Advances in Experimental Medicine and Biology 838
Neuroscience and Respiration

Mieczyslaw Pokorski *Editor*

Allergens and Airway Hyperreactivity

Springer

Advances in Experimental Medicine and Biology

Neuroscience and Respiration

Volume 838

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Mieczyslaw Pokorski
Editor

Allergens and Airway Hyperreactivity

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Editor
Mieczyslaw Pokorski
Institute of Psychology
University of Opole
Poland

ISSN 0065-2598 ISSN 2214-8019 (electronic)
ISBN 978-3-319-10008-1 ISBN 978-3-319-10009-8 (eBook)
DOI 10.1007/978-3-319-10009-8
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014957143

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

This is a new book series entitled Neuroscience and Respiration, a subseries of Springer's renowned Advances in Experimental Medicine and Biology. The book volumes present contributions by expert researchers and clinicians in the field of pulmonary disorders. The chapters provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of pulmonary disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with respiratory medicine, neural and chemical regulation of respiration, and the interactive relationship between respiration and other neurobiological systems such as cardiovascular function or the mind-to-body connection. In detail, topics include lung function, hypoxic lung pathologies, epidemiology of respiratory ailments, sleep-disordered breathing, imaging, and biomarkers. Other needful areas of interest are acute respiratory infections or chronic inflammatory conditions of the respiratory tract, exemplified by asthma and chronic obstructive pulmonary disease (COPD), or those underlain by still unknown factors, such as sarcoidosis, respiratory allergies, lung cancer, and autoimmune disorders involving the respiratory system.

The prominent experts will focus their presentations on the leading-edge therapeutic concepts, methodologies, and innovative treatments. Pharmacotherapy is always in the focus of respiratory research. The action and pharmacology of existing drugs and the development and evaluation of new agents are the heady area of research. Practical, data-driven options to manage patients will be considered. The chapters will present new research regarding older drugs, performed from a modern perspective or from a different pharmacotherapeutic angle. The introduction of new drugs and treatment approaches in both adults and children will be discussed. The problem of drug resistance, its spread, and deleterious consequences will be dealt with as well.

Lung ventilation is ultimately driven by the brain. However, neuropsychological aspects of respiratory disorders are still mostly a matter of conjecture. After decades of misunderstanding and neglect, emotions have been rediscovered as a powerful modifier or even the probable cause of various somatic disorders. Today, the link between stress and respiratory health is undeniable. Scientists accept a powerful psychological connection that can directly affect our quality of life and health span. Psychological approaches,

by decreasing stress, can play a major role in the development and course of respiratory disease, and the mind-body techniques can aid in their treatment.

Neuromolecular aspects relating to gene polymorphism and epigenesis, involving both heritable changes in the nucleotide sequence and functionally relevant changes to the genome that do not involve a change in the nucleotide sequence, leading to respiratory disorders will also be tackled. Clinical advances stemming from basic molecular and biochemical research are but possible if the research findings are "translated" into diagnostic tools, therapeutic procedures, and education, effectively reaching physicians and patients. All that cannot be achieved without a multidisciplinary, collaborative, "bench-to-bedside" approach involving both researchers and clinicians, which is the essence of the book series Neuroscience and Respiration.

The societal and economic burden of respiratory ailments has been on the rise worldwide leading to disabilities and shortening of life span. COPD alone causes more than three million deaths globally each year. Concerted efforts are required to improve this situation, and part of those efforts are gaining insights into the underlying mechanisms of disease and staying abreast with the latest developments in diagnosis and treatment regimens. It is hoped that the books published in this series will fulfill such a role by assuming a leading role in the field of respiratory medicine and research and will become a source of reference and inspiration for future research ideas.

Titles appearing in Neuroscience and Respiration will be assembled in a novel way in that chapters will first be published online to enhance their speedy visibility. Once there are enough chapters to form a book, the chapters will be assembled into complete volumes. At the end, I would like to express my deep gratitude to Mr. Martijn Roelandse and Ms. Tanja Koppejan from Springer's Life Sciences Department for their genuine interest in making this scientific endeavor come through and in the expert management of the production of this novel book series.

Opole, Poland Mieczyslaw Pokorski

Volume 7: Allergens and Airway Hyperreactivity

Respiratory allergy is constantly encountered and is sharply on the rise, particularly in the two most vulnerable age groups: young children and seniors. Allergy results in airway hyperreactivity and increased airway resistance, with ensuing inflammatory sequelae. The chapters show how respiratory allergy research is interconnected with other disciplines by discussing neurotransmitter, membrane receptor, and ionic channel mechanisms of allergy and by giving diagnostic and pharmacological cues on desensitization and therapy.

Contents

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 7: 1–10
DOI 10.1007/5584_2014_62

Published online: 25 September 2014

The Influence of L-NAME on iNOS Expression and Markers of Oxidative Stress in Allergen-Induced Airway Hyperreactivity

M. Antošová, A. Strapková, P. Mikolka, J. Mokrý, I. Medveďová, and D. Mokrá

Abstract

Nitric oxide (NO) effects in airways are influenced by the activity of NO-synthase isoforms and NO metabolism. Inducible NO-synthase (iNOS), which produces large amounts of NO, is active during the inflammatory process. NO quickly reacts, producing reactive oxygen species (ROS). In this study we attempted to detect the expression of iNOS and markers of ROS in the airway hyperreactivity (AHR) condition. The study was performed in guinea pigs, divided into four groups. Two groups were treated with the non-selective inhibitor of NO-synthase L-NAME. The other two groups were used as controls. Exhaled NO was monitored *in vivo,* AHR was assessed both *in vivo* and *in vitro*, and the expression of iNOS in lung homogenate, and oxidative stress markers were measured in the venous blood. L-NAME significantly affected the AHR only in *in vitro* condition, blocked the expression of iNOS in control but not in sensitized animals, and decreased the level of exhaled NO. The results concerning the oxidative stress markers are equivocal. The study confirmed that NO is involved in the regulation of AHR; the effects being mediated *via* iNOS and ROS activity.

Keywords

Airway hyperreactivity • Inducible NO-synthase • Nitric oxide • Oxidative stress

M. Antošová (✉), P. Mikolka, and D. Mokrá
Department of Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, 4 Malá Hora St., 036 01 Martin, Slovakia
e-mail: antosova@jfmed.uniba.sk

A. Strapková, J. Mokrý, and I. Medveďová
Department of Pharmacology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, 26 Sklabinska St., 036 01 Martin, Slovakia

1 Introduction

One of the most important aspects of human physiology is clarifying the status of nitric oxide (NO) in a wide range of different systems and organs, including the airways. NO acts as a neurotransmitter of inhibitory non-adrenergic non-cholinergic neurotransmission. Its sources

are different respiratory cells – neural, endothelial, epithelial, vascular, bronchial smooth muscle, inflammatory, and other cells (Ricciardolo 2003). NO is synthesized from L-arginine by three isoforms of NO synthase (NOS). The constitutive isoforms – neuronal (nNOS) and endothelial (eNOS) – produce NO in relatively small amounts, which are mainly involved in the regulation of physiological functions, e.g., bronchodilation, mucous secretion, mucociliary transport, gas exchange, or non-specific defense mechanisms. On the other hand, increased production of NO associated with high activity of inducible isoform (iNOS) is considered to underlie the development of pathological processes. The expression and activity of iNOS is increased in such conditions as exposure to exogenous irritants (smoking, allergens, etc.), bacterial products, or proinflammatory cytokines (Barnes and Belvisi 1993). Symptoms accompanying large NO amounts are proinflammatory changes, vasodilatation, plasma exudation, mucus hypersecretion, free radical productions, and airways hyperreactivity. A disordered balance of NO level in airways contributes to changes in airway smooth muscle tone. Activation of the iNOS is mainly responsible for changes in the body NO level including that in exhaled air.

iNOS is a cytoplasmic enzyme that particularly increases in response to proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin 2 and 10 (IL-2, IL-10), interferon gamma (IFN-γ), or lipopolysaccharides. NO generated by iNOS has antimicrobial, antitumor, cytostatic, or cytotoxic effects associated with free radicals production. Although NO has a protective role in a variety of infectious and inflammatory conditions (Kröncke et al. 1998), it also may play a major role, along with free radicals, in the pathogenesis of airway inflammation. A hallmark of inflammation is airway hyperreactivity, the pathogenesis of which is still unclear.

In pathology, iNOS can produce up to 1,000-fold greater amounts of NO, whose role shifts from regulatory and protective to cytotoxic. NO can be characterized as a free radical with a very short biological half-life. This property gives NO an opportunity to interact with a number of molecules, such as proteins containing thiol groups or superoxide ($\cdot O_2^-$). High amounts of NO produce, through a reaction with superoxide anion, cytotoxic peroxynitrite ($\cdot ONOO^-$), from which the biologically destructive hydroxyl radical originates (Anderson et al. 2011). ROS formed during airway inflammation accelerate the signaling processes of inflammatory mediators and as a consequence underlie the pathogenesis of airway hyperreactivity (Ghosh and Erzurum 2011; De Boer et al. 2001).

To clarify the involvement of NO and ROS pathways in airway hyperreactivity, in the present study we set out to determine the influence of pre-treatment with L-NAME, a non-selective inhibitor of NO-synthase, on the expression of iNOS and markers of oxidative stress (3-nitrotyrosine and thiobarbituric acid reactive substances – TBARS) in experimental allergic inflammation.

2 Methods

The study design was approved by the Ethical Committee of Jessenius Faculty of Medicine in Martin, Slovakia. All experiments were realized in accordance with the recommendations of Helsinki Declaration of the World Medical Association, Directive of European Commission on the protection of animals used for experimental and other scientific purposes adopted in 1986 (86/609/EEC) and the regulations of the Slovak Republic (Law No. 289/2003 Statute-book Regulation of Slovak Republic).

Thirty two pathogen-free, adult male TRIK strain guinea pigs weighing 180–250 g were used in the experiments. The animals were obtained from the Department of Toxicology and Breeding of Experimental Animals of the Institute of Experimental Pharmacology and Toxicology of the Slovak Academy of Sciences, Dobrá Voda, Slovak Republic, a certified breeding facility. They were group-housed in individual cages in the climate-controlled commercial cages, with a 12/12 h light/dark cycle in place,

and had access to water and chow *ad libitum*. Room temperature was maintained at 21 ± 1 °C.

2.1 Study Design

Guinea pigs were divided into four groups, each consisting of eight animals: two control and two experimental groups:

- healthy animals which received saline in a dose of 1 ml/kg;
- animals which were sensitized with ovalbumin (OVA, Sigma Aldrich, St. Louis, MO) in accordance with the sensitizing scheme described below;
- animals which received L-NAME (N^{ω}-nitro-L-arginine methylester) in a dose of 40 mg/kg daily for 14 days, without sensitization;
- animals which received L-NAME in a dose of 40 mg/kg daily throughout a 14-day sensitization time.

All injections were intraperitoneal unless otherwise indicated.

2.2 Airway Hyperreactivity Provocation

The animals were sensitized with the allergen ovalbumin. The sensitizing scheme was the following. A hundred micrograms of ovalbumin, dissolved in 1 ml saline, were injected on Day 1 (0.5 ml – subcutaneously in the neck and the other 0.5 ml intraperitoneally). On Day 3, the animals received only the intraperitoneal dose of OVA. Then, on Day 14, the animals inhaled 0.1 % OVA solution for 5 min. The inhalation of OVA was realized in a rodent whole-body plethysmograph consisting of glass thoracic and nasal chambers (type 885, Hugo Sachs Electronik; March-Hugstetten, Germany).

2.3 Measurement of Specific Airway Resistance

Specific airway resistance (RxV) was measured plethysmographically 2 min after inhalation of saline solution and subsequently 2 min after inhalation of the bronchoconstrictive mediator histamine in a concentration of 10^{-6} mol/l (Sigma-Aldrich, St. Louis, MO). The measurement was performed a day before OVA sensitization and on the last day of sensitization, 5 h after ovalbumin administration.

2.4 Airways Smooth Muscle Reactivity

Airway smooth muscle reactivity was recorded *in vitro*. The animals were euthanized with an overdose of an anesthetic 24 h after the last OVA administration. The trachea and lungs were removed and small tissue strips were prepared and placed into an organ bath consisting of the Krebs-Henseleit solution (110.0 mmol/l NaCl, 4.8 mmol/l KCl, 2.35 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 25.0 mmol/l $NaHCO_3$, and 10.0 mmol/l glucose in glass-distilled water), which was exchanged every 10 min. 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. The measurement of smooth muscle reactivity was conducted in the isolated tissue bath system (Experimetria, Budapest, Hungary). Tissue strips were initially exposed to the tension of 4 g for 30 min (loading phase). Thereafter, tension was reduced to the baseline level of 2 g for 30 min (adaptation phase). Tension changes induced by contracted tracheal and lung tissue strips in response to cumulative doses of histamine and acetylcholine (10^{-8}–10^{-3} mol/l; Sigma-Aldrich, St. Louis, MO) were recorded by computer after an hour's incubation time. A cumulative concentration-response curve was determined for every strip.

2.5 Exhaled Nitric Oxide Detection

Exhaled NO (eNO) was detected using a chemiluminescence method. The method consists of detection of the luminescence arising during a chemical reaction of NO and ozone, which

progresses in the analyzer (NIOX, Aerocrine; Solna, Sweden), which was adapted for small laboratory animals with a hermetic plexiglass chamber (MR Diagnostic; Prague, Czech Republic). The resulting output signal corresponds to the concentration of NO in the input sample (exhaled air). Each guinea pig was placed in the chamber individually. After 5 min of spontaneous breathing, the air from the chamber was discharged through a two-way valve into the analyzer. The off-line mode was used for analysis of all air samples. eNO analysis corresponded with the sensitization scheme. It was performed on Day 1, Day 3, and Day 14 in all groups of animals; in the sensitized ones 30 min before OVA administration.

2.6 Inducible NO-Synthase Expression

Distal parts of the lungs were taken for RT-PCR analysis. Lung tissue was homogenized in a solution consisting of ß-mercaptoethanol and RLT buffer for 20–40 s. Total RNA was isolated using RNeasy Micro Kit (QIAGEN Group; Hilden, Germany) and was then eluted in the RNase-free water. We used 1 μg of total isolated RNA for reverse transcription. cDNA synthesis was conducted using a QuantiTect® Reverse Transcription Kit (QIAGEN Group; Hilden, Germany). The transcription lasted at 42 °C and the reaction time was 20 min. The concentration of total DNA and cDNA was determined using a NanoPhotometer (Implen; Munich, Germany). Isolation of RNA and reverse transcription were performed according to the manufacturer's instruction.

iNOS primer sequences (forward: GCAGCAGCGGCTTCACA; reverse: ACATCCAAACAGGAGCGTCAT) used for the guinea pig were previously published (Yamada et al. 2005) and checked for a sequence homology against the known sequence of Cavia porcellus nitric oxide synthase-2, inducible (NOS2) mRNA (NCBI Reference Sequence: NM_001172984.1). Hypoxanthine phosphoribosyltransferase (HPRT) was used as a reference gene. The primer sequences were previously published as well (Cho et al. 2005). All data were normalized to HPRT mRNA expression in the same sample.

RT-PCR was performed with QuantiTect® SYBR® Green PCR Kit (QIAGEN Group; Hilden, Germany) according to the manufacturer's instruction, using 1 μl of cDNA (from RT) in a final volume of 25 μl containing 0.3 μM final F, R primer concentration. Quantitative PCR was performed using iCycler iQ®5 (Bio-Rad Laboratories; Hercules, CA) for 45 cycles at 95 °C for 15 s, primer-specific annealing temperature of 60 °C for 1 min, and 72 °C for 30 s. The crossing point, or the cycle number at which the fluorescence of the sample exceeded that of the background, was determined by the Bio-Rad iQ5–Standard Edition Optical System Software 2.0 using the second derivative method. RT-PCR assays of cDNA samples were performed in triplicate. A relative quantification method was used to assess the differences between tissue samples, employing the cycle number at which the fluorescence signal associated with a particular amplicon accumulation crosses the threshold, referred to as the (ΔCt).

2.7 Detection of Oxidative Stress Markers

We detected 3-nitrotyrosine and thiobarbituric acid reactive substances (TBARS) in the plasma. The presence of the former points to protein damage evoked by modification of tyrosine with peroxynitrite or other oxidative stress products, and the latter is indicative mostly of the level of lipid peroxidation. The blood was drawn from the heart to EDTA tubes immediately after animals' death and was then centrifuged for 15 min at 1,000 × *g*. Plasma was stored at −80 °C. The markers were detected with an enzyme-linked immuno-sorbent (ELISA) method using Oxi Select Nitrotyrosine and Oxi Select TBARS Assay kits (Cell Biolabs; San Diego, CA), respectively, according to the manufacturer's instructions. Plasma 3-nitrotyrosine was expressed

in nM and TBARS was expressed as quantitative values of malonedialdehyde (MDH) in μM.

2.8 Statistical Elaboration

All results are expressed as means ± SE. Statistical analysis was performed using one-way analysis of variance (ANOVA). Comparisons of baseline values between groups were performed with a *t*-test. Differences were considered statistically significant when p-value was below 0.05. A commercial statistical package was used for all calculations (Microsoft Excel and IBM SPSS Statistic Standard; Czech Republic).

3 Results

3.1 Specific Airway Resistance

Specific airway resistance (RxV) was significantly higher in the OVA-sensitized animals than in the control group of healthy non-sensitized L-NAME untreated animals ($p < 0.05$). L-NAME pretreatment had no effect on RxV in the healthy animals, but it decreased the enhanced RxV in the animals with allergic inflammation, although the effect did not reach statistical significance and the RxV remained above that present in the healthy animals (Fig. 1).

3.2 In Vitro Airway Reactivity

Changes in *in vitro* airway reactivity caused by L-NAME pretreatment in the non-sensitized and OVA-sensitized animals are exemplified by the presentation of the tracheal smooth muscle response to histamine only (Fig. 2). Tracheal smooth muscle contractile reactivity significantly increased in response to 10^{-6}–10^{-3} mol/l histamine in the healthy non-sensitized L-NAME pretreated animals compared with L-NAME untreated animals (Fig. 2a; $p < 0.05$*). Likewise, tracheal smooth muscle reactivity strongly increased in the OVA-sensitized L-NAME pretreated compared with L-NAME untreated animals (Fig. 2b; $p < 0.05$*, $p < 0.01$**). Thus, L-NAME pretreatment markedly enhanced tracheal smooth muscle reactivity in both non-sensitized and OVA-sensitized animals. Similar tracheal changes were observed in response to acetylcholine; however, lung tissue showed no reaction to L-NAME pretreatment in the OVA-sensitized condition (data not shown).

3.3 Exhaled Nitric Oxide

The level of eNO in healthy non-sensitized guinea pigs amounted to 3.4 ± 1.0 ppb. OVA sensitization had no appreciable effect on eNO which remained at 3.6 ± 0.4 ppb. Pretreatment with L-NAME in non-sensitized guinea pigs exerted a dual effect on eNO; it first significantly increased to 4.8 ± 0.6 ppb on Day 1 of sensitization and then decreased to 1.7 ± 0.8 ppb on Day 14 (Fig. 3a; $p < 0.05$‡), the level lower than that in the control untreated guinea pigs. In the OVA sensitized animals, L-NAME pretreatment caused a decrease in eNO throughout the experiment, to 1.9 ± 0.4 and 1.5 ± 0.6 ppb on Day 3 and Day 14, respectively. The decreases were significant compared with the OVA L-NAME untreated group ($p < 0.05$*) (Fig. 3a).

