influx inhibition in both healthy and asthmatic rat tracheas. We found that both SNP and TERPY are capable of inhibiting the contraction provoked by extracellular Ca^{2+} influx stimulated by an agonist, which suggests that the two NO-donors can block the Ca^{2+} influx. TERPY, however, was more effective than SNP in promoting this inhibition in both healthy and asthmatic tracheas. In the case of asthma, TERPY totally inhibited the contraction induced by the Ca^{2+} influx. Using these results as a basis, we can suggest that TERPY mediates relaxation through mechanisms that alter the mobilization to extracellular Ca^{2+} , principally in asthmatic rats.

The airways contract in the presence of a cholinergic stimulus and such an effect can vary according to anatomical site, when one considers that the bronchioles are more responsive than the proximal bronchial airways.²⁷ Our results demonstrated that while carbachol caused bronchoconstriction and reduced the area, TERPY was able to reverse the contraction and induce bronchodilation.

Our results show an important therapeutic effect of TERPY in airways from asthmatic rats. However, the main criticism of rodents as asthma models is the tolerance they develop with increasing allergen challenges.^{28,29} Although the development of tolerance is considered a disadvantage, some research consider it to be a good model for understanding allergic disease.^{28,30} OVA, the most commonly-used allergenic compound in immunology, is known to cause occupational asthma in both humans and animals, which can lead to inflammation with cell migration.^{28,31} Aluminium adjuvants (especially $\text{Al}(\text{OH})_3$) develop a chronic inflammatory response.³² Thus, OVA and $\text{Al}(\text{OH})_3$ are able to generate inflammation in the airways of rodents similar to that in human asthma.²⁸

Despite the fact that rats are used in popular disease models of allergic airways, the possibility of extrapolating from animal studies to humans depends on several factors, including the type and degree of purity of animals, sensitization, and challenge methods.²⁸ Guinea pigs, mice, rats, sheep, dogs, and others, are used as animal models of asthma. Rats have advantages over certain animals, such as mice as they present features for developing airway allergies and allergic asthma similar to those in humans.^{4,32} It is known that rats develop both immediate and late asthmatic responses after an allergen or nonspecific challenge.³³ In addition, rats are easier than mice to sensitize when using OVA and to challenge with $\text{Al}(\text{OH})_3$.^{4,29,31}

In summary, the new NO-donor TERPY is more effective in inducing relaxation in both asthmatic and healthy rat tracheas than the SNP. In healthy trachea, in contrast to SNP, NO released from the ruthenium complex, induces airway smooth muscle relaxation through cGMP-independent mechanisms and appears to involve Ca^{2+} and K^+ fluxes across the membrane (Fig. 8A). In asthmatic tracheas, in addition to these mechanisms, TERPY also provoked relaxation through activation of the sGC/cGMP pathway (Fig. 8B). SNP-induced relaxation in asthmatic and healthy tracheas also appears to involve Ca^{2+} and K^+ fluxes across the membrane and activation of NO/sGC/cGMP pathways. However, the activation of sGC pathways appears to be reduced in airway inflammation, as is the role of SERCA, which further impairs the relaxing effect of the SNP. Therefore, the biological effects induced by TERPY shown in our study will contribute to the body of knowledge about metallic-based NO-donors and their medicinal use for constructive pulmonary illnesses.

MATERIALS AND METHODS

Animals

Male Wistar rats (180–220g) from the Central Animal House, at the Federal University of Goiás, were used for the experiments. They were housed in a temperature- and light-controlled room ($22\pm 2^{\circ}\text{C}$; 12h light/dark cycle) in our laboratory with free access to water and rodent chow and acclimatized for at least one week before starting the experiment. They were handled in accordance with internationally accepted standard guidelines for the use of animals. All procedures were approved by the Animal Research Ethics Committee at the Federal University of Goiás, Goiânia, Brazil, under protocol number 029/2013.

