Advances in Experimental Medicine and Biology 935
Neuroscience and Respiration

Mieczyslaw Pokorski *Editor*

Pulmonary Infection and Inflammation

Advances in Experimental Medicine and Biology

Neuroscience and Respiration

Volume 935

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Pulmonary Infection and Inflammation

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-319-44484-0 ISBN 978-3-319-44485-7 (eBook)
DOI 10.1007/978-3-319-44485-7

Library of Congress Control Number: 2016948844

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Preface

The book series *Neuroscience and Respiration* presents 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. The authors focus 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. New research is presented 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 also is discussed.

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 therapy of respiratory diseases.

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 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.

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 assume a leading role in the field of respiratory medicine and research and will become a source of reference and inspiration for future research ideas.

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2016) 26: 1–11
DOI 10.1007/5584_2016_28
© Springer International Publishing Switzerland 2016
Published online: 23 June 2016

Prevalence of Pulmonary Infections Caused by Atypical Pathogens in non-HIV Immunocompromised Patients

E. M. Grabczak, R. Krenke, M. Przybylski, A. Kolkowska-Lesniak, R. Chazan, and T. Dzieciatkowski

Abstract

Although atypical bacteria are important causes of lower airway infections, data on their role in immunocompromised patients are scarce. The aim of the study was to evaluate the prevalence of atypical pulmonary infections in patients with various types of immunosuppression, and to analyze clinical characteristics of these infections. Eighty non-HIV immunocompromised patients with different underlying diseases and clinical and radiological signs of pulmonary infection were enrolled. Due to incomplete data on eight patients, 72 patients were eligible for final analysis (median age 58 years). All patients underwent fiberoptic bronchoscopy and bronchoalveolar lavage. Bronchoalveolar lavage fluid (BALF) fluid samples were sent for direct microscopy, cultures, and fungal antigen detection, when appropriate. Commercial qualitative amplification assay (PNEUMOTRIS oligomix Alert Kit®), based on nested PCR method, was used to detect specific DNA sequences of *L. pneumophila*, *C. pneumoniae,* and *M. pneumoniae* in BALF. There were 61 (84.7 %) patients with hematologic diseases, 3 (4.2 %) after solid organ transplantation, and 8 (11.1 %) with miscellaneous diseases affecting immune status. Specific sequences of *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila* DNA were found in 7 (9.7 %), 2 (2.8 %), and 0 patients, respectively. In 8 of these patients co-infections with different microorganisms were demonstrated. Co-infection with *A. baumanii* and *P. aeruginosa* was diagnosed in three patients who died. We conclude that

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atypical lower airway infections are uncommon in immunocompromised patients. The majority of these infections are co-infections rather than single pathogen infections.

Keywords
Atypical bacteria • Bronchoalveolar lavage fluid • *Chlamydophila pneumoniae* • *Legionella pneumophila* • *Mycoplasma pneumoniae* • Immunodeficiency • Respiratory infections

1 Introduction

The incidence of lower airway infections in immunocompromised patients is high and the course of a disease is usually more severe than that in immunocompetent hosts (Sousa et al. 2013; Bonatti et al. 2009). Mortality rate largely depends on the type and severity of immunosuppression, with the highest rate reported after hematopoietic stem cell transplantation (HSCT) and somewhat lower in solid organ transplant (SOT) recipients and patients with hematologic malignancies (HM) (Cervera et al. 2006; Rañó et al. 2001; 2002). It has also been shown that the outcome of pulmonary infections is significantly affected by a delay in diagnosis of specific etiology. An increase in mortality rate from 29 to 71 % has been reported in patients in whom the etiology of infection was ascertained within the first 7 days of onset of symptoms compared with those with later diagnosis (Rañó et al. 2001). The etiology of lower respiratory tract infections in immunocompromised patients is diverse. It includes common bacteria, uncommon bacterial agents, and opportunistic pathogens such as various fungal species and viruses. Although atypical bacteria are important causes of pulmonary infections in the general population, data on the role of these pathogens in immunocompromised patients are relatively scarce. In the immunocompetent hosts *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* are responsible for 1–36 % and 3–22 % of community acquired pneumonia (CAP) cases, respectively (Singanayagam et al. 2014; Masiá et al. 2007; Gleason 2002). The majority of these infections affect children and young adults and present as mild, self-limiting disease (Capelastegui et al. 2012). However, even 26 % of patients may require hospital admission and in-hospital death rate may be as high as 5 %. The prevalence of *Legionella pneumophila* pneumonia in the general population is slightly lower (2–16 %), but this infection is usually more severe. In two studies, *L. pneumophila* was responsible for 2–9 % of CAP that required hospitalization (Yu and Stout 2008; Gleason 2002). On the other hand, recent data do not confirm the relation between *L. pneumophila* infection and increased in-hospital mortality rate (Capelastegui et al. 2012).

It might be hypothesized that the course of atypical pulmonary infections in immunocompromised patients can be more severe than that in the general population and that the co-infection with atypical pathogens can aggravate the course of pulmonary disease caused by typical bacteria or fungi. Surprisingly, there are little data on the incidence and clinical features of atypical pulmonary infections in immunocompromised patients. According to the available publications, the incidence of these infections is quite low (Corti et al. 2009; Jain et al. 2004; Perez and Leigh 1991). However, a few cases of life threatening pneumonia caused by *C. pneumoniae* and *L. pneumophila* have been described (Di Stefano et al. 2007; Heinemann et al. 2000). Whether the true prevalence of atypical pathogen infections in immunocompromised hosts is low or it is underestimated due to low sensitivity of the diagnostic methods seems to be an interesting issue. It must be realized that the culture of atypical bacteria is difficult and demanding and can be offered by few

laboratories only. Serological methods, including specific IgM and IgG antibodies detection in the serum, have limited clinical application due to a delay in the diagnosis and suboptimal sensitivity in patients with immunoglobulin deficiency (false negative results) (Bartlett 2008; Hammerschlag 2000; Welti et al. 2003). Likewise, *L. pneumophila* antigen detection in the urine has limited sensitivity as a negative result of this test does not exclude infection with other than serotype 1 *L. pneumophila* strains. The introduction of polymerase chain reaction (PCR)-based methods that can identify specific genetic material in different biological samples, including broncholaveolar lavage fluid (BALF), throat swabs, and nasopharyngeal samples, enables a rapid, sensitive, and specific diagnosis of atypical pathogen infection even if patients are already treated with an antibiotic (Murdoch 2004; Welti et al. 2003; Murdoch 2003). Therefore, the aims of this study were to evaluate the prevalence of atypical lower airway infections using nested PCR (nPCR) method in patients with various types of immunosuppression and to analyze clinical characteristics of these infections.