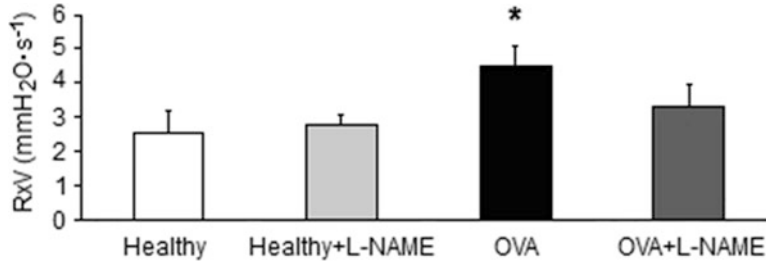

Fig. 1 Effects of L-NAME on specific airway resistance (RxV) (reactivity *in vivo*). $p < 0.05$ *vs.* healthy and healthy + L-NAME groups

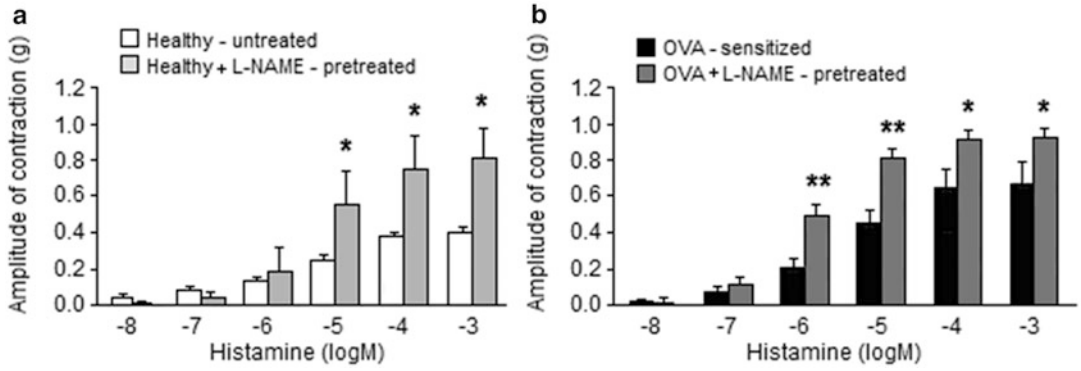

Fig. 2 Effects of L-NAME on tracheal smooth muscle reactivity (reactivity *in vitro*). (**a**) Healthy non-sensitized animals; $p < 0.05$* for L-NAME pretreated *vs.* L-NAME untreated and (**b**) OVA-sensitized animals; $p < 0.05$*, $p < 0.01$** for L-NAME pretreated *vs.* L-NAME untreated

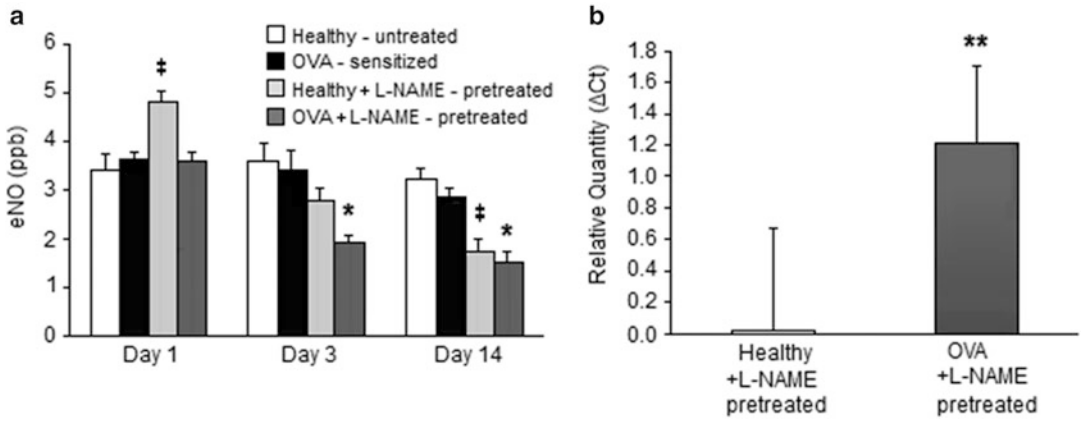

Fig. 3 Exhaled NO (a) and expression of iNOS in lung homogenate (b) before and after L-NAME pretreatment in non-sensitized and OVA-sensitized guinea pigs. The levels of eNO decreased gradually with therapy duration. OVA-sensitization markedly enhanced iNOS expression in lung tissue. See text for details

3.4 Inducible NO-Synthase

The expression of iNOS in lung tissue of healthy non-sensitized L-NAME pretreated animals was very low. It was strongly enhanced in OVA-induced hyperreactivity and stayed at the enhanced level despite L-NAME pretreatment in this group (Fig. 3b, $p < 0.01$**). In general, expression of iNOS in lung tissue was rather variable, which likely reflected local differences in inflammatory changes in various parts of lungs, such as epithelium, bronchi smooth muscle cells, blood vessels, etc.

3.5 Oxidative Stress Markers

Both markers of oxidative stress, 3-nitrotyrosine and malondialdehyde (MDA; one of the end products of lipids peroxidation in the TBARS assay), changed in like manner in response to the pharmacological procedures used (Fig. 4a, b). Their levels were significantly higher in the OVA-sensitized animals compared with the healthy non-sensitized ones ($p < 0.05$*). Pretreatment with L-NAME resulted in a dual effect; it enhanced the markers in the non-sensitized animals ($p < 0.05$*), but reversed the enhancement due to sensitization (Fig. 4a, $p < 0.01$‡).

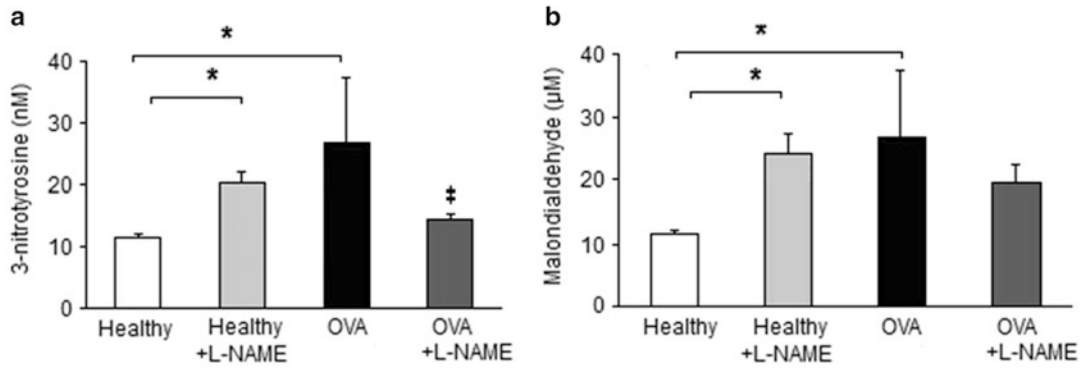

Fig. 4 Markers of oxidative stress in the experimental conditions used: (**a**) 3-nitrotyrosine and (**b**) malondialdehyde. Both markers were significantly increased by OVA-sensitization ($p < 0.05$*). L-NAME pretreatment enhanced the markers in non-sensitized ($p < 0.05$*) but decreased them in OVA-sensitized animals; the decrease was significant in case of 3-nitrotyrosine ($p < 0.05$‡)

4 Discussion

Airway hyperreactivity (AHR) is characterized as uncontrolled bronchoconstriction in response to various endogenous and exogenous stimuli (Grootendorst and Rabe 2004). It is a hallmark of asthma and chronic obstructive pulmonary disease (COPD), but can also be present in upper respiratory tract infections, rhinitis, or gastroesophageal reflux. Bronchoconstriction may be caused by allergens, chemical irritants, cold air, hypoxia, etc. The genesis of airway hyperreactivity after exposure to such stimuli involves various types of cell which release mediators. The most important of these mediators is nitric oxide.

The literature shows that the role of NO in airways is still largely uncertain. Therefore, we deemed it worthwhile to investigate NO changes in airway hyperreactivity. To invoke AHR we used OVA sensitization, a model of allergic inflammation (Antošová and Strapková 2008; Mokrý et al. 2013), which was confirmed in the present study by increases in specific airway resistance. The NO homeostasis was modulated with L-NAME, an inhibitor of NO-synthase, in both healthy and OVA-sensitized, and thus suffering from airway inflammatory hyperreactivity, animals. Pretreatment with L-NAME did not appreciably affect airway resistance in healthy non-sensitized animals. This finding is at variance with that of Rubini (2011) who found an increase in airway resistance after L-NAME in healthy rats. On the premise that NO causes bronchodilation, the inhibition of NO-synthesis by L-NAME could indeed have been expected to increase airway resistance. That was not the case in the present study. We surmise that there is no tonic NO action at the physiological level of airway resistance, in other words no need to bronchodilate in healthy animals, and thus the lack of appreciable action of L-NAME. In contrast, in allergic inflammation, where airway resistance is increased, which is accompanied by overproduction of NO, L-NAME clearly acts to decrease airway resistance. Jiang et al. (2008) did not report an inhibitory action of L-NAME in lipopolysaccharide-induced airway hyperresponsiveness in guinea pigs, but recorded a significant decrease in airway resistance after aminoguanidine (a selective iNOS inhibitor) pretreatment. In the present study, non-selective inhibition of NO-synthase sufficed to demonstrate a drop in the allergy-enhanced airway resistance.

In the present study L-NAME increased reactivity of tracheal and lung tissue smooth muscles on the background of bronchoconstrictive action of histamine and acetylcholine in non-sensitized animals. In OVA-sensitized animals we observed an increase only in tracheal smooth muscle reactivity; lung tissue did not react. The reason for

OVA-sensitized lung tissue not responding with increased reactivity on the background of bronchoconstriction is not readily explicable, but might have to do with a differential action of NO-synthase isoforms in response to airway allergy, depending on the level of the respiratory tract (trachea or lung tissue), and therefore a differential expression of the blocking effect of L-NAME. Plausibly, in OVA-sensitized animals L-NAME blocked predominantly cNO-synthase in lung tissue, but not iNOS through which NO could still be formed. These results correspond with the previously reported data on selective and non-selective inhibitors of NO-synthase (Antošová and Strapková 2008). It also is possible that in OVA-sensitized animals others mediators, such as cytokines, interleukines, or leukotrienes played a role in airway hyperreactivity which was not controlled for in the present study.

Subsequently we examined eNO, a marker of eosinophilic inflammation, in animals without and with L-NAME pretreatment. Detection of eNO is a specific noninvasive method, predominantly used in humans. In our department we used a standard allergic animal model, in which allergic inflammation was extensively confirmed by histology in our previous studies (Antošová 2007). We assumed that the values of eNO in allergic guinea pigs would be increased. Our results showed that after 14 days of OVA sensitization eNO was about the same as that in healthy animals. These results did not correspond well with increased airway reactivity found. However, eNO strongly increased the day following administration of L-NAME in healthy animals to decrease in later days in both healthy and OVA-sensitized animals. This dichotomous response of eNO to L-NAME pretreatment is not readily explainable. The literature data on the topic are scarce and controversial. Samb et al. (2001) described similar results; there was a reduction in eNO and an increase in hyperresponsiveness of tracheal smooth muscle in guinea pigs sensitized with ovalbumin. In the control group, these authors found an average value of eNO of 3.53 ppb, close the 3.37 ppb of the present study, and 2.52 ppb in sensitized animals. The findings were explained by a reduction in nNOS expression and activity in ovalbumin-sensitized animals, as no changes were observed in the other isoforms – eNOS and iNOS. A change in the activity of arginase, an enzyme that competes with the NO synthases for the common substrate – L-arginine, could contribute to a deficit of NO production. In contrast, Sethi et al. (2008) found in healthy mice that the baseline eNO of 7 ppb doubled in response to ovalbumin sensitization; the increase abated down to 10 ppb after 72 h. Likewise, Ahmad et al. (2009) showed a significant increase in eNO that correlated with allergic inflammation. After weeks of ovalbumin sensitization, eNO increased from 10.6 to 18 ppb in mice. The initial increase of eNO after L-NAME pretreatment in healthy animals in the present study could be evoked by pharmacokinetics properties and delayed onset of action of the inhibitor. The following eNO decrease confirms that the inhibition of NO was effective in both healthy and sensitized animals. To this end, our results are similar to those of Hori et al. (2011) who described a suppressive effect of L-NAME in both sensitized and non-sensitized animals. The question, however, remains, which NO synthase isoform is affected in relation to eNO changes.

The literature shows that asthmatic airway hyperreactivity has to do with increased expression of iNOS (Koarai et al. 2002; Schuiling et al. 1998), which suggests that this isoform is involved in the pathogenesis AHR and asthma. We confirmed the role of iNOS in the present study, finding that after L-NAME pretreatment in healthy animals its expression was very low, but it dramatically increased after allergen exposition. It seems that iNOS is the predominantly active NO isoform in airway hyperactivity and L-NAME is unable to fully inhibit it, which would be in line with a decrease in airway resistance in L-NAME-pretreated OVA-sensitized animals. The lack of the assessment of iNOS expression in the healthy untreated animals in the present study makes, however, the final judgment on the role of iNOS difficult.

ROS and reactive nitrogen species are increased in airway hyperreactivity and asthma in bronchoalveolar lavage, blood, and lung tissue, and ROS production is connected to a higher

expression of iNOS in airways (Andreadis et al. 2003). In the present study we assessed two markers – 3-nitrotyrosine, a marker of protein damage, and malondialdehyde, a marker of lipid peroxidation. Both markers were enhanced in OVA-sensitized animals. In these animals, expectedly, NO inhibition due to L-NAME pretreatment decreased the oxidative markers. However, in healthy untreated animals, L-NAME pretreatment increased both oxidative markers, which is less readily explainable. If, however, we assume that L-NAME is a predominant cNOS inhibitor, then NO would be formed *via* iNOS pathway and produced free radicals. Our results correspond with those of Sugiura et al. (1999) who detected an enhanced level of 3-nitrotyrosine during late allergic reaction in guinea pigs. Likewise, Capellier et al. (1996) founded that in acute lung injury TBARS substances are elevated in the plasma, but decreased in lung tissue, with no appreciable effect of L-NAME found.

In conclusion, the present study confirmed that NO is involved in the regulation of airway hyperactivity in the allergic model of asthma in guinea pigs; the effects having to do with iNOS and ROS activity. There is a need for alternative study designs, using specific selective NO synthase inhibitors to sort out the exact interactions between ROS and NO in allergic airway hyperactivity.

Acknowledgments This work was supported by the project "The increasing opportunities for career growth in research and development in the medical sciences", co-financed from the EU sources: Grant MZ SR 2007/46-UK-11 and VEGA 1/0062/13.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 7: 11–18
DOI 10.1007/5584_2014_77

Published online: 14 October 2014

Influence of Roflumilast on Airway Reactivity and Apoptosis in Ovalbumin-Sensitized Guinea Pigs

I. Medvedova, M. Prso, A. Eichlerova, D. Mokra, P. Mikolka, and J. Mokry

Abstract

Chronic inflammatory diseases, associated with airway obstruction and cough, are usually treated with bronchodilating and anti-inflammatory drugs. Inhibition of phosphodiesterases (PDE) leads to both of these effects and influences apoptosis of immune cells. In chronic obstructive pulmonary disease, roflumilast, a selective PDE4 inhibitor, has been recently approved for pharmacotherapy. The aim of this study was to evaluate the effect of long-term administration of roflumilast in experimental allergic inflammation in guinea pigs. Male adult guinea pigs were used in the study. There were four experimental groups sensitized with ovalbumin for 14 days and thereafter treated *per os*, by inhalation, and intraperitoneally for 7 days with roflumilast or vehicle. A control group was left without sensitization. Roflumilast reduced specific airway resistance after nebulization of histamine, as measured in a double-chamber whole-body plethysmograph. This effect was confirmed in *in vitro* organ bath, with significant decreases in tracheal and lung smooth muscle contractility after cumulative doses of histamine. Suppression of hematological and immunological markers of inflammation and enhanced apoptosis in animals treated with roflumilast points to the possibility of a beneficial effect of roflumilast in allergic inflammation.

Keywords

Apoptosis • Asthma • Inflammation • Phosphodiesterase-4 • Roflumilast

I. Medvedova (✉), M. Prso, A. Eichlerova, and J. Mokry
Department of Pharmacology, Jessenius Faculty of Medicine, Comenius University, 26 Sklabinska, 03601 Martin, Slovakia
e-mail: medvedova@jfmed.uniba.sk

D. Mokra and P. Mikolka
Department of Physiology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovakia

1 Introduction

Allergic asthma is a chronic inflammatory disorder of airways, characterized by allergen-induced, IgE-mediated early and late bronchial obstructive reactions, development of acute and transient airway hyperresponsiveness, and

infiltration of inflammatory cells, particularly eosinophils and Th_2 lymphocytes, in the airways (Maarsingh et al. 2009). The accumulation of eosinophils and subsequent release of their potent mediators, including cytotoxic granule proteins, lipid mediators, cytokines, and chemokines are thought to contribute to airway inflammation, which underlies the asthma pathogenesis (Duncan et al. 2003). In addition, Th_2 cells produce the interleukins IL-4 and IL-13, which promote IgE production by B cells, whereas IL-5 acts to promote airway eosinophilia, and IL-9 and IL-13 contribute to airway hyperresponsiveness (Robinson 2005).

Apoptosis, a form of natural or physiological cell death different from necrosis, can remove somatic cells without causing an inflammatory response; so that one way to eliminate inflammatory cells from lesions would be the induction of apoptosis in immune cells (Raouf 2007).

A primary goal of asthma therapy is to achieve a control of clinical symptoms by improving lung function, reducing airway hyperresponsiveness, and eliminating inflammatory cells (Schalkwyk et al. 2005). Cyclic nucleotide hydrolyzing phosphodiesterases (PDEs) comprise a still-growing superfamily of isoenzymes with 11 members known at present. Among the cAMP-specific isoenzymes, PDE4 has received particular attention due to the fact that all of the inflammatory and immunomodulatory cells contain this isoform. Enhanced PDE4 function may play a role in the pathogenesis of asthma due to increased protein expression or activity (Spina 2008). Immunomodulatory function of inflammatory cells is broadly inhibited by selective PDE4 inhibitors. Thus, PDE4 inhibitors show a pronounced anti-inflammatory effect in various animal models, and as such, they have been proposed as a new therapeutic approach in a variety of inflammatory diseases, including asthma and chronic obstructive pulmonary disease (COPD) (Hatzelmann and Schudt 2001).

The mostly clinically tested PDE4 inhibitors, cilomilast and roflumilast, have a favorable side effect profile compared with the first generation of similar compounds or theophylline (Vignola 2004). Roflumilast and roflumilast N-oxide, a major metabolite in humans, are highly potent, competitive, and selective inhibitors of PDE4, having essentially no activity against PDE1, PDE2, PDE3, and PDE5 isoforms (Giembycz 2002). Roflumilast represents the first PDE4 inhibitor registered in the market for COPD therapy (Keravis and Lugnier 2011).

The aim of the present study was to compare the effects of different routes of roflumilast administration in an animal model of bronchial asthma, associated with eosinophil inflammation, on airway reactivity and apoptosis of inflammatory cells in bronchoalveolar lavage fluid (BALF).

2 Methods

The study protocol was approved by the Ethics Committee of Jessenius Faculty of Medicine, Comenius University in Martin, Slovakia. Male guinea pigs (TRIK tribe) aged 1–3 months, weighing 150–350 g were used in the study. The animals were kept in the animal house and obtained food and water *ad libitum*. They were divided into six groups consisting of eight animals each. One of the groups was left without sensitization and served as a non-sensitized healthy control group. In the other five groups the hyperresponsiveness was induced by exposure to ovalbumin antigen. The animals of one of the five sensitized groups were given physiological saline as a vehiculum only and served as an ovalbumin-sensitized control group. The other four groups were treated with roflumilast from the 14th day of sensitization for 7 days *per os* at a dose 1.0 mg/kg in saline (3.0 ml/kg), roflumilast by inhalation for 3 min (1.0 mg/ml in saline), roflumilast i.p. (1.0 mg/kg in saline; 3.0 ml/kg), and roflumilast combined with salbutamol by inhalation for 3 min (0.5 mg/ml of roflumilast + 0.5 mg/ml of salbutamol in saline), respectively.

2.1 Antigen-Induced Airway Hyperresponsiveness

Sensitization was performed for 21 days to cause tissue injury and subsequent structural changes accompanied with inflammation (Tagaya and

Tamaoki 2007). The ovalbumin allergen (1 % solution in *aqua pro injectione*) was administered on the 1st day of sensitization by two routes: 0.5 ml i.p. and another 0.5 ml s.c., and on the 3rd day 1.0 ml, i.p. Afterward, on the 14th and 21st day the ovalbumin solution was inhaled for 30 s in an inhalation chamber.