Asthma was induced in the rats following the Kanikadan and coworkers model but with some modifications.²⁹ In brief, they were sensitized with an intraperitoneal injection of 100 μg of chicken ovalbumin (OVA) mixed with 10 mg of aluminum hydroxide as adjuvant, 1 mL in total (Day 0). After seven days, the animals were subcutaneously injected with 100 μg of OVA dissolved in saline (0.5 mL). On Day 14 the rats were subjected to a single exposure of aerosolized OVA 1% dissolved saline for 30 min using a nebulizer device (0.15-0.25 mL/min, Inalar Compact NS, São Paulo, Brazil) coupled to a plastic inhalation chamber (30 cm x 20 cm x 13 cm). This group was called OVA. Nonsensitized rats were submitted to intraperitoneal and subcutaneous injection with sterile saline and exposed to aerosolized saline in the same manner as the OVA group. This group was called the control group. On Day 15 of the experiments both groups were anaesthetized and sacrificed by aortic exsanguinations. The trachea was removed, dissected, cleaned and cut into 3-4 mm rings.

Tracheal Reactivity Studies

The tracheal rings were placed between two stainless steel stirrups and connected to an isometric force transducer. The responses were recorded using a computerized system and WinDaq Resource (DATAQ Instruments, Akron, OH, USA, data acquisition unit) to measure tension in the preparations. They were kept in a chamber containing a Krebs solution of the following composition (mmol/L): 130 NaCl, 4.7 KCl, 1.2 KH_2PO_4 , 1.2 MgSO_4 , 14.9 NaHCO_3 , 5.5 glucose, and 1.6 CaCl_2 . The solution was maintained at pH 7.4, and gassed with 95% O_2 and 5% CO_2 at $37 \pm 1^{\circ}\text{C}$. The rings were initially stretched to a basal tension of 1.0 g over 60 min for stabilization. During this period, the tissues were washed every 15 min. After equilibration, the tracheal rings were exposed to cholinergic agonist carbachol (0.5 $\mu\text{mol/L}$, EC_{50} previously determined in our laboratory) to obtain 50% of the maximum contractility. Each ring was sequentially washed, re-equilibrated and then left

to relax. Again, the preparations were pre-contracted with carbachol (EC_{50}) and cumulative concentration–response curves for SNP or TERPY (both 10 nmol/L to 100 μ mol/L) added to the organ bath in the absence or presence (30 min) of a pharmacology tool: TEA – tetraethylammonium, non-selective K^+ channel blocker (5 mmol/L), ODQ - 1H-[1,2,4] oxadiazolo[4,3a]quinoxalin-1, guanylyl cyclase inhibitor (1 μ mol/L), IBMX - 3-isobutyl-1-methylxanthine, non-selective phosphodiesterase (PDE) inhibitor (10 μ mol/L) and CPA - cyclopiazonic acid, selective inhibitor of sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) (10 μ mol/L) to investigate the mechanism(s) responsible for SNP- or TERPY-induced relaxation in the tracheal rings of the control and OVA groups of rats.

Participation of SNP and TERPY in the extracellular Ca^{2+} influx

To further analyze the relative contribution of SNP or TERPY to inhibiting Ca^{2+} influx in the tracheal smooth muscle, the role of extracellular Ca^{2+} mobilization stimulated by a contractile agonist was investigated by $CaCl_2$ -induced contraction in the presence of carbachol. Tracheal rings from control and OVA rats were first contracted with a concentrated solution of KCl (75 mmol/L) to produce maximal contraction (100% contraction). Then, the bath chamber was rinsed with a Ca^{2+} -free solution and the rings contracted with carbachol (EC_{50} , 0.5 μ mol/L) to deplete the intracellular Ca^{2+} stores until no contractile response remained (approximately 60 min). After that, the cumulative concentration–response curves for $CaCl_2$ (0.0 – 1.6 mmol/L) were obtained in a Ca^{2+} -free solution containing carbachol (0.5 μ mol/L), in the absence (control) or after a 20 min incubation period with SNP or TERPY (100 μ mol/L).