2 Methods

2.1 Patients

The study protocol was approved by an Institutional Bioethics Committee. The study group consisted of 80 non-HIV immunocompromised patients with different underlying diseases and clinical and radiological signs of pulmonary infection. Due to incomplete data on eight patients, 72 patients were eligible for final analysis (median age 58; range 16–79 years; F/M – 21/51). The patients were treated in a large multidisciplinary university hospital and in a specialized center for hematology and hematologic oncology in Warsaw, Poland. All met the following inclusion criteria: (1) known immunosuppression; (2) clinical or radiological signs and symptoms of pulmonary infection; and (3) signed informed consent for diagnostic bronchoscopy. Immunosuppression was defined as: (1) hematologic diseases or malignancies (HDM); or (2) immunosuppressive chemotherapy due to any malignant disease; or (3) immunosuppressive treatment due to solid organ or hematologic stem cell transplantation (SOTR); or (4) immunosuppressive therapy due to autoimmune or other diseases; or (5) miscellaneous chronic diseases that could affect the immune state (MISC group).

Clinical signs and symptoms suggestive of lower airway infection included recent cough, fever, dyspnea, or auscultatory findings. Radiological findings consistent with pulmonary infection were defined as the presence of the following pulmonary abnormalities: single or multifocal consolidations, areas of ground glass opacity, pulmonary nodules, interstitial pattern which could not have been explained by other causes, such as e.g. progression of lung tumors or new lung metastases. Exclusion criteria were the following: (1) known AIDS or positive result of HIV test; (2) contraindications to diagnostic bronchoscopy, i.e., unstable hemodynamic status, gas exchange abnormalities resulting in hypoxemia (SaO_2 below 92 %) despite low flow oxygen therapy; and (3) respiratory failure requiring mechanical ventilation.

2.2 Bronchoscopy Procedure

All patients underwent fiberoptic bronchoscopy under local anesthesia. The insertion of a bronchoscope (Olympus BF 1 T180 or Pentax EB 1970 K; Tokyo, Japan) was preceded by premedication with atropine sulphate 0.5 mg s.c. and midazolam 7.5 mg p.o., and by local anesthesia of the upper airways with 2 % lidocaine. Suction was avoided in the upper airways and trachea to minimize contamination of the working channel of the bronchoscope. Additional portions of lidocaine were applied to the lower airways when necessary. After visual inspection of the lower airways, bronchoscope was wedged in segmental or sub-segmental bronchus in accordance with the localization of radiological abnormalities. In case of no relevant radiological abnormalities, bronchoscope was

wedged in the medial or lateral segment of the right middle lobe (RB4 or RB5). Two hundred milliliters of sterile, pre-warmed (37 °C) 0.9 % saline solution were instilled either in ten 20 ml portions or four 50 ml portions and withdrawn by gentle suction. Bronchoalveolar lavage fluid (BALF) was collected in sterile polypropylene tubes.

2.3 Microbiological Procedure

Samples of BALF were sent for microbiological examination including direct microscopy, cultures, and fungal antigen detection, when appropriate. One milliliter samples of BALF were frozen at −20 °C. Total DNA was extracted from 200 μl of BALF, using EXTRAcell® isolation kit. Commercial qualitative amplification assay (PNEUMOTRIS oligomix Alert Kit®), based on nested PCR method, was used to detect specific DNA sequences of *L. pneumophila, C. pneumoniae*, and *M. pneumoniae* in defrozen BALF samples. Also BETA-GLOBIN oligomix Alert Kit®, which uses the human β-globin gene as a standard, was used as an external control of DNA extraction and amplification. All reagents described above were supplied by Nanogen Advanced Diagnostics S.r.L. (Turin, Italy), and all investigations were performed in accordance to the manufacturer's instructions. A presumed limit of detection (LOD) of the PCR assay used was established as a few dozen copies/ml.

2.4 Data Collection and Analysis

Data on clinical and radiological signs and symptoms, and the results of microbiological examination of BALF were retrospectively collected and loaded in an electronic database. Additionally, results of other microbiological studies, including blood samples, throat swabs, sputum, urine, or stool were also analyzed.

Consistently with the aim of the study, results were assessed in patients with different types of immunosuppression.

Quantitative variables were presented as median, interquartile range (IQR) and/or ranges, while qualitative variables were presented as number and percentage. A non-parametric Mann-Whitney *U* test or Chi-squared test was used to assess the difference between variables in different groups. A p-value below 0.05 was considered statistically significant. Statistical analysis was performed using a statistical software package (STATISTICA, ver. 9.0, StatSoft Inc., Tulsa, OK).

3 Results

Demographics and data on the underlying diseases are presented in Table 1. Patients were unevenly distributed, with 61 (84.7 %) in the HDM group, 8 (11.1 %) in the MISC group, and 3 (4.2 %) patients in the SORT group. The most common underlying disease was acute myeloid leukemia (AML) which was responsible for almost one third of all causes of immunosuppression. AML was followed by chronic lymphocytic leukemia (n = 10; 13.9 % of causes) and non-Hodgkin lymphoma (n = 9; 12.5 % of causes).

Clinical signs and symptoms as well as radiographic data are demonstrated in Table 2. The major clinical symptoms were fever found in 54 (75.0 %) patients and cough reported by 30 (41.6 %) patients. There were no typical signs and symptoms of lower airway infection in 9 (12.5 %) patients, and pulmonary disease in these patients was diagnosed based on the new radiological findings. Chest radiographs and thorax CT scans were available in 71 (98.6 %) and 66 (91.7 %) of patients, respectively. The most common radiographic manifestation was lung consolidation, found in 50 (69.4 %) patients. There was a predominance of bilateral radiographic lung involvement, which was

Table 1 Underlying diseases in relation to demographic data in 72 immunocompromised patients