2.2 In Vivo Airway Reactivity Assessment

Specific airway resistance (RxV) was measured as a marker of in vivo airway reactivity The animals were placed in a double-chamber whole body plethysmograph and an aerosol of histamine in the concentration of 10^{-6} mol/l in saline was used for the evaluation of airway reactivity. As a control, inhalation of saline was used. Airways hyperresponsiveness was evaluated after 2 min of histamine or saline inhalation.

2.3 In Vitro Airway Reactivity Assessment

Smooth muscle reactivity of tissue strips from the lungs and trachea was recorded in a tissue bath in an organ chamber. The contractile response to cumulative doses of histamine (10^{-8}–10^{-3} mol/l) was used as a marker of *in vitro* airway smooth muscle reactivity (Mokry et al. 2008).

2.4 Evaluation of White Blood Cells Viability

After sacrificing the animals, BALF was collected by lavaging the lungs with pre-heated saline (2 × 10 ml/kg, 37 °C). Subsequently, BALF sample of 5.0 μl was mixed with tryptan blue solution (5.0 μl) to stain white blood cells. An automatic cell counter (Countess™, Invitrogen, Carlsbad, CA) was used to estimate the cell viability and the total cell count of in BALF.

2.5 Statistical Analysis

Data are shown as means ± SE. For statistical analysis, one-way ANOVA was used. Statistical significance was defined as $p < 0.05$.

3 Results

Roflumilast caused a significant decrease in RxV after histamine nebulization in the guinea pigs treated with it orally or by inhalation of a half dose roflumilast together with salbutamol, compared with the control OVA-sensitized animals (Fig. 1).

The *in vitro* contractile response of airway smooth muscle to cumulative doses of histamine was suppressed in lung and tracheal tissue strips; the effect was more pronounce in lung tissue.

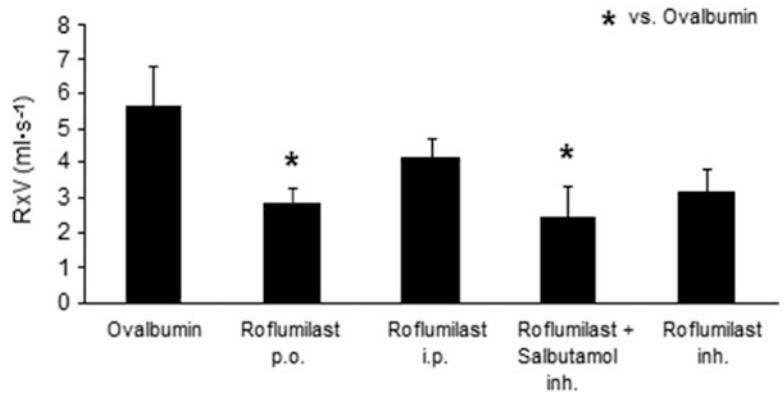

Fig. 1 Specific airway resistance after 7-day treatment with roflumilast by various routes of administration, *p.o* per os, *i.p.* intraperitoneally, *inh.* inhalation

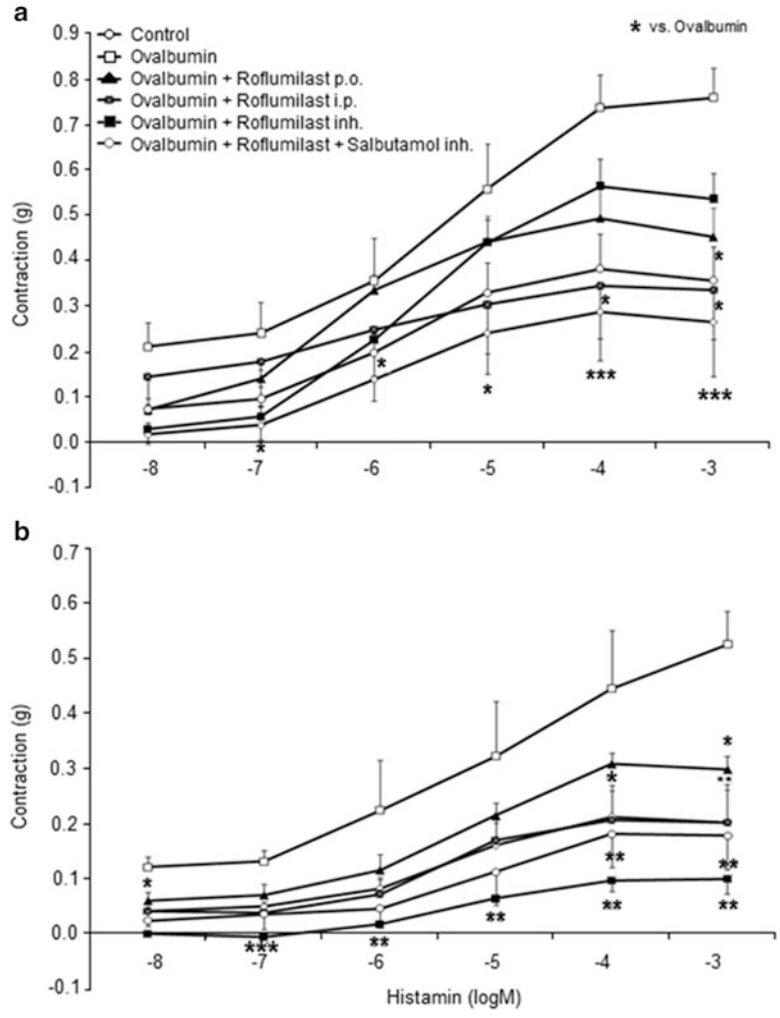

Fig. 2 Changes in tracheal (a) and lung (b) tissue reactivity in response to cumulative doses of histamine after 7-day treatment with roflumilast administered by various routes of administration; *p.o* per os, *i.p.* intraperitoneally, *inh.* inhalation

Roflumilast given alone by inhalation or in combination with salbutamol counteracted the histamine-induced suppression of tracheal and lung smooth muscle reactivity (Fig. 2a, b, respectively).

White blood cell viability in BALF in the ovalbumin-sensitized guinea pigs was significantly lower than that in the non-sensitized ones. In all roflumilast-treated groups, regardless of the route of administration, further suppression of blood cell viability was observed, with the most pronounced effect seen after i.p. roflumilast (Fig. 3a). However, the number of white blood cells in BALF significantly increased in the ovalbumin-sensitized guinea pigs, compared with the non-sensitized group. The increases were reverted by roflumilast given i.p. or by inhalation together with salbutamol (Fig. 3b).

4 Discussion

Airway responsiveness is usually described as the ability of airways to narrow after exposure to constricting agents, usually some inhaled allergens or antigens. Airway hyperresponsiveness is a hallmark of asthma. Histamine and metacholine are routinely used to assess the contractile airway response in bronchial asthma and COPD. Furthermore, fluctuations in airway hyperresponsiveness correlate with the severity

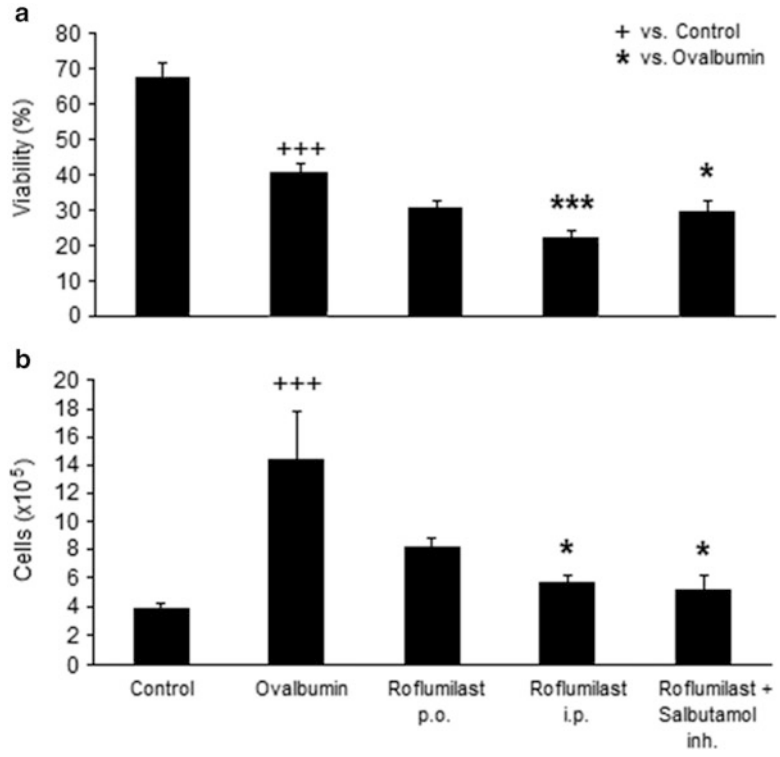

Fig. 3 Changes in white blood cells viability (a) and in the number of white blood cells in BALF (b) in control healthy and ovalbumin-sensitized animals after 7-day treatment with roflumilast by various routes of administration; *p.o* per os, *i.p.* intraperitoneally, *inh* inhalation

of disease and the requirement for drugs needed to control symptoms (O'Byrne and Inman 2003). Histamine is an inducer of bronchoconstriction, exerting its effect through a direct interaction with smooth muscle cells (Louw et al. 2007).

Roflumilast is a selective inhibitor of phosphodiesterase 4 (PDE4) with anti-inflammatory and immunomodulating activities demonstrated previously in several asthma and COPD models (Hatzelmann et al. 2010). In the present study, roflumilast was administered for 7 days to animals either orally, intraperitoneally, by inhalational alone or in combination with salbutamol. Reduction in specific airway resistance after nebulization of histamine was observed in animals treated with roflumilast orally and by inhalational together with salbutamol. Similar results were obtained in a clinical study conducted by Louw et al. (2007), who confirmed a decrease in airway hyperresponsiveness after a single oral dose of 1,000 μg of roflumilast in patients with bronchial asthma. Therefore, the PDE4 inhibitor roflumilast may reduce airway hyperresponsiveness both in experimental animal models and in patients with bronchial asthma. The majority of studies on PDE4 inhibitors, as therapeutic tools, were done in patients with COPD, where there is a different kind of inflammation involved, consisting predominantly of neutrophils. Studies on the role of roflumilast and on the PDE4 involvement in eosinophil inflammation are thus essential.

The existing clinical data demonstrate that the efficacy of roflumilast is relatively well confirmed. However, several other issues remain to be clarified, including the safety profile and systematic anti-inflammatory effects of roflumilast. A major safety issue is the emetogenic potential of roflumilast rarely reported after oral administration (Antoniu 2011). Based on these facts, we chose multiple application ways to test the ability of roflumilast to influence airway reactivity and viability of inflammatory white blood cells. Inhibition of PDE4 has been previously confirmed as a suitable target to decrease airway inflammation and contractility of airway smooth muscle. In a study of Mokry et al. (2008), however, citalopram, a first generation PDE4

inhibitor, was used with multiple other mechanisms of action, e.g., inhibition of serotonin reuptake in central nervous system.

Initially, PDE4 inhibition was expected to induce bronchodilation and reduction of the underlying eosinophil inflammation in bronchial asthma models, which was confirmed in preclinical tests (Antoniu 2011). In the present study, a significant reduction in contractility of airway smooth muscle in each group of animals treated with roflumilast, compared with untreated ones, was observed, regardless of the route of administration. The most significant decrease in smooth muscle reactivity was recorded in lung tissue strips of obtained from animals treated with roflumilast by inhalational alone or combined with salbutamol. Thus, inhalative administration could be considered a suitable way of roflumilast administration. This suggestion needs further testing.

We observed similar effects in tracheal tissue strips. However, the intraperitoneal administration here showed a more significant suppression of *in vitro* airway reactivity. That roflumilast reduced airway smooth muscle reactivity in lung strips from the allergen sensitized animals more than in control non-sensitized animals speaks for its direct bronchodilating effect and for the involvement of PDE4 not only in the regulation of inflammation, but also of smooth muscle contraction and relaxation.

The differences we observed between the reactivity of lung and tracheal smooth muscles are due likely to increased content of vascular smooth muscle in lung tissue compared with tracheal tissue. In a previous study by Bundschuh et al. (2001) roflumilast inhibited the ovalbumin-evoked contractions of tracheal strips prepared from ovalbumin-sensitized guinea pigs. Roflumilast administered intravenously displayed bronchodilatory activity and it dose-dependently attenuated allergen-induced bronchoconstriction in guinea pigs. In a study by Chapman et al. (2007) a robust effect of inhaled roflumilast was observed, leading to improvement of lung function in the allergen-challenged Brown Norway rats. The protective effects of inhaled roflumilast on lung function appeared to be superior to its effect when given orally. These results support the hypothesis that inhaled PDE4 inhibitors could be efficacious in inflammatory lung diseases, particularly due to improvement of lung functions.

As mentioned above, roflumilast is an anti-inflammatory drug leading to the elevation of intracellular cAMP and subsequently down-regulation of a variety of inflammatory cells activities, predominantly neutrophils and eosinophils. In an animal model of asthma, PDE4 inhibitors reduced inflammatory cell infiltration, mainly eosinophils (Bundschuh et al. 2001). Novel therapies aimed at enhancing apoptosis and phagocytic removal of immune cells might become a reality for patients with bronchial asthma (Walsh 2008). In the present study, we demonstrate that roflumilast affected the viability of inflammatory cells in BALF. The highest viability was observed in the healthy non-sensitized group, while in the OVA-sensitized group the cellular viability was significantly reduced. On the other hand, the number of inflammatory cells in BALF significantly increased in OVA-sensitized group compared with the non-sensitized one.

We found that roflumilast led to a significant reduction of both the number of total inflammatory cells and living inflammatory cells. However, the viability of inflammatory white blood cells was reduced compared with the healthy non-sensitized group. These results suggest that roflumilast induced apoptosis of inflammatory cells in the sensitized animals as a way of keeping in check inflammatory changes induced by OVA sensitization. Roflumilast-induced apoptosis was the strongest in animals treated intraperitoneally or by inhalation with salbutamol. In these groups, a concentration of viable (living) inflammatory cells in BALF was even lower than in the non-sensitized group.

IL-5 appears to be a specific survival factor for eosinophils, at least within the human system. Not surprisingly then, eosinophilia and high IL-5 expression have often been associated with one another, especially in chronic allergic disorders such as bronchial asthma. Moreover, it appears that the severity of asthma negatively

correlates with the amount of eosinophil apoptosis in the airways. A study by Simon (2001) suggests that delayed apoptosis represents one important mechanism leading to tissue eosinophilia. PDE inhibition has been shown to accelerate spontaneous apoptosis in both eosinophils and neutrophils, as well as partially to block IL-5-mediated delayed eosinophil apoptosis. Results from human subjects have also demonstrated significant pulmonary anti-inflammatory effects of roflumilast. Roflumilast treatment has been associated with an approximately 40 % reduction in sputum leukocyte number *versus* placebo (McIvor 2008). The animal studies with roflumilast demonstrate that it reduced the accumulation of inflammatory cells in BALF following a short-term exposure of guinea pigs, mice, or rats to allergen (Rabe 2011).

In conclusion, our experimental data suggest the potential of roflumilast to improve lung function and to exert anti-inflammatory, bronchodilating, and pro-apoptotic effects in ovalbumin-induced airway hyperresponsiveness, predominantly after intraperitoneal and inhalative administration. As a combination of the processes above outlined could be useful in therapy of patients with bronchial asthma, further studies are necessary to verify these results in clinical conditions.

Acknowledgments This work was supported by the Slovak Research and Development Agency under the contract No. APVV-0305-12, by Grant MZ2012/35-UKMA-12, Grant VEGA 1/0030/11, and by project UK/159/2013.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 7: 19–25
DOI 10.1007/5584_2014_79

Published online: 25 September 2014

Antitussive Activity of *Withania somnifera* and Opioid Receptors

Gabriela Nosálová, Veronika Sivová, Bimalendu Ray, Soňa Fraňová, Igor Ondrejka, and Dana Flešková

Abstract

Arabinogalactan is a polysaccharide isolated from the roots of the medicinal plant *Withania somnifera L.* It contains 65 % arabinose and 18 % galactose. The aim of the present study was to evaluate the antitussive activity of arabinogalactan in conscious, healthy adult guinea pigs and the role of the opioid pathway in the antitussive action. A polysaccharide extract was given orally in a dose of 50 mg/kg. Cough was induced by an aerosol of citric acid in a concentration 0.3 mol/L, generated by a jet nebulizer into a plethysmographic chamber. The intensity of cough response was defined as the number of cough efforts counted during a 3-min exposure to the aerosol. The major finding was that arabinogalactan clearly suppressed the cough reflex; the suppression was comparable with that of codeine that was taken as a reference drug. The involvement of the opioid system was tested with the use of a blood-brain barrier penetrable, naloxone hydrochloride, and non-penetrable, naloxone methiodide, to distinguish between the central and peripheral mu-opioid receptor pathways. Both opioid antagonists acted to reverse the arabinogalactan-induced cough suppression; the reversion was total over time with the latter antagonist. We failed to confirm the presence of a bronchodilating effect of the polysaccharide, which could be involved in its antitussive action. We conclude that the polysaccharide arabinogalactan from *Withania somnifera* has a distinct antitussive activity consisting of cough suppression and that this action involves the mu-opioid receptor pathways.

Keywords

Codeine • Cough • Opioid receptors • *Withania somnifera*

G. Nosálová (✉), V. Sivová, and S. Fraňová
Department of Pharmacology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovakia
e-mail: Nosalova@jfmed.uniba.sk

B. Ray
Natural Products Laboratory, Department of Chemistry, The University of Burdwan, Bardhaman, India

I. Ondrejka and D. Flešková
Department of Psychiatry, Jessenius Faculty of Medicine, Comenius University, Martin, Slovakia

1 Introduction

Coughing is a common symptom of respiratory diseases and related non-respiratory conditions such as nasal disease or gastro-oesophageal reflux disease (Davis 1997). A persistent cough can be distressing to patients, leading to depression (in up to 53 % of patients, Dicpinigaitis et al. 2006; McGarvey et al. 2006), insomnia, vomiting, exhaustion, and rib fractures (Irwin 2006). Coughing has a significant human and socioeconomic burden, as it is linked with absenteeism from work, impaired quality of life, and can affect daily activities (Irwin et al. 2006). Synthetic drugs, such as codeine, dextromethorphan, and other antitussive drugs are used extensively for the treatment of coughing (Chung 2005; Irwin et al. 2000, 2006). Current antitussive drugs do not solve this problem sufficiently as they can cause unwanted side-effects (Belvisi and Hele 2009; Pratter et al. 2006). This is the main reason behind the search for new natural substances that influence the cough reflex. Due to the fact that the use of herbal medicines is now a significant part of modern health care throughout the world (Wills et al. 2000; Patel and Goyal 2012), we focused on the extract of *Withania somnifera*.

Ashwagandha, *Withania somnifera L.* Dunal (Solanaceae family), is an Ayurvedic medicinal plant which is a popular home remedy for several diseases (Patwardhan and Hopper 1992). It is mentioned in Vedas as a herbal tonic and health food. The chemical composition and pharmacological and therapeutic efficacy have been established (Ziauddin et al. 1996; Buddhiraja and Sudhir 1987). It is the main component of a variety of formulations prescribed for common diseases of the respiratory tract (Kirtikar and Basu 1935). The roots of the plant are the main source of drug preparation. Pharmacologically active constituents are mostly secondary metabolites. *Withania somnifera* contains phytochemicals of great interest to researchers, including steroidal lactones and phytosterols, as well as alkaloids, a variety of amino acids and polysaccharides (Kulkarni and Dhir 2008; Nosálová et al. 2005, 2007). In our experiments we investigated the effectiveness of the last component, polysaccharides, on experimentally induced cough reflex.

The rapid growth in the herbal market has been stimulated in part by a greater scientific understanding of how herbs work. From this point, we wanted to experimentally demonstrate if opioid receptors play a role in cough suppressive activity of polysaccharides from *Withania somnifera*. This receptor system was chosen because it is known to be involved in the mechanism of action of codeine cough suppressants (Brown et al. 2004; Kotzer et al. 2000; Karlsson et al. 1990).