Histological Studies

The tracheal rings of the control and OVA rats were fixed in 10% buffered formaldehyde. 10 mm wide slices were then stained using the standard method with haematoxylin and eosin, Masson's trichrome and Toluidine blue for transmitted-light microscopy (Axio Scope.A1 with AxioCam MRc, Carl Zeiss – 200 and 400X) to identify the absence or presence of an inflammatory process, smooth muscle, and mast cells, respectively. In the Toluidine blue slices, mast cell density (μm^2) was calculated across the trachea using an integration grid (Carl Zeiss 474068000000-Net micrometer 12.5) with 40x objective.

Relaxation Bronchi by TERPY

This model was based on studies by Kott and coworkers but with some modifications.⁵⁰ In brief, after sacrificing the asthmatic rats, a lung was removed, kept in a Krebs solution, dissected and cut into 1-2 mm slices with the aid of a scalpel. In the light microscopy (Axio Scope.A1 with AxioCam MRc, Carl Zeiss – 50 X), the bronchioles were identified and their area measured (time zero). The carbachol (0.5 $\mu\text{mol/L}$) was added and the contraction was measured at 180s. Immediately after that, the TERPY (100 $\mu\text{mol/L}$ – EC_{100}) was added and relaxation was measured again at 360s.

Chemicals

The TERPY complex was synthesized and described by de Lima et al.¹⁹, in *Laboratory of Analytical Chemistry* by Dr. Roberto Santana da Silva. The drugs which included OVA, SNP, carbachol, ODQ, TEA, used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in the study were commercially available and of reagent grade. OVA was dissolved in 0.9% sterile saline. ODQ and CPA were dissolved in DMSO while the other drugs were dissolved in distilled water.

Statistical analysis

The data in the graphics are presented as means $\pm$ SEM and “n” indicates the number of rats studied in each set of experiments. The values for reactivity and responses to TERPY or SNP are expressed as percentages of the preceding contraction. The concentration of the compound producing a half-maximal response (EC_{50}) was determined after logarithmic transformation of the normalized concentration–response curves and reported as the negative logarithm ($-\log \text{EC}_{50} = \text{pD}_2$ values) of the mean of individual values for each tissue, using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). The maximum relaxant effect (E_{max}) was considered as the maximal amplitude response reached in concentration–effect curves for relaxant agents. The statistical analysis was performed using the GraphPad Prism version 5.0 (GraphPad Software Corporation San Diego, CA). Comparisons were made between groups using one-way ANOVA (post-test: Newman–Keuls) and Student’s t test, and values of $p < 0.05$ were considered statistically significant.

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Table 1: $E_{\max}$ (% relaxation) and pD_2 (potency) for SNP and TERPY in control and OVA rat tracheas in absence (control) or presence of different pharmacological tools: ODQ (guanylyl cyclase inhibitor), TEA (non-selective K^+ channels blocker), IBMX (non-selective phosphodiesterases inhibitor) and CPA (sarcoplasmic reticulum Ca^{2+} -ATPase inhibitor)

	SNP Control		SNP OVA	
	$E_{\max}$	pD_2	$E_{\max}$	pD_2
Control	68.3 ± 3.1%, n=9	6.1 ± 0.07, n=9	49.9 ± 2.7%, n=6	6.2 ± 0.06, n=6
ODQ	35.0 ± 7.7%, n=7***	5.2 ± 0.22, n=7***	44.5 ± 4.1%, n=5	5.8 ± 0.05, n=5*
TEA	81.6 ± 5.2%, n=9 *	6.3 ± 0.09, n=9	81.5 ± 7.9%, n=5***	6.2 ± 0.26, n=5
IBMX	83.7 ± 3.8%, n=6**	6.3 ± 0.09, n=6	103.1 ± 4.8%, n=6***	6.6 ± 0.07, n=6*
CPA	36.7 ± 6.4%, n=5***	5.5 ± 0.09, n=5**	63.7 ± 4.1%, n=6	5.9 ± 0.09, n=6
	TERPY Control		TERPY OVA	
	$E_{\max}$	pD_2	$E_{\max}$	pD_2
Control	110.2 ± 3.2%, n=9	5.1 ± 0.06, n=9	106.1 ± 1.5%, n=9	5.0 ± 0.09, n=9
ODQ	98.7 ± 1.3%, n=5	5.2 ± 0.10, n=5	108.7 ± 8.0%, n=5	4.3 ± 0.07, n=5 ***
TEA	48.3 ± 2.7%, n=7***	4.9 ± 0.18, n=7	15.2 ± 1.9%, n=6***	4.8 ± 0.35, n=6
IBMX	96.8 ± 1.5%, n=5	5.5 ± 0.19, n=5**	109.8 ± 32.1%, n=6	6.1 ± 0.19, n=6***
CPA	97.2 ± 3.4%, n=5	4.7 ± 0.06, n=5	116.1 ± 1.8%, n=5	3.7 ± 0.24, n=5***