Causes of immunosuppression	All patients (n)	Male (n)	Female (n)	Age[a]
Hematologic diseases and malignancies	61	44	17	56 (45–66)
Lymphoproliferative disorders	29	23	6	56 (47–63)
Chronic lymphocytic leukemia	10	8	2	63 (57–73)
Non-Hodgkin lymphoma	9	8	1	55 (52–58)
Hodgkin lymphoma	5	3	2	38 (34–50)
Multiple myeloma	4	3	1	55 (49–60)
Waldenstrom macroglobulinemia	1	1	0	61
Acute leukemias	24	15	9	52 (43–61)
Acute myeloid leukemia	23	14	9	53 (45–61)
Acute lymphoblastic leukemia	1	1	0	22
Myeloproliferative disorders	4	2	2	68 (66–70)
Chronic myeloid leukemia	1	1	0	67
Essential thrombocythemia	1	0	1	61
Idiopathic myelofibrosis	2	1	1	69, 72
Other diseases	4	4	0	74 (60–78)
Bone marrow hypoplasia	2	2	0	78, 79
Bone marrow aplasia	1	1	0	29
Thrombocytopenia treated with steroids	1	1	0	70
Solid organ transplant recipients	3	3	0	46, 61, 70
Kidney	1	1	0	70
Liver	1	1	0	61
Kidney and pancreas	1	1	0	46
Various diseases that affected immune status	8	4	4	63 (55–68)
Rheumatoid arthritis	3	0	3	46, 67, 74
Granulomatosis with polyangiitis	1	1	0	58
Idiopathic pulmonary fibrosis	1	1	0	69
Liver cirrhosis	1	1	0	63
Diabetes mellitus	1	1	0	63
Porphyria	1	0	1	28

Data on patients age are presented as median and interquartile range (IQR)
[a]Age of individual patients was presented when fewer than four patients with respective diagnosis were evaluated

Table 2 Clinical and radiological characteristics of patients with pulmonary infections in relation to different underlying conditions

Variable	All patients (n = 72)	HDM group (n = 61)	SOTR group (n = 3)	MISC group (n = 8)	p
Signs and symptoms					
Fever, n (%)	54 (75.0)	47 (77.0)	2 (66.6)	5 (62.5)	0.600
Cough, n (%)	30 (41.6)	25 (41.0)	0	5 (62.5)	0.700
Dyspnea, n (%)	13 (18.0)	10 (16.4)	0	3 (37.5)	0.300
Hemoptysis, n (%)	6 (8.3)	3 (4.9)	0	3 (37.5)	0.016
No symptoms, n (%)	9 (12.5)	8 (13.1)	0	1 (12.5)	0.600
Radiological pattern					
Nodular pattern, n (%)	20 (27.8)	19 (31.1)	0	1 (12.5)	0.300
Consolidations, n (%)	50 (69.4)	40 (65.6)	3 (100.0)	7 (87.5)	0.200
Ground glass, n (%)	18 (25.0)	17 (27.9)	0	1 (12.5)	0.400
Other abnormalities (atelectasis, pleural effusion), n (%)	13 (18.0)	10 (16.4)	1 (33.3)	2 (25.0)	0.600

(continued)

Table 2 (continued)

Variable	All patients (n = 72)	HDM group (n = 61)	SOTR group (n = 3)	MISC group (n = 8)	p
Lung involvement in chest radiograph					
Bilateral, n (%)	37 (52.1)	30 (50.0)	2 (66.7)	5 (62.5)	0.700
Right lung only, n (%)	23 (32.4)	21 (35.0)	0	2 (25.0)	0.400
Left lung only, n (%)	8 (11.3)	6 (10.0)	1 (33.3)	1 (12.5)	0.400
No abnormalities, n (%)	3 (4.2)	3 (5.0)	0	0	0.800
No chest radiograph, n (%)	1 (1.4)	1 (1.6)	0	0	0.900
Lung involvement in CT scan					
Bilateral, n (%)	46 (69.7)	38 (69.1)	2 (66.7)	6 (75.0)	0.800
Right lung only, n (%)	13 (19.7)	11 (20.0)	0	2 (25.0)	0.600
Left lung only, n (%)	7 (10.6)	6 (10.9)	1 (33.3)	0	0.300
No CT scan, n (%)	6 (8.3)	6 (9.8)	0	0	0.600
Various data					
Duration from hospital admission to FOB, days	14 (8–28)	14 (8–30)	14, 23, 38[a]	15 (11–21)	0.500
Antibiotic treatment prior to FOB; n/n of pts with DA (%)	51/65 (78.5)	45/55 (82.0)	1/2 (50.0)	5/8 (62.0)	0.300
Treatment with antibiotic active against APs; n/n of pts with DA (%)	28/65 (43.1)	24/55 (44.0)	0/2 (0)	4/8 (50.0)	0.400
Neutropenia; n/n of pts with DA (%)	39/49 (79.6)	38/46 (82.6)	0/0 (0)	1/3 (33.3)	0.200
GCS therapy; n/n of pts with DA (%)	31 (43.0)	24/61 (39.3)	3/3 (100.0)	4/8 (50.0)	0.100
Outcome					
Cured/improved, n (%)	40 (55.5)	36 (59.0)	1 (33.3)	3 (37.5)	0.400
Failure, not fatal, n (%)	5 (6.9)	4 (6.5)	NA	1(12.5)	0.900
Fatal, n (%)	11 (15.2)	9 (14.7)	1 (33.3)	1 (1.25)	0.700
Data not available, n (%)	16 (22.2)	12 (13.1)	1 (33.3)	3 (37.5)	0.500

Data are presented as median and interquartile range (IQR) or number (%)

APs atypical pathogens, *CT* computed tomography, *pts* patients, *DA* data available, *FOB* fiberoptic bronchoscopy, *GCS* glucocorticosteroid, *HDM* hematologic disease/malignancy, *MISC* miscellaneous chronic diseases, *SOTR* solid organ transplant recipients, *NA* non-applicable

[a]Data of individual patients were presented instead of median and IQR when fewer than four patients were evaluated

demonstrated in about half of patients, i.e., in 37/71 (52.1 %) and 46/66 (69.7 %) patients as based on chest radiograph and thorax CT scan, respectively. Isolated right lung involvement was found in 23 chest radiographs and 13 thorax CT scans.

Table 3 presents the clinical, radiological and microbiological characteristics of 9 patients in whom DNA of atypical pathogens was identified in BALF. In none of 72 samples specific *L. pneumophila* DNA sequences were found. *M. pneumoniae* specific DNA was identified in samples collected from 7 (9.7 %) patients. Two samples (2.8 %) tested positively for *C. pneumoniae* DNA. In all patients with identified atypical pathogens, fever was the most commonly reported symptom. In 6 out of the 9 patients bilateral lung involvement was demonstrated. In 8 patients, co-infections with different microorganisms were detected based on BALF or blood microbiological studies. Despite broad spectrum antibiotic and antifungal therapy, 3 patients died. All those patients had positive results of blood culture, with *A. baumanii* and *P. aeruginosa* found in two and one patients, respectively.