2 Methods

2.1 Experimental Protocol

The study protocol was approved by the institutional Ethics Committee of Jessenius Faculty of Medicine, Comenius University in Martin, Slovakia (permission IRB 00005636). The experiment complied with the Slovakian and European Community regulations for the use and care of laboratory animals. The study was performed in accordance with the revised Declaration of Helsinki (1983) and followed the criteria of experimental animals' well fare.

A total of 56 guinea pigs were used in the study. The animals were randomly assigned into seven groups, consisting of eight guinea pigs each. Group 1 received vehicle (water for injection) as a 'negative' control in a dose of 1 ml/kg; Group 2 received codeine as a 'positive' control (codeine phosphate; Slovakofarma Hlohovec, Slovakia) in a dose of 10 mg/kg; Group 3 received the polysaccharide arabinogalactan from *Withania somnifera* in a dose 50 mg/kg; Group 4 received naloxone hydrochloride (Sigma-Aldrich, St. Louis, MO) in a dose of 3.0 mg/kg dissolved *ex tempore* in 1 ml of water for injections; Group 5 was administered naloxone hydrochloride in a dose of 3.0 mg/kg; Group 6 was administered naloxone methiodide

(Sigma-Aldrich, St. Louis, MO) in a dose of 10 mg/kg dissolved in 1.0 ml of water for injections; Group 7 was given the same dose of naloxone methiodide as the previous group. All injections were made intraperitoneally.

2.2 Plant Material

In the experiments we used water extracted polysaccharide from *Withania somnifera* purified from the crude powder from roots (Batch no. AL0054, Dabur India Ltd., New Delhi, India). It contained 83 % sugars (arabinose 52 % and galactose 22 % out of the sugar content) and 22 % proteins on basis of fraction dry weight (Sinha et al. 2011).

2.3 Antitussive Activity

Conscious adult male guinea pigs, weighing 200–350 g, were used for the study. The animals were supplied by the Department of Experimental Pharmacology, Slovak Academy of Science, Dobrá Voda, Slovakia. They were kept in quarantine for at least 1 week before starting the experiments in the animal house with food and water *ad libitum*, and with standard air conditioning system.

Then, guinea pigs were individually placed in a body plethysmograph box (HSE type 855, Hugo Sachs Elektronik, Germany) and restricted, so that the head protruded into the head chamber and the neck was sealed with a soft diaphragm. Cough was induced by an aerosol of citric acid in a concentration 0.3 mol/l, generated by a jet nebulizer (PARI jet nebulizer, Paul Ritzau, Pari-Werk GmbH, Germany; output 5 l/s, particle mass median diameter of 1.2 μm), and delivered for 3 min to the head chamber of the plethysmograph. The intensity of cough response was defined as the number of cough efforts counted during a 3-min exposure to the aerosol. The cough effort was defined as a sudden PC-recorded enhancement of expiratory flow associated with typical cough motion and sound observed by a trained experimenter.

The cough response was measured before (baseline measurement; N value in the graphs) and then after administration of the agents outlined above at the following time intervals: 30, 60, 120, and 300 min. The minimum time difference between two sets of measurements was 2 h in order to prevent cough receptors adapting to the irritant (citric acid), which could influence their response (Šutovská et al. 2009).

2.4 Specific Airway Resistance In Vivo

Reactivity of airway smooth muscles *in vivo* was expressed as specific airway resistance calculated according to Pennock et al. (1979). The value of specific airway resistance is proportional to the phase difference between nasal and thoracic respiratory airflows recorded in the head and thoracic chambers of the plethysmograph, respectively. The bigger phase difference the higher is the value of specific airway resistance and also a greater degree of bronchoconstriction. Airway resistance was registered before administration and subsequently 30, 60, 120, and 300 min after application of the compound.

2.5 Statistical Analysis

Data were presented as means ± SE and were statistically evaluated using a *t*-test. A p-value <0.05 was considered a threshold of statistical significance; $p < 0.05$, $p < 0.01$, and $p < 0.001$ are shown by one, two, and three asterisks, respectively.

3 Results and Discussion

3.1 Antitussive Activity of Arabinogalactan from *Withania somnifera*

In Indian systems of traditional medicine, extracts from *Withania somnifera* are a common ingredient in many prescribed drugs for

treatment of respiratory tract diseases. The justification for this use is not always based on scientific evidence. Our previous results of the antitussive test showed that oral administration of the polysacharid (arabinogalactan) from *Withania somnifera* in a dose 50 mg/kg brought a significant decrease in the number of citric acid induced cough efforts (NE) in conscious, healthy adult guinea-pigs. The onset of the cough suppression action was observed within 30 min after application of the arabinogalactan from *Withania somnifera*. Furthermore, this positive effect was observed during all the study time intervals. Notably, the suppression of cough efforts by the polysaccharide after 120 min was quantitatively higher than that induced by administration of codeine in the same conditions. These results supported our previous finding that several naturally occurring polysaccharides possess antitussive activity without adverse reactions (Nosálová et al. 2005, 2007, 2011, 2013). We assume that this effect might be associated with the ability of the polysaccharide arabinogalactan from *Withania somnifera* to prevent chemical irritation (in this case, citric acid) of cough receptors on the surface of the lining of the airways, although we also searched for other possible mechanisms of cough reflex suppression.

3.2 Opioid Receptor Blockade and Cough Reflex

In the present study, in an attempt to gain insight into the mechanisms behind cough suppressive activity of the polysaccharide from *Withania somnifera*, we blocked the mu-opioid receptors using a penetrating, naloxone hydrochloride, and a non-penetrating through the blood-brain barrier, naloxone methiodide, antagonists.

3.2.1 Naloxone Hydrochloride and Antitussive Activity of Polysaccharide from *Withania somnifera*

The effects on antitussive activity of pretreatment with Naloxone hydrochloride, 15 min before administration of the polysaccharide extract from *Withania somnifera*, are presented in Fig. 1. Naloxone caused a reduction in the cough suppressive power of arabinogalactan; the probability p, a measure of likelihood of occurrence of cough due to arabinogalactan, increased from 0.1 % to 1 % before naloxone hydrochloride to 5 % after it. Pretreatment with naloxone hydrochloride caused a decrease of total antitussive activity of applied arabinogalactan by ~11 %.

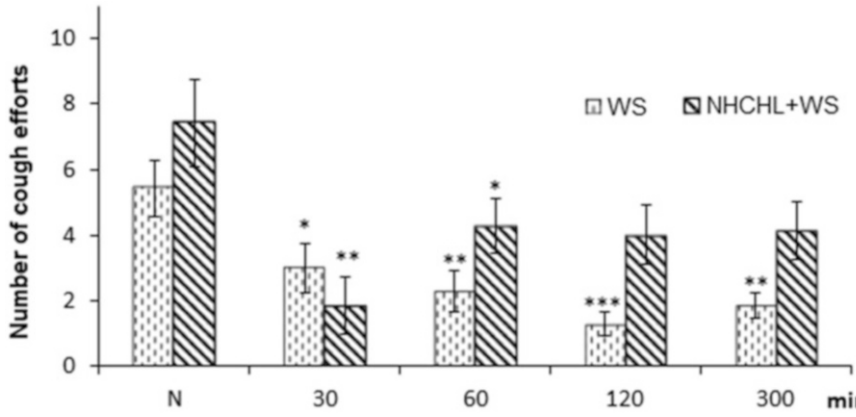

Fig. 1 The number of cough efforts recorded in response to citric acid aerosol in conscious guinea pigs after oral administration of the polysaccharide arabinogalactan from *Withania somnifera* (WS) alone (50 mg/kg) compared with the effect of arabinogalactan in other animals pretreated with naloxone hydrochloride (NHCHL; 3 mg/kg) 15 min before arabinogalactan administration. N – initial baseline values. Values are means ± SE; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ *vs.* the corresponding N value.

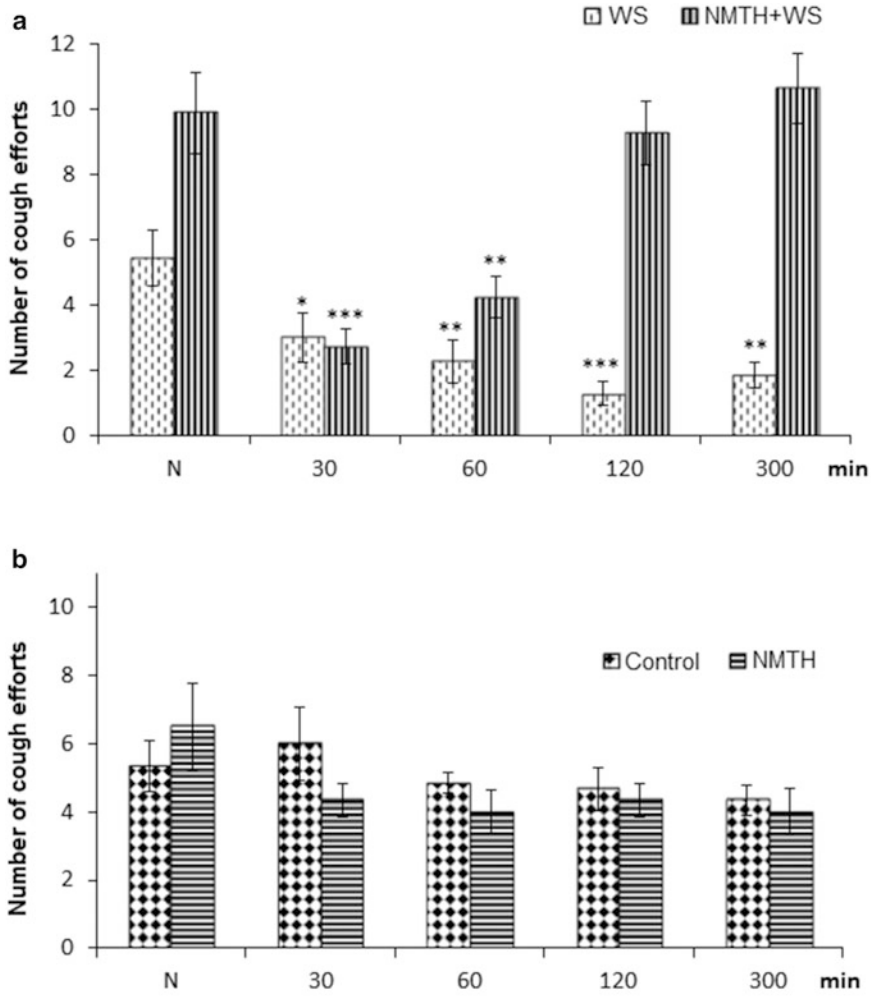

Fig. 2 The number of cough efforts recorded in response to citric acid aerosol in conscious guinea pigs after oral administration of the polysaccharide arabinogalactan from *Withania somnifera* (WS) alone (50 mg/kg) (**a**) and of the control saline-vehicle (1 ml/kg) (**b**) compared in each panel with the effect of arabinogalactan in other animals pretreated with naloxone methiotide (NMTH; 10 mg/kg) 15 min before arabinogalactan or saline administration. N – initial baseline values. Values are means ± SE; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ *vs*. the corresponding N value

Injection of naloxone hydrochloride alone, before the application of *Withania somnifera* extract, taken as the control effect of naloxone, failed to cause any measurable effect on the cough reflex; the number of coughs was comparable with that after saline (data not shown).

These results suggest that a suppressive effect on cough of polysaccharide from *Withania somnifera* could be partly mediated by the activity of central mu-opioid receptors.

3.2.2 Naloxone Methiodide and Antitussive Activity of Polysaccharide from *Withania somnifera*

As opposed to the brain-blood barrier penetrable mu-opioid receptor antagonist above outline, the non-permeating naloxone methiodide failed to affect the suppression of cough reflex induced by the application of arabinogalactan from *Withania somnifera* at the beginning time mark

of 30 min, but the cough suppressive effect of arabinogalactan was entirely reverted at later time marks (Fig. 2a).

Again, injection of naloxone methiodide alone, before the application of *Withania somnifera* extract, taken as the control effect of naloxone, failed to cause any measurable effect on the cough reflex; the number of coughs was comparable with that after saline (Fig. 2b).

Taken together, the results differentiating between the effects on arabinogalactan-induced cough suppression of brain-blood barrier penetrable and non-penetrable mu-receptors antagonists demonstrate the preponderance of the peripheral opioid receptor system in mediating cough suppression. The present findings are in line with the previous literature reports that also demonstrate that in cough reflex inhibition, synthetic drugs which inhibit cough, such as the archetype codeine, can affect both central and peripheral opioid receptor pathways (Barnes 2007; Zhu and Pan 2005; Nosálová 1998).

3.3 Changes in Specific Airway Resistance

Pavord and Chung (2008) argue that bronchoconstriction causes or enhances sensitivity to cough, while bronchodilation does the opposite. It follows that herbs with bronchodilator activities should have antitussive properties. Our present findings show that the cough suppressive effect of arabinogalactan from *Whitania somnifera* was not linked to significant changes in airway resistance. Therefore, we conclude that the antitussive activity of arabinogalactan from *Withania somnifera* does not involve bronchodilation.

4 Conclusion

Arabinogalactan extracted from the root of *Withania somnifera*, administered orally, demonstrated antitussive activity *in vivo* which was manifested as a reduction in the number of cough efforts induced by citric acid in the guinea pigs. The total intensity of cough suppression after application of arabinogalactan was the same as that of codeine (codeine 62 % and arabinogalactan 62 %). The results also show that the antitussive activity of arabinogalactan from *Withania somnifera* is in part mediated by the opioid receptor system, particularly by its peripheral pathway, but this activity is not appreciably connected with bronchodilation.

Acknowledgements Supported by the Slovak Research and Development Agency (APVV) Grant LPP-0317-09, by the project 'Carcinogenic and toxic metals in working environment', and co-financed from EU sources.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 7: 27–34
DOI 10.1007/5584_2014_75

Published online: 15 October 2014

Effects of Provinol and Its Combinations with Clinically Used Antiasthmatics on Airway Defense Mechanisms in Experimental Allergic Asthma

I. Kazimierová, M. Jošková, O. Pecháňová, M. Šutovská, and S. Fraňová

Abstract

Our previous studies show that provinol, a polyphenolic compound, has anti-inflammatory activity during allergic inflammation. In the present study we investigated the effects of provinol and its combinations with clinically used antiasthmatics: budesonide or theophylline on airway defense mechanisms during experimental allergic asthma. Separate groups of guinea pigs were treated during the course of 21-day ovalbumin sensitization with provinol (20 mg/kg/day, p.o.), or budesonide (1 mM by inhalation), or theophylline (10 mg/kg/day, i.p.), and with a half-dose combination of provinol +budesonide or provinol+theophylline. Airways defense mechanisms: cough reflex and specific airway resistance (sRaw) were evaluated *in vivo*. Tracheal smooth muscle reactivity and mucociliary clearance were examined *in vitro*. The findings were that provinol caused significant decreases in sRaw and in tracheal smooth muscle contractility, a suppression of cough reflex, and positively modulated ciliary beat frequency. The bronchodilatory and antitussive effects of provinol were comparable with those of budesonide and theophylline. Provinol given as add-on treatment significantly potentiated the effects of budesonide or theophylline, although the doses of each were halved. We conclude that provinol not only has bronchodilatory and antitussive effects, but also potentiates similar effects exerted by budesonide and theophylline.

Keywords

Red wine • Flavonoids • Cough • Bronchial hyperreactivity • Ciliary movement

I. Kazimierová (✉), M. Jošková, M. Šutovská, and S. Fraňová
Department of Pharmacology, Jessenius Faculty of Medicine, Comenius University, 26 Sklabinská St., 036 01 Martin, Slovakia
e-mail: ivana.kazimierova@jfmed.uniba.sk

O. Pecháňová
Department of Normal and Pathological Physiology, Slovak Academy of Science, Bratislava, Slovakia

1 Introduction

Asthma is a chronic inflammatory disease of lower airways that affects 300 million people worldwide (Bossé 2012; Umetsu and DeKruyff 2006). Currently, treatment of asthma includes β2-adrenergic antagonists, anticholinergics, corticosteroids, leukotriene antagonists, theophylline, and antiIgE medicines. These therapies provide relief only to a fraction of patients and the side effect profiles limit their use (Kandhare et al. 2013). Therefore, other substances with and anti-inflammatory efficiency are searched for. One such group is polyphenolic substances contained in fruits, vegetables, nuts, herbs, cocoa, tea, and red wine (Tangney and Rasmussen 2013). Polyphenols are recognized to possess antioxidant properties and to positively affect pathophysiological processes in the cardiovascular system (Boyer and Liu 2004). Antiallergic, anti-inflammatory and bronchodilatory properties of polyphenols have also been recorded in the respiratory tract (Homma et al. 2000).

Red wine is a rich source of a variety of polyphenols with multiple biological activities (Šeruga et al. 2011). In previous studies, we monitored the effects of provinol (dry powder, mixture of polyphenolic compounds from red wine) on experimental model of allergic asthma. Franova et al. (2011) confirmed that oral administration of provinol positively influenced the airway inflammation by reducing the level of inflammatory cytokines. Furthermore, provinol inhibited histamine-induced airway smooth muscle hyperreactivity in guinea pigs sensitized with ovalbumine (Franova et al. 2007).

The aim of the present study was to evaluate the long-term effect of provinol and its combinations with clinically used antiasthmatics: budesonide and theophylline on allergen-induced experimental asthma in guinea pigs. The outcome measures in this study were the airway defense mechanisms: cough reflex and specific airway resistance *in vivo* and tracheal smooth muscle reactivity and mucociliary clearance *in vitro*.

2 Methods

The study was approved by the Ethics Committee of Jessenius Faculty of Medicine in Martin, Slovakia (permit No. EK 1178/2012). Provinol (dry powder of red wine polyphenolic compounds) was provided by D. Ageron (Société Francaise de Distillerie, Vallont Pont d' Arc, France). Ovalbumin (OVA, egg albumin, grade III), histamine (histamine- 2HCl), citric acid, and other chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO).

2.1 Animals

Male guinea pigs (TRIK, 200–350 g) were randomly divided into seven experimental groups consisting of ten animals each:

- Group 1: control – treated for 21 days with saline
- Group 2: sensitized 21 days with OVA
- Group 3: sensitized 21 day with OVA and treated with provinol (20 mg/kg, daily, p.o.)
- Group 4: sensitized 21 day with OVA and treated with budesonide (1 mM during 5 min nebulization)
- Group 5: sensitized 21 day with OVA and treated with theophylline (10 mg/kg, daily, i.p.)
- Group 6: sensitized 21 day with OVA and treated daily with provinol plus budesonide, with half-doses of the above outlined both drugs
- Group 7: sensitized 21 day with OVA and treated daily with provinol plus theophylline, with half-doses of the above outlined both drugs.

2.2 Sensitization of Guinea Pigs

Guinea pigs were sensitized with 5 mg ovalbumin (OVA) and 1 mg aluminium hydroxide, administered in 1 ml of physiological saline. The allergen was injected both s.c. and i.p. on Day 1; and then i.p. only on Days 4, 11, and 15 and s.c. only on Days 9 and 14. The guinea pigs were

daily exposed to nebulized OVA (1 % in 0.9 % NaCl) in an aerosol chamber for the last 6 days of sensitization. The animals were used for *in vivo* experiments and were sacrificed 24 h after the last day of sensitization.

2.3 Airway Reactivity In Vitro

Reactivity of tracheal smooth muscles was estimated 21 days after sensitization. Tracheal strips were placed in a 20 ml organ baths containing Krebs-Henseleit buffer at 36.5 ± 0.5 °C, pH of 7.5 ± 0.1, and being continuously aerated with a mixture of 95 % O_2 and 5 % CO_2. The amplitude of contraction (mN) of muscle strips in response to the cumulative doses of histamine (10^{-8}–10^{-3} mol/l) was used as a measure of smooth muscle reactivity.

2.4 Airway Reactivity In Vivo

Airway resistance was evaluated on Days 7, 14, and 21 of sensitization. The guinea pigs were placed individually in a double-chamber whole-body plethysmograph box (type 855 with Pulmodyn Pennock software; Hugo Sachs Elektronik-Hardvard, Hugstetten, Germany) and specific airway resistance (sRaw) was measured in response to inhalation of the bronchoconstricting mediator histamine (10^{-6} mol/l) aerosolized in physiological saline. Reactivity after nebulization of saline alone was used as control. There was an interval of 5 min between exposures, during which fresh air was insufflated into the nasal chamber.