Values are mean ± SEM. Significant difference when compared to control of each group (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

LIST OF LEGENDS

Fig. 1. Structure of $[Ru(terpy)(bdq)NO]^+{}^{3+}$.

Fig. 2. Photomicroscopy are transverse histological sections of nonsensitized rat trachea – control group - (A-D) and sensitized with OVA – OVA group - (E-H). Hematoxylin- and eosin-staining showed intense mononuclear inflammatory infiltrate subepithelial permeated by blood vessels of small arms

in (E) and (F) (see arrows) when compared to (A) and (B). Masson Trichrome-staining showed smooth muscles colored in red (C) and (G) (see arrows). Toluidine blue-staining showed mast cells colored in purple (see arrows) with increased density in sensitized trachea in OVA (H) as compared to control (D).

Fig. 3. Relaxant concentration–response curves induced by SNP and TERPY in isolated tracheas of control and OVA groups. Tracheas were pre-contracted with carbachol and both NO-donors were cumulatively added. Data are the mean $\pm$ SEM in preparations obtained from different animals. Statistics *** $p < 0.001$.

Fig. 4. Relaxant concentration–response curves to SNP (A) and TERPY (B) in carbachol-contracted rat trachea of control and OVA groups in absence or presence (30 min) of ODQ (1 $\mu\text{mol/L}$). SNP-induced (C) and TERPY-induced (D) relaxation were constructed in absence or presence (30 min) of TEA (5 mmol/L) prior to the addition of carbachol (0.5 $\mu\text{mol/L}$). Each point represents means $\pm$ SEM and expresses the percentage relaxation induced by SNP and TERPY. Statistics * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 5. Relaxant concentration–response curves to SNP (A) and TERPY (B) in carbachol-contracted rat trachea of control and OVA groups in absence or presence (30 min) of IBMX (10 $\mu\text{mol/L}$). SNP-induced (C) and TERPY-induced (D) relaxation were constructed in absence or presence (30 min) of CPA (10 $\mu\text{mol/L}$) prior to the addition of carbachol (0.5 $\mu\text{mol/L}$). Each point represents means $\pm$ SEM and expresses the percentage relaxation induced by SNP and TERPY. Statistics * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 6. Contraction-response curves to extracellular calcium (CaCl_2) in control (A) and OVA (B) groups in presence of 0.5 $\mu\text{mol/L}$ carbachol in Ca^{2+} -free Krebs solution in the absence (Control) or presence (20 min) of SNP or TERPY (both 100 $\mu\text{mol/L}$). Each point represents the mean $\pm$ SEM and expresses as maximal change from the contraction exerted by 75 mmol/L KCl, which was taken as 100%. Statistics *** $p < 0.001$.

Fig. 7. Photomicroscopy are transverse slices of asthmatic bronchioles. The images show cuts (approximately 1 mm) before (A) the addition of carbachol with an area of 14,456.36 μm^2 ; 180s after addition of carbachol (B), with an area of 11,603.65 μm^2 , and TERPY relaxation (C) 180s after addition of carbachol with an area of 13,196.14 μm^2 . (D) represents the percentage contraction exerted by carbachol (180s) and TERPY-induced relaxation (360s) in asthmatic bronchiole slices ($n=4$). Statistics ** $p < 0.01$.

Fig. 8. Cellular action mechanisms to TERPY in tracheal smooth muscle from the control group (A) and OVA group (B).