Table 3 Characteristics of patients with atypical bacteria DNA in bronchoalveolar lavage fluid (BALF)

Patient No	Age (yr)	Gender	Group	Fever	Cough	Dyspnea	Chest X-ray	CT scan	Blood samples (culture/tests)	BALF culture	BALF fungal antigens or PCR	Immunosuppression factor	Outcome
Patients with *Mycoplasma pneumoniae* infection													
1	64	M	HDM	+	–	–	NA	BL	Negative	Negative	Negative	Neutropenia	Improvement
2	46	M	HDM	+	–	+	RL	RL	*S. aureus*	Negative	Negative	Neutropenia	Improvement
3	39	M	HDM	–	+	–	BL	BL	Negative	*S. pneumoniae*	Negative	CHTH	Improvement
4	75	M	HDM	+	–	–	RL	RL	*A. fumigatus* PCR (+)	*S. viridians, C. glabrata*	Negative	Underlying disease (CLL)	Improvement
5	51	M	HDM	+	+	+	BL	BL	*P. aeruginosa*	*E. faecium*	*Candida* antigen	Neutropenia, SC	Fatal (ARF due to pneumonia)
6	46	M	SOTR	+	–	–	BL	BL	Negative	*E. faecium*	Negative	SC	Improvement
7	58	M	MISC	+	+	+	BL	RL	*A. baumani*[a], *A.* fumigatus[a] *A. fumigatus* antigen	*A. fumigatus*	*A. fumigatus* antigen *Candida* antigen	Neutropenia CHTH, SC	Fatal (ARF due to IPA)
Patients with *Chlamydophila pneumoniae* infection													
8	63	M	HDM	+	–	–	RL	RL	Negative	Negative	*Candida* PCR (+)	Neutropenia	Improvement
9	67	M	HDM	+	–	–	BL	BL	*A. baumanii*	*S. viridans,* MRSA, *E. coli*	Negative	SC	Fatal (ARF due to pneumonia)
Patients with *Legionella pneumophila* infection													
–	–	–	–	–	–	–	–	–	–	–	–	–	–

ARF acute respiratory failure, *BALF* bronchoalveolar lavage fluid, *BL* bilateral lung involvement, *CHTH* chemotherapy, *CLL* chronic lymphocytic leukemia, *HDM* hematologic disease/malignancy, *IPA* invasive pulmonary aspergillosis, *M* male, *MRSA* methicillin resistant *Staphylococcus aureus*, *MISC* miscellaneous chronic diseases, *NA* not available, *PCR* polymerase chain reaction, *RL* right lung involvement, *SOTR* solid organ transplant recipients, + symptom present, − symptom absent, *SC* systemic corticosteroids

[a]Also in tissue samples

4 Discussion

The present study demonstrates a low prevalence of atypical pulmonary infections in non-HIV immunocompromised patients. *M. pneumoniae, C. pneumoniae* and *L. pneumophila* were found in 9.7 %, 2.8 %, and 0 % of patients, respectively. Thus, the prevalence of these infections in this study was somewhat lower than that usually reported in immunocompetent patients with CAP (Capelastegui et al. 2012; Masiá et al. 2007). On the other hand, the percentage of patients in whom atypical pathogens (except *L. pneumophila*) were identified was slightly higher as compared with other studies in immunocompromised hosts (Cervera et al. 2006; Hohenthal et al. 2005; Jain et al. 2004; Danés et al. 2002). This difference can be easily explained by multiple factors that can influence the results of various studies. These include: environmental factors (community or hospital acquired infection), seasonal and local epidemiological situation, type, severity and duration of immunosuppression, methods applied for pathogen detection and identification, reporting method (per entire study group or per subgroup with specific cause of immunoincompetence), and treatment applied prior to microbiological sampling. Despite all these conditions, most authors agree that atypical pulmonary infections in immunocompromised hosts are rather uncommon. Depending on the source of data, typical bacteria, fungi, and viruses have been responsible for 18–51 %, 8–38 %, and 9–23 % of pulmonary infections in non-HIV immunocompromised patients, respectively (Camps Serra et al. 2008; Jain et al. 2004; Danés et al. 2002; Rañó et al. 2001). In addition, polymicrobial infections caused by the pathogens outlined above have been diagnosed in 7–13 % of patients. Atypical pathogens have been found in single cases only.

In this study, diagnosis of pulmonary infection caused by atypical bacteria was based on a sole microbiological test, i.e., identification of specific DNA sequences in lavage fluid collected directly from the site of infection. The role of fiberoptic bronchoscopy and bronchoalveolar lavage as a diagnostic tool in immunocompromised patients with pulmonary infiltrates is well established. It has been shown that an early bronchoscopy (<5 days) has a significantly higher diagnostic yield for pulmonary infections than the late bronchoscopy (78 *vs.* 23 %; p = 0.02) (Lucena et al. 2014). The role of diagnostic methods other than culture in the work-up of immunocompromised patients with pulmonary infections has also been positively verified, albeit ELISA tests for the detection of *C. pneumoniae* and/or *M. pneumoniae* antibodies have some limitations, due to well-known cross reactions with other *Chlamydia* and *Mycoplasma* species. Hohenthal et al. (2005) have shown that the use of PCR and antigen detection to identify infectious agents in BALF from patients with hematological malignancies significantly improves the diagnostic yield. Unfortunately, although *M. pneumoniae* and *C. pneumoniae* PCR tests were performed in 37 and 29 BALF samples, respectively, the authors have neither presented nor discussed these results. Similar to the present study, none of the BALF samples evaluated by Hohenthal et al. (2005) tested positively for *Legionella spp*. in PCR tests. There are, however, two points which should be mentioned when comparing the results of these two studies. Firstly, the number of BALF samples evaluated by Hohenthal et al. (2005) has been almost two-fold higher than that in the present study. Secondly, In the Finnish study both PCR method and cultures have been applied and there was one patient with a positive culture but negative *Legionella spp*. PCR test. Thus, we cannot exclude that some patients with legionellosis could have been found in the present study, had other than PCR diagnostic methods been used. Nevertheless, the results of both studies point to a very low prevalence of *L. pneumophila* pulmonary infection in immunocompromised patients. That seems inconsistent with the results of some earlier studies which showed that hematological malignancies are a significant risk factor (rate ratio 22.4) for *L. pneumophila* pneumonia (Marston et al. 1994). Furthermore, as the course