2.5 Chemically Induced Cough

Cough was induced by inhalation of 0.3 M citric acid in conscious guinea pigs placed in the double chamber of a body plethysmograph as described above. Cough was monitored by two independent observers and the number of cough efforts was recorded during 3 min of citric acid inhalation. Cough was detected from typical changes in the airflow curve, and cough movements and sounds. Codeine was used as an antitussive standard for comparisons with the effects of the substances tested.

2.6 Evaluation of Mucociliary Clearance

Ciliary beat frequency (CBF) was evaluated by a "brushing" method *in vitro*. Samples were taken from trachea by a cytology brush and placed on the slides kept at 36.5 ± 0.5 °C. Subsequently, the movement of ciliated epithelium was recorded by BASLER A504KC camera (Interflex Camera Link, Germany) using 256–512 framers per second. The recordings acquired were analyzed with LabVIEW software to generate ciliary regions of interest. The median CBF for the each region of interest in a preparation was selected, followed by the calculation of an arithmetic mean for the preparation.

2.7 Statistical Analysis

All results were expressed as means ± SE. Differences between mean data were assessed with one-way analysis of variance ANOVA. Significant differences were defined as $p < 0.05$.

3 Results

Provinol alone and in combinations with clinically antiasthmatics decreased sRaw in response to 10^{-6} mol/l histamine nebulization during the in vivo allergic condition. In all tested groups, a significant decrease of sRaw values, enhanced due to sensitization, was already observed on Day 7 (Fig. 1A). The effects were similar on Day 14 (data not shown), but the strongest sRaw reduction took place on Day 21 of therapy. The bronchodilatory effect of

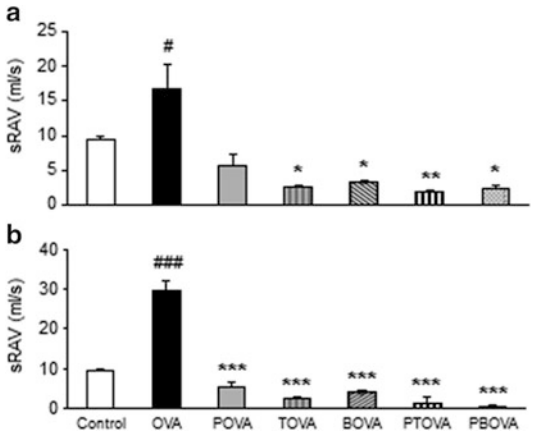

Fig. 1 Specific airway resistance (sRAV) after inhalation of histamine (10^{-6} mol/l) in control healthy guinea pigs; ovalbumin-sensitized (OVA); OVA-sensitized treated with provinol (POVA); OVA-sensitized treated with theophylline (TOVA); OVA-sensitized treated with budesonide (BOVA); OVA-sensitized treated with a combination of provinol+theophylline (PTOVA); and OVA-sensitized treated with provinol+budesonide (PBOVA). Changes were evaluated on Day 7 (*Panel A*) and Day 21 (*Panel B*) of sensitization. Data are means ± SE; n = 10 in each group; $^{\#}p < 0.05$; $^{\#}p < 0.001$ for OVA *vs.* control and $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ for test substances *vs.* OVA

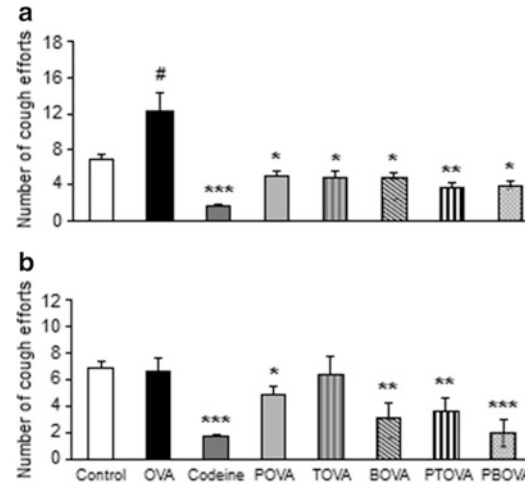

Fig. 2 Cough reflex after inhalation of citric acid (10^{-3} mol/l) in control healthy guinea pigs); ovalbumin-sensitized (OVA); OVA-sensitized treated with codeine; OVA-sensitized treated with provinol (POVA); OVA-sensitized treated with theophylline (TOVA); OVA-sensitized treated with budesonide (BOVA); OVA-sensitized treated with a combination of provinol + theophylline (PTOVA); OVA-sensitized treated with provinol + budesonide (PBOVA). Changes were evaluated on Day 7 (*Panel A*) and Day 21 (*Panel B*) of sensitization. Data are means ± SE; n = 10 in each group; $^{\#}p < 0.05$; $^{\#}p < 0.001$ for OVA *vs.* control and $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ for test substances *vs.* OVA

provinol was comparable to those of theophylline and budesonide. However, the add-on provinol potentiated the effects of theophylline or budesonide, although the doses of each were halved (Fig. 1B).

Provinol suppressed the number of cough efforts induced in guinea pigs by citric acid aerosol (10^{-3} mol/l) on Day 7 of OVA-sensitization (Fig. 2A). Provinol's cough suppressive effect, although significant, was less than that of the clinical antitussive codeine, but it was comparable with budesonide and theophylline. Furthermore, the half-dose combination of provinol +budesonide exceeded the antitussive effect of either substance used in monotherapy on Day 21 of sensitization (Fig. 2B).

The effects of provinol, budesonide, and theophylline on tracheal smooth muscle reactivity, after their administration over the 21-day OVA-sensitization period were evaluated in response to cumulative doses of histamine (10^{-8}–10^{-3} mol/l). Provinol inhibited the OVA-enhanced contractile airway response to histamine. The combination therapy provinol+budesonide and provinol +theophylline failed, however, to show a further smooth muscle contraction easing compared with monotherapy (Fig. 3).

Mucociliary clearance was assessed 24 h after the last challenge with OVA. Provinol as well as its combination with budesonide led to a significant reduction in ciliary beating frequency. The other substances tested failed to significantly affect the mucociliary clearance; except for theophylline after which ciliary beating frequency was increased (Fig. 4).

4 Discussion

Activation of inflammatory cells in allergen-induced bronchial asthma leads to the release of mediators which, cause bronchial smooth muscle

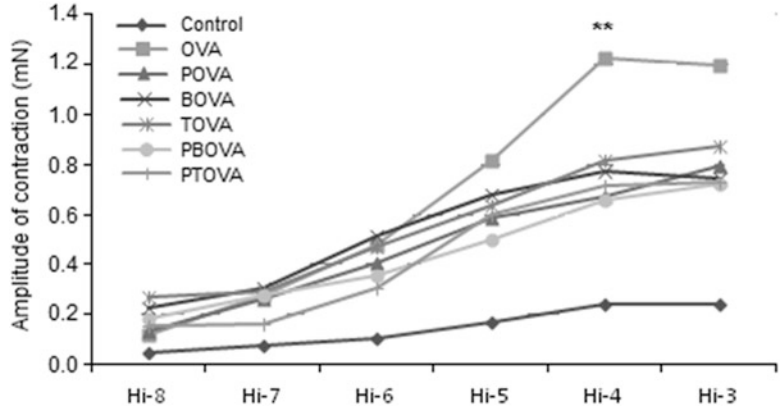

Fig. 3 Tracheal smooth muscle contractile response to cumulative doses of histamine (10^{-8}–10^{-3} mol/l) in control healthy guinea pigs; OVA-sensitized (OVA); OVA-sensitized treated with provinol (POVA); OVA-sensitized treated with theophylline (TOVA); OVA-sensitized treated with budesonide (BOVA); OVA-sensitized treated with a combination of provinol +theophylline (PTOVA); and OVA-sensitized treated with provinol+budesonide (PBOVA). Data are means ± SE; n = 10 for each group; $^{**}p < 0.01$ for OVA *vs*. control

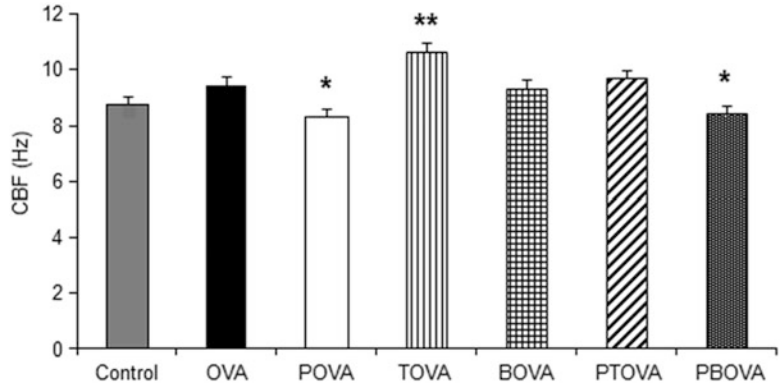

Fig. 4 Ciliary beat frequency in control healthy guinea pigs; OVA-sensitized (OVA); OVA-sensitized treated with provinol (POVA); OVA-sensitized treated with theophylline (TOVA); OVA-sensitized treated with budesonide (BOVA); OVA-sensitized treated with a combination of provinol+theophylline (PTOVA); and OVA-sensitized treated with provinol+budesonide (PBOVA). Data are means ± SE; n = 10 for each group; $^{*}p < 0.05$; $^{**}p < 0.01$ for test substances *vs*. OVA

contraction, inflammatory cell infiltration, mucus hypersecretion, airway hyperresponsiveness and, ultimately, airway remodeling (Jung et al. 2010; Bousquet et al. 2000). In the present study, we evaluated the effects of long-term administration of provinol, a polyphenolic compound mixture present in red wine, on experimental allergic asthma in guinea pigs. Provinol contains more than 95 % polyphenols consisting of 480 mg proantocyanins, 61 mg anthocyanins, 38 mg catechins, 18 mg hydroxycinnamic acid, 14 mg flavonols, and 370 mg polymeric tannins per g of dry powder. We found that provinol exerted a bronchodilating effect, as judged from its being a reducer of bronchial smooth muscle reactivity in vitro, an antitussive effect, consisting of decreased cough efforts, and it positively modulated mucociliary clearance in the bronchial tree. Provinol also mitigated bronchial hyperreactivity assessed from the response to

histamine. These actions were grossly comparable with those of budesonide and theophylline used as reference drugs. Moreover, a mixture of provinol with either budesonide or theophylline appreciably enhanced the antitussive and lessening bronchial hyperactivity effects, compared with the effects of monotherapy, which in addition was observed at a half-dose of each compound enabling at the same time to lower the dose of each drug in half. The possibility to lower the dose of drugs used in allergic asthma, with an apparent gain in beneficial efficacy, by the add-on treatment with provinol, or, by inference, by the long-term use of dietary supplements containing provinol or related compounds, seems the most worthwhile conclusion drawn from the present study.

Wine phenolics have been shown to posses several health promoting activities (Ali et al. 2013). These protective effects could be due to one or many components of the complex mixture of bioactive compounds present in red wine including resveratrol, flavonols, anthocyanins, phenolic acids, as well as their metabolites (Rodrigo et al. 2011). Cruz et al. (2012) showed that quercetin reduces airway hyperreactivity and inflammation by suppressing mast cell degranulation. Chlorogenic acid present in wine may help reduce asthmatic symptoms and incidence asthma (Kim et al. 2010). Provinol has a notable anti-inflammatory and antioxidant activity that may protect against asthma (Franova et al. 2011). It decreases IL-4 and IL-5 in bronchoalveolar lavage fluid. IL-4 is a cytokine directing B lymphocytes to synthesize IgE. In an allergic disease, IgE activates mast cells through interactions with receptors (FcεRI, FcεRII) on the cell surface, which leads to the release of the bronchoconstricting mediators histamine and leukotrienes. IL-5 is a critical cytokine in regulating the function and recruitment of eosinophils, which underlies the progression inflammatory processes of allergic asthma (Mauad et al. 2011).

There are other possible ways to affect bronchodilator activity in allergic asthma. Nitric oxide appears to play a key role in airway muscle tone regulation. Inflammatory mediators reduce the function of constitutive NO synthesis (cNOS); thereby negatively affecting the bronchodiling effects of NO. Provinol activates cNOS and inhibits inducible NOS in experimental allergic asthma (Franova et al. 2007). Meeyoung et al. (2013) demonstrated that resveratrol inhibits OVA-induced airway hyperreactivity. The present findings showed that long-term administration of provinol, better yet together with clinically used antiasthmatics, caused a decline in both airway smooth muscle reactivity *in vitro* and in specific airway resistance after nebulization of histamine *in vivo*. These findings are of applicable importance considering that enhanced contractility or hypertrophy of airway smooth muscles resulting from inflammation leads to decreased lung function (Wenzel 2006). Bronchodilatators play a central role in asthma treatment. They provide relief of symptoms, but evidence of their adverse effects underlines the need for caution in use. Research develops that involves different combinations of clinical antiasthmatics with substances that may contribute to a dose reduction and to alleviation of adverse effects (Bateman and Boulet 2011). Provinol, a mixture of polyphenolics of red wine, significantly enhanced the antitussive and bronchidilatory effects of theophylline and budesonide in the present study. The antitussive effect of provinol in a dose of 20 mg/kg was comparable with the efficacy of codeine. Cough is a hallmark of respiratory diseases and is an airway defense mechanism closely associated with bronchoconstriction. Although there is no direct evidence that polyphenols act on the cough reflex, Widdicombe (2003) showed that rapidly adapting lung receptors are fully enabled by bronchospasm in asthma patients. Therefore, antitussive effects of provinol might plausibly be attributed to its bronchodilatatory activity.

Mucociliary clearance is yet another defense mechanisms in allergic asthma acting to clear the lungs of bacteria and foreign particulate matter. It is a well-coordinated system consisting of airway secretory cells that produce a mucus layer on the airway surface and ciliated cells that propel the mucus up and out of the lungs (Bennett 2002). In the present study we found

that ovalbumine induced a significant increase in ciliary beat frequency. Likewise, theophylline increased the frequency of cilia beating during the allergic inflammation. The frequency of ciliary beating is a phenomenon not yet fully elucidated. Therefore, we cannot evaluate the importance of this effect. However, provinol alone and in combination with budesonide reversed the frequency of cilia beating to the baseline level, which is presumed to be related to the anti-inflammatory activity of these substances. Goh et al. (2012) reported that ovalbumin-challenged mice developed goblet cell hyperplasia and mucus hypersecretion in the bronchi; the latter being suppressed by fisetin, a flavonol belonging to the group of polyphenols. That supports the notion of that polyphenolic substances are at play in muco-ciliary clearance.

In conclusion, we found that a polyphenolic compound mixture of red wine affected the airway defense mechanisms in a beneficial way, mostly consisting of bronchodilatory and antitussive effects. Further more, provinol as add-on to the standard drugs budesonide and theophylline allowed slashing the dosage of individual substances in half, with a better therapeutic efficacy achieved than that resulting from monotherapy. The study shows that provinol could be an adjunct treatment of allergic asthma.

Acknowledgments This work was supported by the Slovak Research and Development Agency contract no. APVV-0305-12; CEKR II and by grants VEGA 1/0020/11 and MZ 2012/35-UKMA-12. The project was co-financed from the EU sources for increasing the opportunities for career growth in research and development in medical sciences.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 7: 35–45
DOI 10.1007/5584_2014_76

Published online: 15 October 2014

Potassium Ion Channels and Allergic Asthma

M. Kocmalova, M. Oravec, M. Adamkov, V. Sadlonova, I. Kazimierova, I. Medvedova, M. Joskova, S. Franova, and M. Sutovska

Abstract

High-conductive calcium-sensitive potassium channels (BK^+_{Ca}) and ATP-sensitive potassium (K^+_{ATP}) channels play a significant role in the airway smooth muscle cell and goblet cell function, and cytokine production. The present study evaluated the therapeutic potential of BK^+_{Ca} and K^+_{ATP} openers, NS 1619 and pinacidil, respectively, in an experimental model of allergic inflammation. Airway allergic inflammation was induced with ovalbumine in guinea pigs during 21 days, which was followed by a 14-day treatment with BK^+_{Ca} and K^+_{ATP} openers. The outcome measures were airway smooth muscle cells reactivity *in vivo* and *in vitro*, cilia beating frequency and the level of exhaled NO (E_{NO}), and the level of pro-inflammatory cytokines in the plasma and bronchoalveolar lavage fluid. The openers of both channels decreased airway smooth muscle cells reactivity, cilia beating frequency, and cytokine levels in the serum. Furthermore, NS1619 reduced E_{NO} and inflammatory cells infiltration. The findings confirmed the presence of beneficial effects of BK^+_{Ca} and K^+_{ATP} openers on airway defence mechanisms. Although both openers dampened pro-inflammatory cytokines and mast cells infiltration, an evident anti-inflammatory effect was provided only by NS1619. Therefore, we conclude that particularly BK^+_{Ca} channels represent a promising new drug target in treatment of airway's allergic inflammation.

Keywords

Asthma therapy • Bronchial hyperreactivity • Ion channels • K^+-channels • Mast cells infiltration

M. Kocmalova (✉), M. Oravec, V. Sadlonova, I. Kazimierova, I. Medvedova, M. Joskova, S. Franova, and M. Sutovska
Department of Pharmacology, Jessenius Faculty of Medicine, Comenius University, Ústav farmakolgie, 26 Sklabinsk St., 036 01 Martin, Slovakia
e-mail: kocmalova@jfmed.uniba.sk

M. Adamkov
Institute of Histology and Embryology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovakia

1 Introduction

Ion channels are a ubiquitous class of specialized membrane protein assemblies that form hydrophilic pores through which ions move down their electrochemical gradients across the membrane (Weiger et al. 2002). Biological systems contain a large variety of channels in order to attain specific conductance properties for different types of ions (Luzhkov and Åqvist 2005). A channel's response to a stimulus is an opening or closing of the pore regulating the passage of specific ions (Carr and Undem 2001). Ion channels regulate many key functions of the cells implicated in the asthma pathophysiology (Valverde et al. 2011). Changes in ion transport are associated with inflammation due to allergy, autoimmune disease, injury, and infection. That implicates ion channels as being mediators of pro-inflammatory conditions (Eisenhut and Wallace 2011).

Potassium calcium-activated channels present in the plasma membrane of the airway smooth muscle cells (ASM) are influenced by ion concentration, membrane potential, metabolites, or receptors. Two of them: high-conductive calcium-sensitive (BK^+_{Ca}) and ATP-sensitive potassium (K^+_{ATP}) channels play a role in modulating the ASM contractility (Pelaia et al. 2002). K^+ channel openers, with their capacity to induce hyperpolarization of smooth muscle and secretory cells, could play a role in the regulation of smooth muscle tone and consequently in bronchial hyperresponsiveness. Experimental and clinical studies with K^+ channels openers demonstrate bronchorelaxation, prevention of bronchocontriction, and a reduction in microvascular leakage and goblet cell secretion; thereby these studies provide the foundation for the therapeutic use of these agents in bronchial asthma and chronic obstructive pulmonary disease (Jahangir and Terzic 2005).

Although acute changes in the membrane potential do not seem necessary to stimulate ASM contraction, it appears that intracellular levels of Ca^{2+} and membrane potential are interconnected by K^+_{Ca} channels, especially by BK^+_{Ca}, and that this regulation is designed to operate over a longer time course to mediate changes in the smooth muscle phenotype. BK^+_{Ca} channels are present in many excitable cells, where they contribute to the integration of changes in intracellular calcium ions with membrane potential (Bissonette 2002). BK^+_{Ca} channels are also activated by nitric oxide (NO) via protein kinase G-dependent phosphorylation of the Slo-11 or beta-1-regulatory subunits, or by the binding of NO or one of its oxidized derivatives to thiols, most likely cysteine residues located on the alpha subunit (Eisenhut and Wallace 2011).

In previous studies we confirmed the K^+ channels' specific role in the pathophysiology of airway allergic inflammation using acute administration of BK^+_{Ca} and K^+_{ATP} channels openers (Kocmalova et al. 2012; Sutovska et al. 2007). In the presented study we focused on the influence of long-term treatment by potassium channels openers on the main markers and symptoms of asthma, using an experimental model of allergic airway inflammation.

2 Methods

The experiments were approved by a local Ethics Committee of the Jessenius Faculty of Medicine in Martin, Slovakia and were performed in accord with the revised Declaration of Helsinki of 1983 and the EU criteria for experimental animal well-fare as described in the document EK 1200/2012.

A total of 60 adult, male TRIK strain guinea pigs, weighing 150–350 g, were used in the study. The animals were obtained from the Department of Experimental Pharmacology of the Slovak Academy of Sciences, Dobra Voda, in Slovakia and from a breeding facility (Velaz Ltd., Prague, Czech Republic), and underwent at least 1 week's adaptation in an animal house having commercial chow and water *ad libitum*.

The animals were subdivided into six groups, each consisting of ten. The airway inflammation was induced in all groups, except for the control healthy animals, by repetitive administration of ovalbumine adsorbed on aluminium hydroxide according to the method previously described by Franova et al. (2013). Sensitization lasted for 21 days and was followed by a 14-day treatment with budesonide, salbutamol, NS1619, and pinacidil. All measurements were performed 24 h after last treatment application.

The groups were the following:

- Control healthy animals;
- Negative control group of sensitized animals, treated with saline for 14 days;
- Sensitized animals treated with pinacidil – 1 mg/kg, s.c. for 14 days;
- Sensitized animals treated with NS1619 – 200 μmol daily by inhalation for 10 min for 14 days;
- Positive control group of sensitized animals treated with salbutamol – 10 mg/kg, i.p. for 14 days;
- Positive control group of sensitized animals treated with budesonide – 3 mg/1 ml of Tween80 suspension by inhalation for 5 min for 14 days.

2.1 Chemicals

Modulators of potassium ion channels, citric acid, histamine, acetylcholine, metacholine, pinacidil, NS1619 budesonide, and salbutamol were obtained from Sigma-Aldrich (Lambda Life, Bratislava, Slovakia). Pinacidil and NS1619 were dissolved in 10 % DMSO. Citric acid, metacholine, and histamine for *in vivo* measurements were dissolved in saline. Salbutamol, histamine, and acetylcholine for *in vitro* measurements were dissolved in water for injection, and budesonide in 1 % Tween80.

2.2 Asthma Model

Sensitization of animals was performed during 21 days by the ovalbumine antigen, which causes airway hyperreactivity on the immunological basis. The method of experimental asthma model in guinea pigs was described previously by Franova et al. (2013). The allergen (ovalbumine 10^{-5} mol/l) (OVA) adsorbed on aluminium hydroxide was administrated on the 1st day of sensitization both i.p. and s.c., on the 4th day i.p., and on the 9th day s.c. Inhalation of the allergen was performed on 12th, 15th, 18th and 20th day for 30–60 s in a double chamber whole body plethysmograph (HSE type 855, Hugo Sachs Elektronik, Germany). Ovalbumine aerosol was generated by jet nebuliser (PARI jet nebuliser, Paul Ritzau, Pari-Werk GmbH, Germany, output 5 l/s, particle mass median diameter 1.2 μm) and delivered to head chamber.

2.3 Airway Smooth Muscle Reactivity In Vivo

In vivo airway smooth muscle reactivity was assessed from changes in specific airway resistance (sRaw), measured in the plethysmographic chamber. sRaw was calculated by a method of Pennock et al. (1979). It is proportional to the phase difference between nasal and thoracic respiratory airflow. The values were measured during 1 min after exposure to bronchoprovocation by citric acid, histamine, or metacholine.

2.4 Contractile Response of Airway Smooth Muscles In Vitro

Animals were sacrificed by transversal interruption of the spinal cord, and respiratory tract organs were removed. We obtained four specimens – two tracheal and two pulmonary strips (20 × 20 × 3 mm). Tissue strips were placed into an organ bath (Multi-Chamber Tissue Bath System with software, ISO-09-TSZ8; Experimetria Ltd., Balatonfüred, Hungary) with Krebs-Henseleit solution (composition: NaCl 112.9; KCl 4.7; $CaCl_2$ 2.8; $MgSO_4$ 0.5; $NaHCO_3$ 24.9; glucose 11.1 mmol/l). The solution in the organ bath was at 37 °C and was saturated with carbogen (5 % CO_2 in O_2). Following 1 h

incubation, contractile responses were measured after application of cumulative doses of acetylcholine or histamine (10^{-8}–10^{-3} mol).

2.5 Measurement of Cilia Beating Frequency

Biological specimens were collected after transversal section of the spinal cord. Tracheal epithelium was obtained by a 'brushing method' through a tracheotomic opening. The epithelial scraping was put into a drop of saline of 37 °C. Intact cilia were detected under light microscopy associated with high-speed camera (Basler 504KC; Adept Turnkey Pty Ltd, Brookvale, Australia). Ten video sequences were made from every preparation, which was evaluated using Ciliary Analysis software, created by the Department of Mechatronics and Engineering, University of Žilina, Slovakia. The median of cilia frequency beating was obtained for each area of interest (ROI). The final value of cilia frequency beating, expressed in Hz, was an average of ten median values obtained from each specimen.

2.6 Measurement of Exhaled Nitric Oxide

Exhaled nitric oxide (E_{NO}) levels were measured by a chemiluminescence method in animals individually placed into a chamber connected with NIOX Flex Offline Start Kit 04-1210-F (Aerocrine, Solna, Sweden). The guinea pigs breathed NO-free air for 5 min. Subsequently, the exhaled gas (flow rate 5 ml/s) was analyzed for 7 s. E_{NO} levels were expressed in ppb.

2.7 Assessment of Cytokines

Selected inflammatory cytokines were measured in the serum and bronchoalveolar lavage fluid (BALF) with a Bio-Plex 200 analyzer (Bio-Rad, Hercules, CA), which is equipped with a flow-based dual-laser system, with excitation at 532 nm for analytes quantification and 635 nm for cytokine identification. We used a Bio-Rad Th1/Th2 Human Cytokine immunoassay. The system detects and quantifies molecules bound to the surface of fluorescent microspheres. Biomarkers bound to a magnetic bead complex with the detection antibody and fluorescent dye marker (streptavidin/phycoerytin).

2.8 Immunohistochemical Staining

Immunohistochemical staining of pulmonary samples (10 × 10 × 5 mm) was performed to assess mast cells infiltration based on the detection of mast cells tryptase. The staining was done in formalin fixed-paraffin embedded tissue. Each paraffin block was cut into 4 μm sections which were subjected to staining. For a greater adherence of tissue to glass slides, we used Flex-slides, which were baked in an oven at 59 °C for 2 h. Then, the slides were treated in a pre-treatment link system (PT Link System; DAKO, Glostrup, Denmark) to optimize staining consistency. The endogenous peroxidase activity was quenched with 3 % hydrogen peroxide. The immunohistochemical reaction was performed using a mouse anti-mast cell tryptase (S640, Clone AA1, Kit K 0609 LSAB system, DAKO) for 20 min. The reaction was visualized by incubation with the chromogen 3, 3′diaminobenzidine for 2–3 min and counterstained with Meyers' haematoxylin.

Double blind labeled microscopic slides were assessed by two independent observers. In case of discrepancy between the observers, the assessment was repeated by both observers using a dual-head microscope to come to a consensus. A degree of mast cells infiltration was determined in a semi-quantitative manner: negative result of infiltration = degrees 0 and 1; positive result of infiltration = degrees 2 and 3, as previously described by Sutovska et al. (2013).

2.9 Statistical Analysis

Data were shown as means ± SE. Statistical comparisons were made with a *t*-test. Fisher's exact test was used for the evaluation of immunohistochemical features. Statistical significance was defined as $p < 0.05$.

3 Results

3.1 Airway Smooth Muscle Reactivity In Vivo

The baseline level of airway smooth muscle reactivity was significantly enhanced in the OVA-sensitized saline-treated condition, compared with that in the control, non-sensitized condition. However, baseline reactivity was significantly reduced after long-term treatment with the K^+ channels openers pinacidil (K^+_{ATP}) and NS1619 (BK^+_{Ca}) (Fig. 1). Long-term 14-day treatment with NS1619 resulted in a significant decrease of values in response to all agents used for bronchoprovocation (citric acid, histamine, or metacholine) in the sensitized group of animals. Furthermore, the effect was stronger than that after the control drug salbutamol during citric acid and histamine irritations. The significant decrease of the sRaw values after long-term treatment with pinacidil decreased the sRaw only in case of bronchoprovocation by citric acid (Fig. 2).

3.2 Contractile Response of Airway Smooth Muscles In Vitro

In vitro tests of contractility of tracheal and pulmonary strips in response to cumulative doses of acetylcholine and histamine after the 14-day treatment with pinacidil and NS1619 gave similar results to those after acute

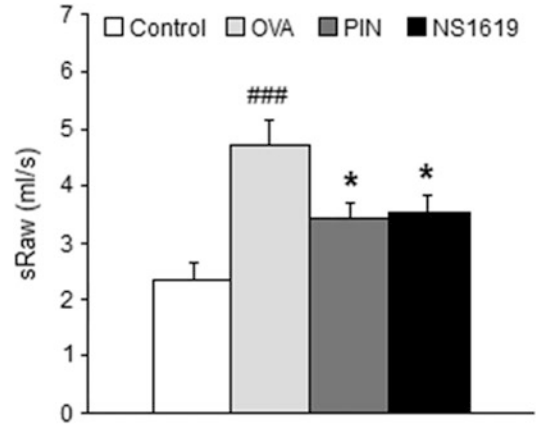

Fig. 1 Changes in specific airway resistance (sRaw) *in vivo* in response to OVA sensitization and then after long-term treatment with pinacidil and NS1619. $^{###}p < 0.001$ *vs.* control, $^{*}p < 0.05$ *vs.* OVA

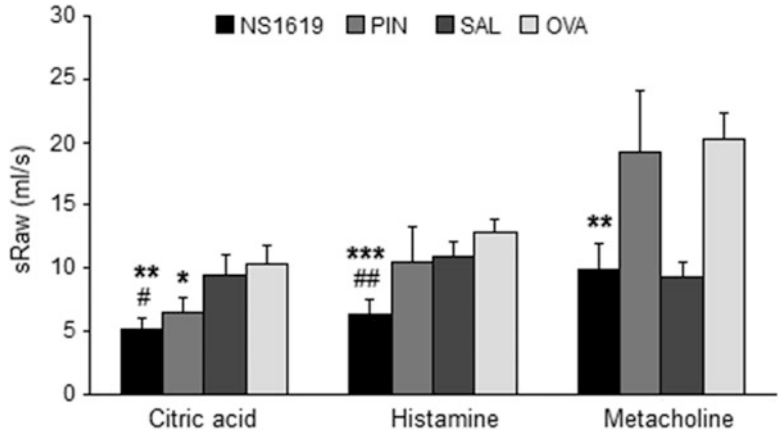

Fig. 2 Effects of long-term treatment with NS1619 and pinacidil on specific airway resistance (sRaw) increased in OVA-sensitized guinea pigs in response to bronchoprovocation with citric acid, histamine, and metacholine compared with the salbutamol (SAL) standard efficacy. $^{\#}p < 0.05$ *vs.* SAL, $^{*}p < 0.05$ *vs.* OVA

Table 1 Effects of acute and long-term treatment with K^+ channels openers on tracheal and pulmonary tissue contractility

Airway allergic inflammation					Airway allergic inflammation			
Acute					Long-term			
Tissue	Trachea		Lung		Trachea		Lung	
Agonist	Ach	Hi	Ach	Hi	Ach	Hi	Ach	Hi
K^+_{ATP} Pinacidil	↓	↓	↓	↓	↓	↓	↓	↓
BK^+_{Ca} NS1619	↓	↓	↔	↓	↓	↓	↔	↓

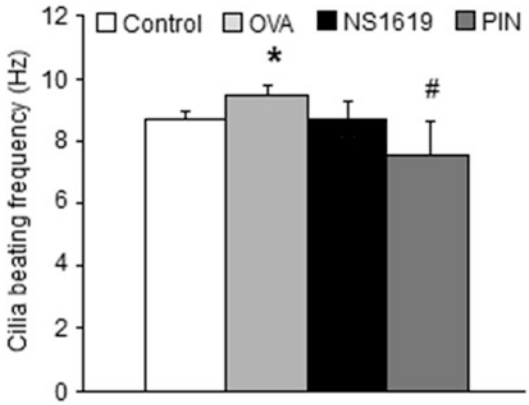

Fig. 3 Ciliary beating frequency in control nonsensitized, ovalbumine (OVA)-sensitized, and in NS1619- and pinacidil-treated sensitized animals. *$p < 0.05$ *vs*. Control and $^{\#}p < 0.05$ *vs*. OVA

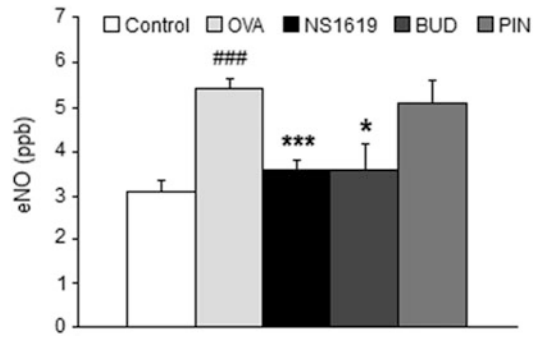

Fig. 4 Changes in exhaled NO in control nonsensitized, ovalbumine (OVA)-sensitized, and in NS1619- and pinacidil-treated sensitized animals. *$p < 0.05$ *vs*. control, ***$p < 0.001$ *vs*. control, and $^{\#\#\#}p < 0.001$ *vs*. OVA

administration of the openers. However, there was a slight reduction in the contractility of tracheal tissue observed in response to acetylcholine after the long-term treatment with these K^+ channels openers (Table 1).

3.3 Cilia Beating Frequency (CBF)

The cilia beating frequency was significantly higher in the group of sensitized animals. Long-term treatment with Pinacidil, a selective opener of K^+_{ATP} channels, significantly reduced the OVA-enhanced beating frequency ($p < 0.05$); the reduction went down below the level present at baseline in the control nonsensitized animals. The effect of NS1619, a selective BK^+_{Ca} opener failed to reach statistical significance (Fig. 3).

3.4 Effect of Long-Term Therapy on eNO

OVA-induced allergic inflammation evoked a marked increase in eNO. Long-term treatment with both NS1619 and a control drug, budesonide, resulted in significant decreases in eNO. On the other hand, pinacidil failed to affect the level of eNO (Fig. 4).

3.5 Assessment of Cytokine Levels

OVA-sensitization strongly enhanced the level of proinflammatory cytokines in both serum and BALF. Long-term treatment with NS1619 and pinacidil caused significant decreases in the cytokines IL-4, IL-5, IL-13, and TNF-α in both serum and BALF, which regarding the serum

tended to be stronger than those observed after a control drug, budesonide. The cytokine decreases in the serum went down beyond the control level. In case of BALF samples in the control healthy animals, the cytokines appeared below the detection limit (Table 2).

3.6 Immunohistochemical Staining

Lung and tracheal tissue specimens investigated immunohistologically after 21 days of OVA sensitization showed an increased number of the samples positive for mast cell infiltration as assessed from the tryptase staining. The number of tryptase identifying reactions was significantly reduced after long-term treatment with budesonide and NS1619, but not pinacidil (Table 3).

4 Discussion

The major finding of this study was that the K^+ channels openers tested, NS1619 (BK^+_{Ca} opener) and pinacidil (K^+_{ATP} opener) had a protective effect on the airway defense mechanisms in both *in vivo* and *in vitro* conditions. The current study was concentrated on the long-term treatment effect of these K^+ channels modulating agents on experimentally-induced asthma. We found that both NS1619 and pinacidil decreased the level of pro-inflammatory cytokines in the plasma and BALF and counteracted the OVA-induced increases in airway resistance or cilia beating frequency. Of the two openers, however, NS1619 acted stronger, particularly in decreasing OVA-induced increases in eNO concentration or airway mast cell infiltration, characteristic of allergic asthma in guinea pigs.

Asthma is a disease that leads from various degrees of airflow obstruction to airway hyperresponsiveness. The inflammation developing in asthmatic lungs contributes to the pathophysiology of the disease. This occurs mostly through the release of inflammatory mediators and airway remodelling. Most of the asthma features are due to an aberrant expansion of Th2 lymphocytes that secrete type-2 cytokines such as IL-4, IL-5, IL-9, and IL-13. Attempts have been made to block selectively cytokines, chemokines, and pathways associated with the adaptive immune pathway of the disease (Deckers et al. 2013). Increased cytokine levels assessed in the serum and BALF samples of sensitized animals in the present study confirmed their role in expression of symptoms of allergic asthma.

K^+ channels contribute to the relaxation of airway smooth muscles by hyperpolarizing the membrane potential and thereby preventing the activation of voltage-gated Ca^{2+} channels (VGCC). Electrophysiological and molecular approaches have identified several K^+ channels in airway smooth muscles such as K^+_{Ca}, voltage-activated K^+, and K^+_{ATP} channels (Valverde et al. 2011). K^+_{Ca} and VGCC outward K^+ current, which are sensitive to charybdotoxin and iberiotoxin, are widely found in isolated tracheal smooth muscle cells. The properties of these currents are consistent with the BK^+_{Ca} channels responsible for spontaneous transient outward currents that are associated with Ca^{2+} sparks. In smooth muscle cells, K^+_{Ca} channels lead to membrane hyperpolarization and inhibition of VGCC L-type channels. This, in turn, reduces the amount of Ca^{2+} influx, and thereby decreases smooth muscle tone (Perez-Zoghbi et al. 2009). In the presented study we found that NS1619 significantly inhibited airway smooth muscle basal reactivity and responses to bronchoprovocative agents (citric acid, histamine, and methacholine) *in vivo* as well as it decreased the strength of contraction of isolated airway smooth muscles strips *in vitro*. Pinacidil also reduced basal reactivity values, but unlike NS1619, it did not have such an evident effect on chemically-induced reactivity. Furthermore, smooth muscle contraction amplitude decreased on cumulative doses of acetylcholine after the long-term administration of these substances. Pelaia et al. (2002) showed a spasmolytic activity of K^+_{ATP} channel activators *in vitro* regarding airway smooth muscles contractile responses

Table 2 Cytokines in serum and bronchoalveolar lavage fluid (BALF) in control healthy and OVA-sensitized guinea pigs, and after long-term treatment with budesonide (BUD) and the K^+ channels openers Pinacidil (PIN) and NS1619

	IL-4		IL-5		IL-13		TNFα	
	Serum	BALF	Serum	BALF	Serum	BALF	Serum	BALF
Control	0.67 ± 0.13	–	0.78 ± 0.24	–	3.93 ± 0.00	–	12.74 ± 1.79	–
OVA	1.57 ± 0.17$^{\#\#}$	4.96 ± 0.77	2.18 ± 0.33$^{\#}$	3.66 ± 0.67	4.82 ± 1.30$^{\#\#}$	6.53 ± 0.74	28.56 ± 4.26$^{\#\#}$	22.6 ± 4.77
BUD	0.97 ± 0.13*	2.32 ± 0.68*	0***	1.58 ± 0.09**	0.36 ± 0.23**	3.40 ± 0.00***	6.81 ± 1.64***	8.40 ± 2.17**
PIN	0.54 ± 0.07***	2.80 ± 0.73*	0***	2.59 ± 0.33	0.09 ± 0.00***	3.82 ± 0.37**	4.29 ± 2.94***	12.21 ± 3.70
NS1619	0.66 ± 0.07***	2.97 ± 0.64*	0***	2.52 ± 0.49	0.09	3.67 ± 0.80*	6.30 ± 1.55***	13.89 ± 4.55
					0.00***			

All data are means ± SE of pg/ml
$^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* control, *p<0.05, **p<0.01, ***p<0.001 *vs.* OVA

Table 3 Immunohistology of lung and tracheal specimens for changes in cellular infiltration evoked by a single dose of the potassium channels agonists pinacidil and NS1619 in ovalbumine (OVA)-sensitized guinea pigs, compared with the effects of a control drug, budesonide

	Positive	Negative
Control	0	9
OVA	5	$2^{\#\#}$
Budesonide	0	8**
Pinacidil	3	4
NS1619	1	7*

$^{\#\#}p<0.01$ for OVA *vs*. Control, and *$p<0.05$, **$p<0.01$ NS1619 and Budesonide *vs*. OVA (Fisher's exact test)

evoked by low concentrations of spasmogenes such as histamine, prostaglandin D_2, leukotriene C_4, and neurokinin A. The spasmolytic activity of K^+_{ATP} channels openers was also confirmed in *in vivo* studies of El-Hashim et al. (2004) in allergen-induced airway hyperresponsiveness in rabbits.