of pulmonary infections in immunocompromised patients is often severe and *L. pneumophila* is a well-known pathogen responsible for severe pneumonias, a higher prevalence of this infection could be expected in immunocompromised patients. Therefore, some methodological issues that could have negatively influenced the prevalence of *L. pneumophila* infections found in the present study should be considered. The hypothesis that extremely low prevalence of *L. pneumophila* infection was related to false negative PCR results is highly unlikely. Contrary to the above mentioned data (positive *L. pneumophila* culture and false negative PCR test) numerous other studies demonstrate that *Legionella* PCR has a sensitivity equal to, or greater than, culture. A PCR test can give false negative results when polymerase inhibitors are present in the biological sample (Hammerschlag 2000). It has been shown that in *M. pneumoniae* infections, throat swabs are preferred over nasopharyngeal samples due to a lower rate of PCR inhibitors (Murdoch 2003). As PCR inhibitors are usually nonspecific, their presence would have caused false negative results not only in terms of *L. pneumophila* infection but also other pathogens, i.e., *M. pneumoniae* and *C. pneumoniae*. This was not the case in our study, as an external control of DNA extraction and amplification was used simultaneously and no inhibition was observed during this study. Early and adequate antibiotic therapy before sample collection can be another cause of false negative results of microbiological studies. In fact, a significant proportion of our patients (65.3 %), including 7/9 patients with atypical bacterial infection, had been treated with macrolides or fluoroquinolones before or at the time of diagnostic bronchoscopy. Prior studies in patients with pneumonia have shown that bronchoalveolar lavage performed within 3 days of antibiotic therapy onset has a diagnostic yield of 63.4 %, while the diagnostic value decreases to 57.6 % and 34.4 %, when lavage is done later on, before and after 14 days of treatment initiation, respectively (Kottmann et al. 2011). The argument against the confounding role of prior treatment for the results obtained in the present study is that PCR tests allow detecting genetic material of causative pathogen even a few weeks after initiation of antibiotic therapy (Welti et al. 2003).

Interestingly, atypical pathogens were identified in the present study exclusively in males. This may be partially explained by a higher proportion of males (71 %). Nevertheless, we believe this is not a sufficient explanation for this finding. Some gender-related differences in the incidence of atypical bacterial infections have also been reported in previous studies. Gutiérrez et al. (2006) have found the incidence of CAP caused by *C. pneumoniae* and *L. pneumophila* in the general population two-fold and ten-fold higher in males than in females, respectively. Age-related differences in the prevalence of atypical pathogen infections should also be mentioned. In the present study, median age of patients with *M. pneumoniae* infection was 51 years. This is somewhat inconsistent with Gutiérrez et al.'s (2006) findings who have reported the highest incidence of *M. pneumoniae* CAP in young and very elderly people, and the lowest between 45 and 64 years of age. To our knowledge, no specific data have been published on the gender-related or age-related differences in the prevalence of atypical pathogen infection in immunocompromised patients. Therefore, we could not confront our observation with any other. We realize that the number of patients with atypical pathogen infections is too small to draw unequivocal conclusions on the relationship between age or gender and the prevalence of *M. pneumoniae* and *C. pneumoniae* infections.

The mortality rate in our nine patients with *M. pneumoniae* or *C. pneumoniae* infection was relatively high (33.3 %), but we believe that neither was the course of disease nor mortality rate related exclusively to atypical bacterial infection. In this context, it should be underlined that in eight of these patients co-infection with other microorganisms was found (positive BALF and/or blood cultures). Systemic bacterial co-infection was proved in all three patients who died (*A. baumanii* and *P. aeruginosa* cultured from blood samples). This finding is

consistent with the results of three earlier studies that have reported co-infection with at least one another pathogen in 33–64 %, 48–74 %, and 54–63 % patients with *M. pneumoniae*, *C. pneumoniae,* and *L. pneumophila* infections, respectively (Welti et al 2003; Gleason 2002; Hammerschlag 2000). Perhaps, destruction of the airway epithelial layer and ciliostatic effect of these pathogens, facilitate other bacterial infections.

We are aware of several limitations of this study. Due to a small sample size, 95 % confidence interval could be calculated as 2.9–17.0 % and 0.0–6.8 % for a proportion of *M. pneumoniae* and *C. pneumoniae* infections, respectively. These values may question the confidence of a low prevalence of atypical pathogen infection in the study group. There is a marked disproportion between the number of patients with different causes of immunosuppression. In fact, our study group included mainly patients with hematological malignancies; hence the results refer mostly to this group of immunocompromised patients. That is also why we could not analyze the relationship between underlying diseases and the prevalence or clinical course of atypical infections.

A significant limitation of our study is associated with the use of PCR only to identify atypical bacteria infection. In consequence, we were unable to assess and discuss potential false positive and false negative results. Previous studies, including that by Pignanelli et al. (2009), have shown that a concomitant use of two or more different tests provides a higher diagnostic accuracy. Thus, the question on the true etiology of lower respiratory tract infection in some of our patients is still pending. In cases in which we did not find any putative etiological agent, it could have been any of the common respiratory viruses (metapneumovirus, coronavirus, or bocavirus) that are not routinely detected. Therefore, use of -wide-range diagnostic tool, e.g., FilmArray® Respiratory Panel based on multiplex nested PCR assay, could be helpful to improve outcome in immunocompromised patients (Dzieciatkowski et al. 2013).

In conclusion, we found that atypical lower airway infections are uncommon in immunocompromised patients. This particularly refers to *L. pneumophila* pneumonia. The majority of atypical pulmonary infections are co-infections rather than single pathogen infections.

Acknowledgment The authors gratefully acknowledge Warsaw Medical University and the Foundation for Patients with Hematological Diseases in Warsaw, Poland for the financial support that enabled the realization of the project.

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2016) 26: 13–23
DOI 10.1007/5584_2016_34
© Springer International Publishing Switzerland 2016
Published online: 23 June 2016

Effects of S-Nitroso-N-Acetyl-Penicillamine (SNAP) on Inflammation, Lung Tissue Apoptosis and iNOS Activity in a Rabbit Model of Acute Lung Injury

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Abstract

Acute lung injury is characterized by lung edema, surfactant dysfunction, and inflammation. The main goal of our study was to evaluate effects of S-nitroso-N-acetyl-penicillamine (SNAP) on migration of cells into the lung and their activation, inducible NO synthase (iNOS) activity, and apoptosis in experimental acute lung injury (ALI) in rabbits. ALI was induced by repetitive lung lavage with saline. The animals were divided into the following groups: (1) ALI without therapy, (2) lung injury treated with SNAP (ALI + SNAP), and (3) healthy animals (Control). After 5 h of ventilation, total and differential counts of cells in the bronchoalveolar lavage fluid (BALF) were assessed. Concentrations of interleukins (IL)-1ß, IL-6, and IL-8, endogenous secretory receptor for advanced glycation endproducts (esRAGE), sphingosine-1-phosphate receptor (S1PR)3, caspase-3, and mRNA expression of inducible NO synthase (iNOS) in lung tissue and nitrite/nitrate in plasma were analyzed. In the right lung, apoptotic cells were evaluated by TUNEL assay. In the animals with ALI, higher counts of cells, mainly neutrophils, in BALF and increased production of pro-inflammatory substances were observed compared with controls. SNAP therapy reduced a leak of cells into the lung and decreased concentrations of pro-inflammatory and apoptotic markers, reduced mRNA expression of iNOS, and decreased apoptotic index in the lung.

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Keywords
Apoptosis • Cytokines • Inflammation • Lung edema • Lung injury • Lung lavage • Oxidative stress • Surfactant • Tissue damage

1 Introduction

Acute lung injury (ALI) can be caused by many reasons including pneumonia, sepsis, trauma or aspiration (Ferguson et al. 2005). The hallmark of this acute event is an increased permeability of the alveolar-capillary membrane resulting from injury to the endothelium and epithelial alveolar cells. Damaged cell surface enables influx of protein-rich edema fluid into the alveoli and migration of inflammatory cells, particularly neutrophils into the lung (Nkadi et al. 2009). Neutrophils are attracted into the interstitial and bronchoalveolar space by chemoattractants, such as interleukin-8 (IL-8). Subsequently, neutrophils are activated and produce immune cell-activating agents, proteinases, cationic polypeptides, and cytokines. Reactive oxygen (ROS) and nitrogen species (RNS) are also produced through the oxidant-generating systems, e.g., phagocyte NADPH oxidase, myeloperoxidase, or nitric oxide synthase (NOS), all of which damages lung tissue (Grommes and Soehnlein 2011).

There are three types of NOS forming nitric oxide (NO), neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS); the last mentioned is highly relevant to the immune system. NO provides a wide array of actions in the body. For instance, NO plays an important role in the regulation of inflammatory responses. In healthy humans, NO acts as an autoregulatory feedback inhibitor, limiting tissue damage after onset of inflammation. NO inhibits expression of pro-inflammatory cytokines by downregulating nuclear factors that bind to the promoter region of the cytokine genes (e.g., NF-kB) (Hogaboam et al. 1997). On the other hand, excessively high NO production leads to post-translational modifications of proteins through S-nitrosylation of thiol groups or *via* generation of peroxynitrite ($ONOO^-$) leading to tyrosine nitration. Dysregulation of NO production in chronically infected host tissues can lead to immunopathology. Production of NO and activity of NOS in the tissue can be indirectly reflected by the concentration of natural oxidation products of NO: nitrite (NO_2^-) and nitrate NO_3^- anions (Ignarro et al. 1993).

Beside RNS, ROS are also produced by lung epithelial cells, neutrophils, and macrophages in abundant levels in ALI (Kinnula et al. 1992). In addition to detrimental effect of ROS and oxidative damage to proteins, lipids, and nucleic acids, superoxide anions react with NO and form the highly potent oxidant peroxynitrite. The complex action of inflammatory processes and oxidative effect of ROS and RNS finally leads to a disruption of the alveolar-capillary barrier, with subsequent formation of interstitial and alveolar edema and progression of lung injury (Lamb et al. 1999).

Similarly to endogenous NO, exogenously delivered NO and NOS inhibitors may have clinical implications in certain conditions as bronchodilators and vasodilators and they can be of benefit in inflammatory lung diseases. For instance, inhaled NO reduces pulmonary hypertension, improves oxygenation, and inhibits transendothelial migration of activated neutrophils in a variety of lung disorders (Miao et al. 2002).

Considering the mentioned favorable properties of inhaled NO, we supposed that administration of a soluble donor of NO directly into the lung may alleviate local inflammation and inflammation-related processes, such as oxidation and apoptosis of cells. Therefore, this study seeks to determine whether and to what extent the soluble NO donor S-nitroso-N-acetyl-penicillamine (SNAP) can influence the transmigration of neutrophils into the lung and their activation at the injury site. To estimate the effectiveness of SNAP, we investigated the following: injury to lung epithelial and endothelial cells, activation of lung cells and leukocytes, and

production of pro-inflammatory cytokines and markers of oxidation, production of NO expressed by iNOS and nitrite/nitrate concentration, and apoptosis of lung cells.

2 Methods

The experimental protocols were authorized by a local Ethics Committee of Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava and by the National Veterinary Board of Slovakia. In the study, we used adult New Zealand white rabbits, supplied by VELAZ Animal Breeding Station in Czech Republic, of both genders with the mean body weight of 3.0 ± 0.3 kg.