Other functions of conducting airway epithelia related to hydroelectrolytic transport, osmo-mechanical responses and mucociliary clearance are also connected with the activity of ion channels and intracellular Ca^+ signaling. K^+_{ATP} controls the volume and composition of the airway surface liquid, which affects ciliary beating and mucociliary clearance (Valverde et al. 2011). Furthermore, Ca^{2+} plays a crucial role in the regulation of ciliary beating frequency. Elevations of intracellular Ca^{2+} are associated with an increase in ciliary beating frequency, and the opposite way around (Salathe 2007). BK^+_{Ca} and K^+_{ATP} ion channels are localized in ciliary cells and act to decrease ciliary beating during allergic inflammation (Valverde et al. 2011). Similar results were obtained in the present study. Sensitization by allergen increased ciliary beating frequency and both pinacidil and NS1619 acted to reverse reversed the decreases toward the baseline levels; the effect of pinacidil was stronger and significant.

NO is known as both a signalling and anti-inflammatory molecule, and it plays an important role in asthma. Inducible NO synthase (iNOS) is a source of NO during allergic inflammation, and also a marker of inflammation found in asthmatic patients (Ten Broeke et al. 2001). BK^+_{Ca} channels are activated by NO via a protein kinase G-dependent phosphorylation of Slo11 or beta1-regulatory subunits or by binding of NO (or one of its oxidised derivatives) to thiols, most likely cysteine residues located on the α subunit of channels. NO can indirectly activate BK^+_{Ca} by preventing the formation of an endogenous inhibitor of these channels. NO also inhibits the enzymatic formation of 20-hydroxyeicosatetraenoic acid (20-HETE), a potent inhibitor of BK^+_{Ca} channels activity (Elsevier and Walace 2011). The present study confirmed the findings above mentioned in that it showed a decrease in exhaled NO after the long-term treatment with NS1619. According to Vaali et al. (1998), NO does not hyperpolarize plasma membrane acting via K^+_{ATP} channels, which is in line with the lack of effect on exhaled NO of pinacidil observed in the present study.

Since K^+ channels of various types are expressed by inflammatory and immune cells, such as T lymphocytes, basophils, and macrophages, it has been proposed that these channels may be involved in airway inflammatory responses. However, although it has been reported that K^+ channels openers may interfere with the activity of inflammatory cells *in vitro*, these drugs do not seem to affect the antigen-induced histamine release or inflammatory cell recruitment into the airways (Pelaia et al. 2002). Mast cells have many biological roles, ranging from maintenance of tissue homeostasis to deleterious activities contributing to the pathogenesis of many diseases including asthma. There is evidence of mast cell infiltration and proliferation in bronchial smooth muscles of asthmatic airways, accompanied by sustained release of a plethora of autacoid mediators, cytokines, and proteases. Human mast cells express the K^+_{Ca} channel that is opened after IgE-dependent activation. A widely proposed role for K^+ channels is to maintain a negative membrane potential during cell activation, counteracting the tendency for Ca^{2+} influx to depolarize the cell membrane. Both VGCC and K^+_{Ca} channels regulate T cell activation and proliferation, and the latter also is involved in mast cell IgE-mediated histamine release (Valverde et al. 2011). This is in line with our present findings from the immunohistochemical staining

of pulmonary sections where we followed mast cells infiltration after long-term administration of both K^+ channels openers.

Activation of K^+ channels results in K^+ influx and hyperpolarization of the plasma membrane. This leads to closure of VGCC and reduced Ca^{2+} influx. The resulting decrease in intracellular Ca^{2+} concentrations is associated with diminished contractile function and hyporeactivity of airway smooth muscle cells. Among the more than 100 K^+ channels identified so far, the opening of K^+_{ATP} channels, VG potassium channels, inwardly rectified K^+ channels, two-pore domain K^+ channels, and K^+_{Ca} channels was shown to be influenced by inflammatory processes. Ca^{2+}-induced mast cell degranulation leads to release of inflammatory cytokines that subsequently activate a cascade of signaling pathways including the potentiation of NF-κB activity, which all leads to cell damage (Bali et al. 2013). Martin et al. (2008) studied the relationship between BK^+_{Ca} channels and IL-4 in human airways. The main conclusion of that study is that IL-4, a cytokine important in the pathophysiology of asthma, rapidly increased BK^+_{Ca} channel activity in normal human airwat smooth muscle cells. Notably, the closely related cytokine, IL-13, a central mediator of asthma, did not share the effect of IL-4 on BK^+_{Ca} channel activity, but did antagonize the effect of IL-4. The IL-4 relaxing effect is antagonized by numerous pro-contractile mediators and cytokines in allergic inflammation. Furthermore, according to Valverde et al. (2011) bronchial hyperreactivity of asthmatic subjects is dependent mostly on IL-13 levels. These findings support prior observations in bovine tracheal cells showing that IL-4, but not IL-13, inhibits contractile agonist signaling, an effect favoring cell relaxation. These results are in significant correlation with our present results. Pinacidil and NS1619 decreased IL-13 concentrations in both serum and BALF samples and that corresponded to a significant bronchodilatory effect of these K^+ channels openers.

In conclusion, the presented results confirmed a significant role of K^+ ion channels in airway defense reflexes as well as in allergic inflammation. The openers of BK^+_{Ca} ion channels, in particular, could be an interesting target for novel antiasthmatics because of their strong bronchodilatory and anti-inflammatory potential.

Acknowledgments The authors thank Ms. Katarina Jesenska for technical support. This work was supported by the project Center of Experimental and Clinical Respirology II, the grants MZ 2012/35-UKMA-12 and VEGA No 1/0020/11 and 1/0062/11, and it was co-financed from EC sources.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 7: 47–52
DOI 10.1007/5584_2014_49

Published online: 26 September 2014

Impulse Oscillometry in the Diagnosis of Airway Resistance in Chronic Obstructive Pulmonary Disease

T. Piorunek, M. Kostrzewska, S. Cofta, H. Batura-Gabryel, P. Andrzejczak, P. Bogdański, and E. Wysocka

Abstract

Spirometry is a standard lung function test for diagnosis and staging of chronic obstructive pulmonary disease (COPD). Impulse oscillometry (IOS) can be complementary to spirometry, especially in patients at advanced age and with physical or mental disorders who cannot be diagnosed through spirometry. The aim of this study was to compare IOS and spirometry in the assessment of airway obstruction in COPD. The study was conducted in 112 stable COPD patients, including 29 females and 83 males of the mean age of 69 ± 11 years. The oscillometric evaluation included total (R5), peripheral (R5-R20), and negative reactance (X5), which were compared with the predicted forced expiratory volume in 1 s (FEV1%pred). The findings show a significantly negative correlation between FEV1%pred and the R5, R5-R20, and X5. COPD patients had increased R5, R5-R20, and X5. The severity of bronchial obstruction found by impulse oscillometry correlated well the spirometric assessment. IOS is a simple to perform test that may be helpful for functional examination of COPD patients.

Keywords

Airflow limitation • Lung function tests • Obstructive lung disease • Respiratory reactance • Respiratory resistance

T. Piorunek (✉), M. Kostrzewska, S. Cofta, and H. Batura-Gabryel
Department of Pulmonology, Allergology, and Respiratory Oncology, University of Medical Sciences, 84 Szamarzewskiego St., 60-185 Poznan, Poland
e-mail: t_piorun@op.pl

P. Andrzejczak
Department of Physics, Adam Mickiewicz University, 85 Umultowska st., 61-614 Poznan, Poland

P. Bogdański
Department of Internal Medicine, Metabolic Disorders, and Hypertension, Poznań University of Medical Sciences, Poznan, Poland

E. Wysocka
Department of Clinical Biochemistry and Laboratory Medicine, University of Medical Sciences, Poznan, Poland

1 Introduction

Spirometry is a standard, objective lung function test for diagnosis of airflow limitation in chronic obstructive pulmonary disease (COPD) in accordance with the Global Initiative of Obstructive Lung Disease classification (GOLD 2013). The essential diagnostic parameter for airway obstruction in COPD is a ratio of forced expiratory volume in 1 s to forced vital capacity FEV1/FVC of less than 0.70. The measurement of FEV1 allows assessing the severity of airway obstruction in COPD patients (Cooper 2005). Spirometry is highly dependent on the performance technique, as the maximal inspiration and forced expiratory maneuver require the patient's active cooperation (Kubota et al. 2009).

Impulse oscillometry (IOS) is a pulmonary function technique that measures airway resistance and reactance, and it is considered as complementary to spirometry. This method can especially be recommended to elderly patients with physical and mental limitations or with poor pulmonary function, who may have difficulty in caring out the flow-volume spirometry properly (Janssens et al. 2001). The measurement of IOS parameters is performed during tidal breathing and does not require respiratory effort. IOS can also detect distal airways disorders that are not measured by spirometry (Crim et al. 2011). Moreover, the IOS enables an assessment of total resistance (R5), proximal resistance (R20) and distal capacitive reactance (X5), as well as to calculate peripheral resistance (R5-R20) (Jaranbäck et al. 2013; Kanda et al. 2010). The aim of this study was to compare IOS and spirometry in the assessment of airway obstruction in COPD patients.

2 Methods

The study protocol involving patients was approved by the Ethics Committee of the University of Medical Sciences in Poznan, Poland, and each participant recruited gave informed consent. The study was conducted in 112 stable COPD patients (F/M – 29/83; mean age of 69 $\pm$ 11 years) and 15 subjects with the spirometry as a control group (mean age 63 $\pm$ 11 years). COPD was diagnosed in accordance with Global Initiative for Chronic Obstructive Lung Disease (GOLD 2013) guidelines including: COPD Assessment Test (CAT) or modified Medical Research Council questionnaire (mMRC), airflow limitation measurements, and the number of exacerbations and hospitalizations in the past 12 months. The control group consisted of 15 healthy subjects with normal spirometry. Spirometry and IOS (MasterScreen; Jaeger; Höchberg, Germany) measurements were evaluated after receiving 400 μg of short-acting beta2-agonist (salbutamol), according to ERS/ATS recommendations (Pellegrino et al. 2005). Flow-volume spirometric assessments included FEV1/FVC (FEV1%FVC), FEV1 (FEV1%pred) and FVC (FVC%pred). The spirometric criterion for airflow limitation was a fixed ratio of FEV1/FVC < 0.70. Classification of airflow limitation severity was based on post-bronchodilator FEV1%pred value according to GOLD standardizations (GOLD 2013). The patients were stratified according to the severity of airway obstruction: mild (FEV1 $\geq$ 80%pred), moderate (50 % $<$ FEV1 $<$ 80%pred), severe (30 % $<$ FEV1 $<$ 50%pred), and very severe (FEV1 $<$ 30 %pred). IOS measurements included: total respiratory resistance at 5 Hz (R5) comprising extrathoracic, central, and peripheral airways, proximal resistance at 20 Hz (R20), comprising mainly extrathoracic and central airways, and distal capacitive reactance at 5 Hz (X5) comprising elastic lung and thorax components. Peripheral resistance at 5 Hz minus that at 20 Hz (R5-R20) was calculated. The oscillometric values of R5, R20, R5-R20, and X5 were correlated with the FEV1%pred results. The relationship between pulmonary function parameters was assessed with Pearson's correlation. $P < 0.05$ was considered significant.

3 Results

The flow-volume spirometry enabled to distinguish 19 subjects with mild airway obstruction, 33 with moderate, 35 with severe, and 25 with very severe obstruction. The general characteristics of the study group showed the patients at older age and lower BMI with increasing airway obstruction severity, but the mean

BMI was normal at each stage of the disease (Table 1). The impulse oscillometry measurements performed in the course of COPD, confirmed the increases in total respiratory resistance (R5) and peripheral resistance (R5-R20), and a decrease in negative reactance (X5) at all stages of airway obstruction (Table 2).

The oscillometric measurements worsened with a higher degree of obstruction as assessed by spirometry (lower FEV1%pred.). A negative correlation between the severity of airway obstruction and total respiratory resistance (R5) was significant ($r = -0.62$, $p < 0.05$) (Fig. 1a). There was no correlation between the airway obstruction and proximal resistance (R20) ($r = -0.8$, $p > 0.05$). The comparative evaluation of peripheral resistance (R5-R20) revealed a negative correlation with the severity of airflow limitation ($r = -0.80$, $p < 0.05$) (Fig. 1b). A positive correlation between the stage of airway obstruction and distal capacitive reactance (X5) was significant as well ($r = 0.75$, $p < 0.05$) (Fig. 1c). The contribution of peripheral resistance (R5-R20) to the total resistance (R5) depends on the severity of airway obstruction. The peripheral resistance was 15.2 % in the control group and its share increased in COPD patients in rapport with increasing airway obstruction, amounting to 17.9 % in mild, 27 % in moderate, 40.8 % in severe, and 52.9 % in very severe obstruction (Fig. 2).

4 Discussion

General assessment of COPD patients in the present study shows that the older age and lower BMI correlated with increasing airway obstruction, although the mean BMI was within the normal range at each stage of the disease. Previous reports showed that 38 % of COPD patients are underweight (BMI < 18.5 kg/m^2), irrespective of the severity of the disease. The mean BMI significantly goes down with COPD worsening (De 2012). Difficulties associated with the proper performance of respiratory maneuvers by elderly patients or patients at advanced stages of COPD, limit the use of spirometry in the assessment of lung function. Additionally, spirometry measures mainly the proximal respiratory airflow, while COPD is notably a disease of peripheral airways, where resistance may reach even 60 % in advanced disease (GOLD 2013; Jaranbäck et al. 2013). An important advantage of spirometry is high repeatability of measurements expressed by coefficients of variations (Miller et al. 2005). The patients included into the present study performed the flow-volume spirometry in accordance with the recommendations on the quality of the examination. Impulse oscillometry (IOS) introduced into the clinical practice in recent

Table 1 Patient characteristics

Study and control group	n	Age (year)	Body weight (kg)	Body height (m)	BMI (kg/m^2)
Control	15	63 ± 11	96.5 ± 34.4	1.69 ± 0.12	33.1 ± 8.6
Mild obstruction	19	63 ± 12	82.5 ± 21.5	1.64 ± 0.09	30.8 ± 8.5
Moderate obstruction	33	66 ± 10	87.6 ± 25.2	1.69 ± 0.10	30.2 ± 7.2
Severe obstruction	35	71 ± 10	78.9 ± 15.0	1.67 ± 0.08	28.5 ± 6.0
Very severe obstruction	25	74 ± 9	76.8 ± 16.9	1.70 ± 0.07	27.0 ± 7.4

Table 2 Mean values of spirometric and oscillometric parameters in study and control group

Patients	n	FEV1 (l)	%pred.	R5 ($cmH_2O/l/s$)	%pred.	R20 ($cmH_2O/l/s$)	%pred.	R5-R20 ($cmH_2O/l/s$)	X5 ($cmH_2O/l/s$)
Control	15	3.0 ± 1.1	116.2	3.8 ± 0.7	108.7	3.0 ± 0.5	110.7	0.5 ± 0.3	−1.2 ± 0.4
Mild	19	2.3 ± 0.6	87.3	5.6 ± 1.4	154.9	3.1 ± 0.4	148.2	1.0 ± 0.6	−2.0 ± 0.6
Moderate	33	1.7 ± 0.6	65.5	5.1 ± 1.5	143.7	3.0 ± 0.4	121.0	1.4 ± 0.9	−2.1 ± 1.0
Severe	35	1.1 ± 0.2	40.1	6.5 ± 1.9	184.9	2.9 ± 0.4	130.0	2.7 ± 1.2	−4.4 ± 1.2
Very severe	25	0.7 ± 0.1	24.6	9.2 ± 1.9	278.7	2.9 ± 0.3	145.6	4.9 ± 1.4	−5.5 ± 1.1

Values are means ± SD

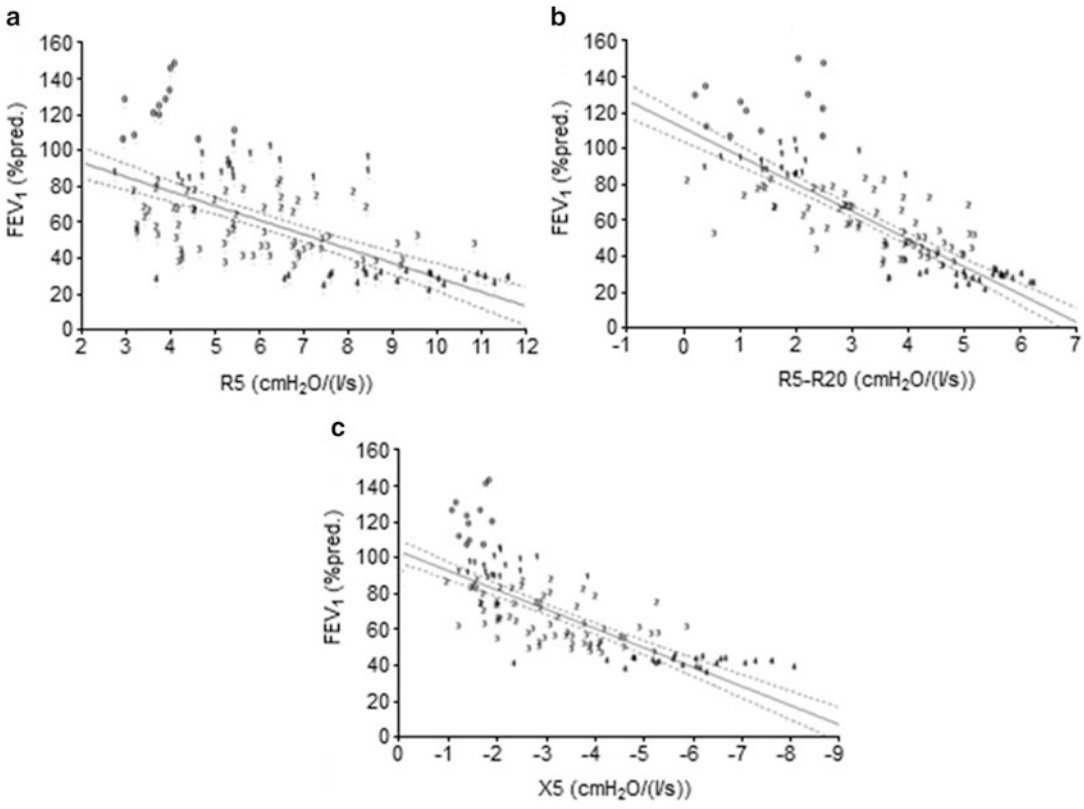

Fig. 1 Correlations between the severity of airway obstruction and respiratory resistances: total resistance – R5 (**a**), peripheral resistance – R5-R20 (**b**), and distal capacitive reactance – X5 (**c**). Control group – 0, mild obstruction – 1, moderate obstruction – 2, severe obstruction – 3, and very severe obstruction – 4

years can be a complementary method to conventional pulmonary function testing and, in selected cases, the method of choice in the evaluation of functional disorders in patients with COPD (Schulz et al. 2013). There is a renewed interest in the IOS because of its non-invasiveness and potential ability to distinguish a small from larger airway disease (Cooper 2005). The repeatability of IOS measurements is lower than that of spirometry, and when expressed by coefficients of variations it does not usually exceed 20 % in obstructive diseases (Oosteveen et al. 2003). In the present study, we found decreasing FEV1 values with increasing severity of obstructive disorders. The FEV1 values were less than 1 L at severe and very severe stages of obstruction. It has been determined that the FEV1 alone is not a good diagnostic and prognostic measure of COPD, since it

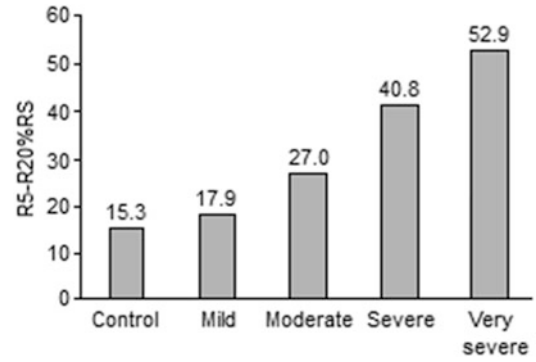

Fig. 2 Contribution of peripheral resistance (R5-R20) to the total airway resistance (R5) depending on COPD severity

does not represent the whole of a respiratory disorder (Jaranbäck et al. 2013). However, FEV1 complemented by IOS in the assessment of airflow limitation may become a measurement

of components of airway resistance (R5). A study by Tanaka et al. (2011) showed an increased in the mean R5 and R5-R20, but not R20, with increasing severity of obstructive disease. A significant increase of proximal resistance (R20) was observed only in bronchial asthma, but never in COPD patients, irrespective of disease severity (Lutchen et al. 2001). The present study shows the contribution of peripheral resistance (R5-R20) to total resistance (R5) depending on the stage of obstruction. This contribution increased from 17.9 % in mild, 27.0 % in moderate, and 40.8 % in severe, to 52.9 % in very severe airway obstruction. Increased negative values of distal capacitive reactance (X5) were also found and they correlated with increasing airflow limitation. The study also showed negative correlations between the severity of airway obstruction (FEV1%pred) and the R5, R5-R20, and X5 of oscillometric measurements. However, correlation between FEV1%pred and R20 was insignificant. Kanda et al. (2010) and Kolsum et al. (2009) reported similar findings. Those authors concluded that none of the IOS parameters alone could distinguish healthy individuals from COPD patients. Similar observations were made by Qi et al. (2013) with respect to functional disturbances in patients with bronchial asthma. Other authors suggest that IOS should be recommended for detecting respiratory abnormalities in COPD earlier than spirometry (Winkler et al. 2009). Moreover, it has been established that pulmonary function tests performed in tandem are more sensitive in detecting airflow limitation in COPD than IOS used separately, but have the same specificity in excluding bronchial obstruction (Jaranbäck et al. 2013). An observation of Anderson and Lipworth (2012) is noteworthy in that the IOS is useful for detecting airway disorders in COPD patients, but does not provide a link between symptoms assessed by the Medical Research Council Dyspnea Score and pulmonary function measurements.