2.1 General Design of Experiments

The animals were anesthetized with ketamine (20 mg/kg, i.m.; Narketan, Vétoquinol, Great Slade, UK) and xylazine (5 mg/kg; Xylariem, Riemser, Greifswald, Germany), followed by a continuous infusion of ketamine (20 mg/kg/h). Catheters were inserted into the femoral artery and right atrium for sampling the blood, and into the femoral vein to administer anesthetics. Tracheotomy was performed and endotracheal cannula was inserted. Animals of one group, which served as healthy non-ventilated controls (Contr group, n = 6), were sacrificed at this stage of experiment by an overdose of anesthetics. Other animals were given pipecuronium bromide (0.3 mg/kg/30 min; Arduan, Gedeon Richter, Budapest, Hungary), subjected to a pressure-controlled ventilator (Beat-2, Chirana, Slovakia) and ventilated conventionally with the following settings: frequency (f) of 30/min, fraction of inspired oxygen (FiO_2) of 1.0, time of inspiration (Ti) 50 %, peak inspiratory pressure (PIP)/positive end-expiratory pressur (PEEP) of 1.5/0.3 kPa, and tidal volume (V_T) of 6–8 ml/kg. After 15 min of stabilization, respiratory parameters were recorded and blood samples were taken for blood gas content (RapidLab 348; Siemens, Munich, Germany). Lung injury was induced by repetitive lung lavage with 0.9 % saline (30 ml/kg of 37 °C) which was instilled into the endotracheal cannula in the semi-upright right and left lateral positions of the animal and was immediately suctioned by a suction device. Lavage was performed 6–10 times, until PaO_2 decreased to <26.7 kPa in two measurements at 5 and 15 min after the lavage at FiO_2 kept at 1.0. When the criteria of the ALI model were fullfilled, animals were treated with S-nitroso-N-acetyl-penicillamine (7 mg/kg; ALI + SNAP group, n = 6) which was given intratracheally by means of inpulsion effect of high-frequency jet ventilation (f 300/min, Ti 20 %; Mokra et al. 2007) to ensure a homogenous distribution of the substance throughout the lung. Other animals were left without therapy (ALI group, n = 6). The animals of both ALI groups were oxygen-ventilated (FiO_2 1.0, f 30/min, PIP/PEEP 1.5/0.3 kPa, V_T 6–8 ml/kg) for an additional 5 h after administration of the treatment. Blood gases and respiratory parameters were measured at 0.5, 1, 2, 3, 4, and 5 h of the treatment. At the end of experiment, blood samples were taken and animals were sacrificed by an overdose of anesthetics.

2.2 Counting of Cells in Bronchoalveolar Fluid (BALF)

After sacrificing the animal, lung and trachea were excised. The left lung was lavaged three times with 0.9 % NaCl (individual dose of 10 ml/kg, 37 °C) and BALF was centrifuged at 1500 rpm for 10 min. A total number of cells in BALF was determined microscopically in a counting chamber. A differential count of cells in the BALF sediment was evaluated microscopically after the May-Grünwald-Giemsa staining.

2.3 Expression of iNOS mRNA Using Quantitative PCR

Stabilized lung tissue was homogenized in a Polytron homogenizer PT 1200 E (Kinematica AG; Lucerne, Switzerland) for 20 s at the maximum speed and isolated using the RNeasy® Mini kit (QIAGEN Group; Hilden, Germany). A total

1 μg mRNA was used to produce a complementary DNA (cDNA) using a random initiator QuantiTect® Reverse Transcription Kit (QIAGEN Group) with a reaction mixture of 20 μL according to the manufacturer's instructions. Hypoxanthine phosphoribosyltransferase (HPRT) was used as a reference gene and all data were normalized to HPRT mRNA expression. The primer sequences for iNOS were following: forward: GCAGCAGCGGCTTCACA; reverse: ACATCCAAACAGGAGCGTCAT and the sequences for HPRT were following: forward: AGGTGTTTATCCCTCATGGACTAATT; reverse: CCTCCCATCTCCTTCATCACAT.

Quantitative real-time PCR (qPCR) was performed with QuantiTect® SYBR® Green PCR Kit (QIAGEN Group) in a total volume of 25 μL reaction mixture composed of 1 μL of cDNA, 0.3 μM final forward and reverse primer concentration, according to the manufacturer's instructions. qPCR was performed using an iCycler iQ® (Bio-Rad Laboratories; Hercules, CA) for 45 cycles at 95 °C for 15 s, followed by a primer-specific annealing temperature at 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 ver. 2.0 using the second derivative method. All qPCR analyses were performed in triplicates.

2.4 Markers of Inflammation and Lung Injury

A sample of arterial blood taken at the end of experiment was centrifuged (3000 rpm, 15 min, 4 °C) and plasma was stored at −70 °C until further use. Samples of right lung tissue were taken and prepared for additional biochemical and immunohistological analyses.

2.4.1 Preparation of Lung Tissue Homogenate

Lung tissue was homogenized (five times for 25 s, 1200 rpm) in an ice-cold phosphate buffer (pH 7.4). Homogenates were freezed three times and centrifuged (12,000 rpm, 15 min, 4 °C). Final supernatants were then stored at −70 °C until further use. Protein concentration in lung homogenates was determined according to the Lowry et al. (1951) method using bovine serum albumin as a standard.

2.4.2 Measurement of Markers of Inflammation and Lung Injury by Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokine concentration (IL-1β, IL-6, and IL-8) and the markers of lung epithelial cells injury (endogenous secretory receptor for advanced glycation end-products, esRAGE) and endothelial cells injury (sphingosine-1-phosphate receptor 3, S1PR3) were measured in lung homogenates using rabbit-specific ELISA kits (Wuhan USCN Business Co., Houston, TX for interleukins, and BioSource, San Diego, CA for sRAGE and S1PR3) according to the manufacturers' instructions. The results were analyzed spectrophotometrically at 450 nm using an ELISA microplate reader.

2.4.3 Measurement of Lipid Peroxidation

Lipid peroxidation expressed as the formation of thiobarbituric acid-reactive substances (TBARS) was assessed from the level of malonaldehyde-bis-dimethylacetal (MDA) in lung homogenates, using an OxiSelect™ TBARS Assay Kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instruction. TBARS concentration was determined from the absorbance at 532 nm.

2.4.4 Measurement of Nitrite/Nitrate Concentration

Plasma concentrations of total nitrite and nitrate were determined using Cayman's Nitrate/Nitrite Colorimetric Assay Kit (Alexis Corp., San Diego, CA), according to the manufacturer's instruction. The results were analyzed spectrophotometrically at 540 nm using an ELISA microplate reader.

2.5 Apoptosis Assays

2.5.1 In Situ Labeling of DNA Strand Breaks by TUNEL Method

The lungs were immersed in 4 % formalin. After paraffin embedding, 4 μm thick slices were cut on a microtome, followed by deparaffinization and pretreatment with a proteinase K. The specimens were further processed by the DeadEnd™ Colorimetric TUNEL System (Promega Corp., Fitchburg, WI), the assay labeling fragmented DNA of apoptotic cells. Biotinylated nucleotide is incorporated at the 3′-OH DNA ends using terminal deoxynucleotidyl transferase (rTdT), a recombinant enzyme. Horseradish peroxidase-labeled streptavidin is then bound to the biotinylated nucleotides. For the detection of nucleotides and blocking endogenous peroxidases, specimens were incubated with 0.3 % H_2O_2 solution and were developed with diaminobenzidine (DAB) chromogen solution. Specimens were then counterstained with Mayer's hematoxylin, mounted with Permount Mounting Medium (Fisher Scientific, Fair Lawn, NJ), and viewed under an Olympus BX41 microscope (Olympus, Tokyo, Japan). The image was captured with Quick Photo Micro software ver. 2.2 (Olympus). The apoptotic index of bronchial and alveolar epithelium was calculated as the percentage of TUNEL immunoreactive (TUNEL-IR) dark-brown stained nuclei in 100 nuclei randomly counted from three sites within each specimen.