In conclusion, COPD patients have increased total and peripheral airway resistance, and decreased negative reactance. The severity of bronchial obstruction assessed by spirometry (FEV1%pred) correlates with the measures obtained from an oscillometric method. The IOS is a simple-to-perform test that is complementary to spirometry and, in some cases may be essential for functional examination of COPD patients.

Conflicts of Interest No conflict of interest was declared by the authors as regards this work.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 7: 53–58
DOI 10.1007/5584_2014_68

Published online: 15 October 2014

Efficacy of Noninvasive Volume Targeted Ventilation in Patients with Chronic Respiratory Failure Due to Kyphoscoliosis

P. Piesiak, A. Brzecka, M. Kosacka, and R. Jankowska

Abstract

Severe kyphoscoliosis can cause chronic respiratory failure. Noninvasive mechanical ventilation (NIMV) is a new optional treatment for such patients. The aim of this study was to evaluate the effectiveness of average volume-assured pressure support (AVAPS) NIMV in patients with kyphoscoliotic chronic respiratory failure. The study was performed in 12 patients (mean age 49 ± 11 years and body mass index 27.5 ± 7.9 kg/m^2) with advanced kyphoscoliosis complicated by severe respiratory failure (PaO_2 6.68 ± 0.34 kPa, SaO_2 81.7 ± 3.1 %, $PaCO_2$ 9.51 ± 1.08 kPa) treated by the NIMV. The short-term, after 5 days, and long-term, after 1 year of home treatment, efficacy of NIMV was evaluated. We found a significant improvement of diurnal PaO_2 and $PaCO_2$ on the 5th day of NIMV (an increase of 1.4 ± 0.3 kPa and a decrease of 1.8 ± 0.8 kPa, respectively; $p < 0.05$) and after one year NIMV (an increase of 2.07 ± 0.46 kPa and a decrease of 2.68 ± 0.85 kPa, respectively; $p < 0.05$). There was a significant increase of mean blood oxygen saturation during sleep on the 5th day (86.2 ± 3.2 %) and after 1 year of treatment (89.4 ± 2.1 %) compared with the baseline level (83.2 ± 3.2 %). The forced vital capacity also increased after 1 year (1,024 ± 258 ml *vs.* the baseline 908 ± 267 ml; $p < 0.05$). The NIMV was well tolerated and no patient discontinued the treatment during the observation period. We conclude that AVAPS NIMV is an effective treatment option in kyphoscoliotic patients with chronic respiratory failure, resulting in a prompt and long-term improvement of daytime and nocturnal blood gas exchange.

Keywords

Hypoxemia • Kyphoscoliosis • Lung function • Pressure support ventilation • Respiratory failure

P. Piesiak (✉), A. Brzecka, M. Kosacka,
and R. Jankowska
Department of Pulmonology and Lung Cancer,
Medical University in Wroclaw, 105 Grabiszynska St.,
53-439 Wroclaw, Poland
e-mail: ppiesiak@tlen.pl

1 Introduction

Kyphoscoliosis is a chronic disease, occurring in 2–3 % of the general population, which leads to a distorted spinal curvature and chest wall deformity. As a consequence, respiratory function is impaired due to reduced chest wall compliance and a restrictive lung function pattern arises (Smyth et al. 1984). There are two factors that contribute to the severity of functional disorders: impairment of chest mechanics and size of 'internal prominence', the latter being related to the loss of physiological thoracic kyphosis (Kotwicki et al. 2009). A significant impairment occurs in the minority of scoliotic patients (0.1–0.3 %) and comes to light when a spinal curvature reaches an angle of 60–80° (Kotwicki et al. 2009) causing a decrease in vital capacity, total lung capacity, functional residual capacity, and, residual volume (Bergofsky 1979). Additionally, deformities of the rib cage cause changes in the length and orientation of respiratory muscles leading to inspiratory muscle insufficiency, mainly that of the diaphragm (Nickol et al. 2005; Gonzales et al. 2003; Smyth et al. 1984). All these factors can, at times, lead to life threatening chronic respiratory failure (CRF) (Kotwicki et al. 2009).

Noninvasive mechanical ventilation (NIMV) used for home mechanical ventilation is a well-established and increasingly used therapeutic option for patients with CRF due to chronic obstructive pulmonary disease (COPD), neuromuscular diseases, or in obese patients with chronic alveolar hypoventilation (Piesiak et al. 2012; Lloyd-Owen et al. 2005). The new mode of NIMV, such as average volume-assured pressure support (AVAPS), has been developed to increase the patient's tolerance and treatment effectiveness. It automatically adapts the pressure support to the patient's ventilation, depending on the average tidal volume. Briones Claudett (2013) and Piesiak et al. (2012) have presented positive effects of AVAPS NIMV among patients with COPD and obesity-hypoventilation syndrome. The aim of the present study was to evaluate the effectiveness of AVAPS NIMV in a short- and long-term treatment of patients with CRF due to severe kyphoscoliosis, the setting in which the effects of this mode of NIMV apparently have not yet been tackled.

2 Methods

The study has been approved by the Commission of Bioethics of Wroclaw Medical University in Poland. There were 12 patients (F/M – 7/5, mean age 49 ± 11 years, BMI 27.5 ± 7.9 kg/m^2) affected by severe kyphoscoliosis complicated by CRF enrolled into the study. The patients gave written informed consent. They were hospitalized in the Department of Pulmonology and Lung Cancer, Wroclaw Medical University in 2008–2013 for the assessment of either stable chronic respiratory failure or treatment following an episode of acute decompensated respiratory failure. To be included into the study the patients had to fulfill at least one of the following criteria: daytime complete respiratory failure with blood partial pressure of carbon dioxide ($PaCO_2$) >7.3 kPa, ≥1 hospitalization caused by exacerbation of CRF in preceding year, and inefficacy of oxygen therapy. The exclusion criteria were the presence of pulmonary parenchymal disease and instable cardiovascular disease.

After inclusion, the history of prior hospitalizations caused by respiratory system disorders was taken. Then, patients underwent baseline assessments of spirometry, arterial blood gas analysis, and polysomnography (PSG) before treatment and on the 5th day of NIMV therapy. PSG was performed with a PSG Aura setup (Grass Technologies; Warwick, RI) and spirometry with a Lung Test 1,000 system (MES; Cracow, Poland), according to guidelines recommended by Quanjer and the European Respiratory Society (Quanjer et al. 1993). NIMV parameters were established during polysomnography in such a way as to increase ventilation and achieve a $SaO_2 \geq 90$ % with a significant decrease in $PaCO_2$. Supplementary oxygen was provided to patients who were

hypoxemic despite the NIMV treatment (PaO_2 < 7.3 kPa, SaO_2 < 90 %).

After the initial evaluation, establishing the NIMV parameters, and achieving clinical improvement, the patients were sent home with an adjusted respirator and were subsequently treated under the supervision of a team of professionals familiar with the NIMV, consisting of the physician, nurse, and physiotherapist. After 1 year of home NIMV therapy, the patients were admitted to the hospital once again. The assessment of the number of the interim hospitalization days caused by respiratory system disorders, pulmonary function tests, blood gas analysis, and PSG were performed.

All patients received NIMV *via* Trilogy 100 ventilators (Philips-Respironics; Andover, MA) in a pressure support, spontaneous/timed mode (PS-S/T). They used the optional average volume assured pressure support (AVAPS) mode that automatically adapts the pressure support-inspiratory positive airway pressure (IPAP) to provide the preset patient's average tidal volume. The IPAP was titrated during ventilation in steps of 1 mbar/min in order to achieve a desired tidal volume and was set between the expiratory positive airway pressures (EPAP) and 30 mbar. Initially, the AVAPS tidal volume was set to 7–10 ml per kg of an ideal body weight. Ventilator settings were changed according to the patient's daytime and nocturnal tolerance, and to a maximal decrease of $PaCO_2$. During the 1 year treatment period, the settings for IPAP, EPAP, respiratory rate, and targeted tidal volume were kept at the same level. Three patients received supplemental oxygen. Ventilatory variables are summarized in the Table 1.

Data are given as means ± SD. A paired *t*-test was used for statistical elaboration to compare the pre- and post-treatment variables. Statistically significant difference were defined as $p < 0.05$.

Table 1 Noninvasive mechanical ventilation parameters

Variables	
IPAP (mBar)	21.3 ± 4.2
EPAP (mBar)	4.9 ± 1.7
Vt (ml)	421 ± 68
Vleak (ml)	55 ± 14
fb (breaths/min)	14.6 ± 3.1
Oxygen (l/min)	1.6 ± 0.9
Patient-triggered breaths (%)	76 ± 23
Compliance (h:min/day)	5:49 ± 01:45
Supplemental oxygen therapy (% of patients)	25

Values are means ± SD
IPAP inspiratory positive airway pressure, *EPAP* expiratory positive airway pressure, *Vt* tidal volume, *Vleak* leakage volume, *fb* breathing frequency

Table 2 Lung function tests and polysomnography at baseline and after a year's NIMV therapy

	Baseline	After 1 year
FEV1 (ml)	728 ± 226	833 ± 204
FEV1 (% predicted)	39 ± 14	42 ± 13
FVC (ml)	908 ± 268	1,024 ± 258*
FVC (% predicted)	38 ± 13	45 ± 12
FEV1/FVC (%)	82 ± 7	76 ± 12
Hospitalization (days)[a]	9.1 ± 3.2	3.1 ± 1.6*

Values are means ± SD
FVC forced vital capacity, FEV_1 forced expiratory volume in 1 s, *FEV1/FVC* Tiffeneau-Pinelli index, SaO_2 arterial oxygen saturation, $PaCO_2$ blood partial pressure of carbon dioxide; PaO_2 blood partial pressure of oxygen, *AHI* apnea/hypopnea index, *DI* desaturation index
[a]Number of hospitalization days caused by respiratory system disorders during preceding year
*$p < 0.05$ compared with baseline

3 Results

We found that idiopathic kyphoscoliosis was the cause of CRF in 11 out of the 12 patients. The remaining patient had structural kyphoscoliosis caused by a neuromuscular disease. Two of the 11 patients with idiopathic kyphoscoliosis had a coexisting obstructive sleep apnea syndrome. Lung function tests revealed severe restrictive ventilatory failure, with the Tiffeneau-Pinelli index of 82 ± 7 % and a chronic hypoxic/hypercapnic condition (Tables 2 and 3).

During the initial hospitalization, just 5 days after the onset of NIMV we found an improvement of patients' clinical status and lung ventilation as assessed by polysomnography and blood gas content (Table 3). There was a significant 8 % increase in diurnal PaO_2 (mean +1.4 ± 0.3 kPa) and 12 % decrease in $PaCO_2$ (mean −1.8 ± 0.8 kPa).

Table 3 Daytime arterial blood gas analysis at baseline on the 5th day of NIMV and after a year's NIMV therapy

	Baseline	After 5 days	After 1 year
PaO_2 (kPa)	6.7 ± 0.3	8.1 ± 0.3*	8.8 ± 0.4*
$PaCO_2$ (kPa)	9.5 ± 1.1	7.8 ± 0.9*	6.9 ± 0.9*
SaO_2 (%)	81.7 ± 3.1	90.1 ± 3.2*	91.3 ± 3.3*
pH	7.34 ± 0.04	7.36 ± 0.04	7.37 ± 0.03
AHI	8 ± 11	7 ± 9	5 ± 10
DI	9 ± 10	8 ± 10	4 ± 11
Mean nocturnal SaO_2 (%)	83.2 ± 3.2	86.2 ± 3.2*	89.4 ± 2.1*
Mean minimal nocturnal SaO_2 (%)	71.0 ± 5.0	75.3 ± 7.8	83.2 ± 6.1*

Values are means ± SD

SaO_2 arterial oxygen saturation, *$PaCO_2$* blood partial pressure of carbon dioxide; *PaO_2* blood partial pressure of oxygen, *AHI* apnea/hypopnea index, *DI* desaturation index

*$p < 0.05$ compared with baseline

Nocturnal ventilation improved, with an increase in the SaO_2 during sleep (+4.1 ± 3.2 %) ($p < 0.05$ for all).

After 1 year of home NIMV therapy, the improvements of daytime and nocturnal blood gas content were evidently pronounced, compared with the very baseline level. Diurnal PaO_2 increased by a mean of 2.1 ± 0.5 kPa and $PaCO_2$ decreased by 2.7 ± 0.8 kPa. The mean and minimum SaO_2 during sleep increased by a mean of 6.2 ± 3.2 % and 8.3 ± 4.1 %, respectively ($p < 0.05$ for all) (Table 3). In addition, NIMV therapy caused a nearly threefold decrease in the number of hospitalization days due to respiratory system disorders (9.1 ± 3.2 days in a year's time prior NIMV *vs.* and 3.1 ± 1.6 days during 1 year of NIMV, $p < 0.05$) (Table 2). During the observation period, tolerance of NIMV was satisfactory; none of the patients quit the treatment.

4 Discussion

The NIMV has become a predominant form of ventilatory support for treatment of CRF resulting from restrictive thoracic disorders, including kyphoscoliosis. Interestingly, there is a large discrepancy between countries concerning the number of patients with restrictive thoracic diseases receiving long-term NIMV, with Poland reporting one of the lowest and Spain the highest number (Lloyd-Owen et al. 2005). One of the new modes of pressure support ventilation – AVAPS has recently been developed in order to increase the patient's tolerance and treatment effectiveness. There are data presenting a higher efficacy of treatment conducted with AVAPS compared with the pure pressure-preset NIMV in obesity-hypoventilation and COPD patients (Briones Claudett et al. 2013; Storre et al. 2006), but there has been lack of studies on AVAPS therapy in kyphoscoliotic CRF. The present study demonstrates that AVAPS NIMV seems efficient therapy also in this group of patients.

All study patients had severe functional ventilatory alterations with advanced restrictive impairment and hypercapnia, accompanying kyphoscoliosis, justifying the prolonged use of AVAPS NIMV. Consistent with the results of previous studies (Adıgüzel et al. 2012; Fuschillo et al. 2003; Gonzales et al. 2003; Jansenns et al. 2003), our results confirmed the effectiveness of NIMV in improving daytime and nocturnal blood gas content in these patients. We observed a significant daytime increase in PaO_2 and a decrease in $PaCO_2$ already in a short-term of 5 days after the onset of NIMV. Nocturnal blood gas exchange also improved with an increase in the mean and minimum SaO_2. The improvements were sustained and were even better after 1 year of therapy. Comparing with the

pretreatment values, PaO_2 increased by an average of 10 % and $PaCO_2$ decreased by 19 %. Our results confirmed the findings of some previous studies on the efficacy of the longer-term, 5 years' NIMV use (Duiverman et al. 2006; Leger et al. 1994). The mechanisms of such positive effects are still debated. Some authors suggest that an increased ventilatory response to carbon dioxide is a principal mechanism underlying the long-term improvement in gas exchange following NIMV in restrictive thoracic diseases (Nickol et al. 2005). Another explanation is a recovery of the global inspiratory muscle strength after NIMV therapy, followed by improvements in lung function, observed in some studies in scoliotic patients (Buyse and Gonzales 2003). In the present study the only significant change in lung function was an increase in vital capacity by 12 % after 1 year of NIMV therapy. In other trials the restrictive pattern of ventilatory insufficiency was stable despite NIMV therapy (Nickol et al. 2005; Schonhofer et al. 2001). Meager changes in lung function could be explained by the fact that NIMV therapy does not affect the structural configuration of the chest, from which substantial improvements in lung function might be expected (Windish et al. 2008).

There is consistent impression that the need for hospitalization decreases during NIMV therapy, but no controlled studies are available to support that notion. Leger et al. (1994) revealed that for patients with kyphoscoliosis the mean number of days spent in hospital decreased from 34 ± 31 before the initiation of NIMV to 6 ± 6 days during the first year of therapy. In a group of 16 scoliotic patients studied by Gonzales et al. (2003), nine experienced at least one hospitalization for respiratory insufficiency during the 6 months before the beginning of NIMV therapy and none required hospitalization after NIMV supported ventilation. These data are consistent with the results of our study. We demonstrate that the mean number of days spent in hospital decreased by 67 % after 12 months of NIMV. After the beginning of NIMV support only two patients were admitted to the hospital due to infectious CRF exacerbations.

There are different approaches to the noninvasive ventilation of CRF patients. Some authors use lower pressure support in scoliotic patients with CRF (Gonzales et al. 2003). We chose to use rather a high inspiratory pressure (mean IPAP of 21.3 ± 4.2 kPa) and low expiratory pressures (mean EPAP 4.9 ± 1.7). This is consistent with other authors' recommendations (Windisch et al. 2008). The only exclusion in our study concerned the two patients with coexisting obstructive sleep apnea who were treated by NIMV with a higher EPAP of 8 cmH_2O in order to eliminate apnea/hypopnea episodes.

The duration of nocturnal NIMV therapy factors in treatment efficacy. In our group the mean daily time of NIMV was longer than 5 h. This is in accordance with a report by Murphy et al. (2012) who showed that nocturnal ventilation lasting for more than 4 h is required to achieve a reduction in daytime carbon dioxide. A long daily duration of NIMV in our study was possible due to a very good tolerance by patients of this mode of treatment.

5 Conclusions

Kyphoscoliosis is a spine disorder that can lead to severe chest deformity, complicated by chronic respiratory failure. In some scoliotic patients, traditional treatment is insufficient and respiratory failure can be life threatening. Our results show that in such patients, the average volume-assured pressure support noninvasive mechanical ventilation is an effective and well tolerated treatment option resulting in a rapid and sustained improvement of the respiratory system functioning during both daytime and sleep. This modern mode of therapy also decreases the risk of hospitalization.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 7: 59
DOI 10.1007/5584_2014

Index