2.5.2 Measurement of Caspase-3 Concentration in the Lung Homogenate by ELISA Method

A concentration of the marker of apoptosis caspase-3 in lung homogenate was measured with an ELISA kit (Cusabio Biotech Co., Newmarket, Suffolk, UK), according to the manufacturer's instruction. The results were analyzed spectrophotometrically at 450 nm using an ELISA microplate reader.

2.6 Statistical Analysis

Data were presented as means ± SE and statistical differences between the groups were determined by analysis of variance ANOVA. A p-value <0.05 was considered significant. Statistical analysis was performed using GraphPad Prism ver 5.1 for Windows (GraphPad Software, La Jolla, CA).

3 Results

3.1 Cells in BALF

In the animals with acute lung injury due to lung lavage (ALI group), a higher number of cells in BALF was found compared with the control group. In the group treated with S-nitroso-N-acetyl-penicillamine (ALI + SNAP), the number of cells in BALF decreased significantly compared with the ALI group ($p < 0.001$ for both; Fig. 1a). The percentage of neutrophils was significantly higher in the ALI group, but monocytes and macrophages decreased in this group compared with the control group ($p < 0.001$ for both; Fig. 1b). In the ALI + SNAP group, there were no significant differences in percentage of neutrophils, monocytes, macrophages, and eosinophils compared with ALI ($p > 0.05$; Fig. 1b).

3.2 Markers of Inflammation and Lung Injury

The level of biomarkers in lung homogenates in the control, ALI, and ALI + SNAP-treated groups is summarized in Fig. 2. The pro-inflammatory cytokines IL-1β, IL-6, and IL-8 increased in the ALI animals compared with controls ($p < 0.001$; Fig. 2a, b, and c), but the concentration of TBARS did not change

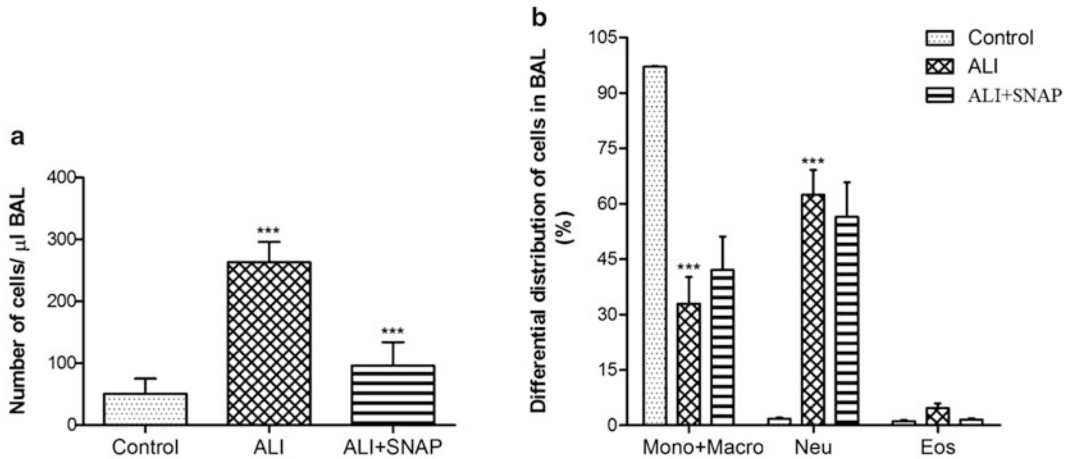

Fig. 1 Total number of cells (**a**) and differential count of cells (**b**) in bronchoalveolar fluid BALF fluid. *Mono-Macro* monocytes-macrophages, *Neu* neutrophils, *Eos* eosinophils; ***$p < 0.001$ for ALI *vs.* control, ALI *vs.* ALI + SNAP; and control *vs.* ALI + SNAP

($p > 0.05$; Fig. 2d). The marker of epithelial injury (esRAGE) and marker of endothelial injury (S1PR3) increased in the ALI animals compared with controls ($p < 0.01$ for both; Fig. 2e and f).

Treatment with the soluble NO donor SNAP decreased the concentration of the inflammatory cytokines IL-1β and IL-8 ($p < 0.05$; Fig. 2a and c) and IL-6 ($p < 0.001$; Fig. 2b), TBARS ($p < 0.01$; Fig. 2d), esRAGE ($p < 0.001$; Fig. 2e), and S1PR3 ($p < 0.05$; Fig. 2f) compared with the untreated ALI group.

3.3 iNOS mRNA in Lung Tissue and Nitrite/Nitrate in Plasma

For the evaluation of a relative change in the mRNA expression of iNOS in lung tissue, healthy control animals were used as a reference group with the iNOS gene expression taken as value 1. Thus, values over or below represented higher or lower gene expression. Expression of iNOS in the ALI group increased compared with controls and it decreased in the ALI + SNAP-treated group compared with the untreated ALI group ($p < 0.05$; Fig. 3a). Likewise, nitrite/nitrate concentration, measured in the plasma at the end of experiment, was significantly higher in the ALI group compared with controls ($p < 0.001$) and it decreased in the ALI + SNAP-treated group compared with the untreated ALI group ($p < 0.05$; Fig. 3b).

3.4 Apoptosis in Lung Tissue

The extent of apoptosis of cells in lung specimens was determined by the apoptotic index, i.e., a ratio of number of TUNEL-positive cells/number of DAPI-stained cells. As shown in Fig. 4a, apoptotic index increased in the ALI animals compared with controls ($p < 0.001$) and it decreased in the ALI + SNAP-treated animals ($p < 0.01$). The ALI animals also displayed a higher concentration of caspase-3 in the lung compared with controls ($p < 0.01$). However, in the ALI + SNAP-treated animals a concentration of caspase-3 decreased compared

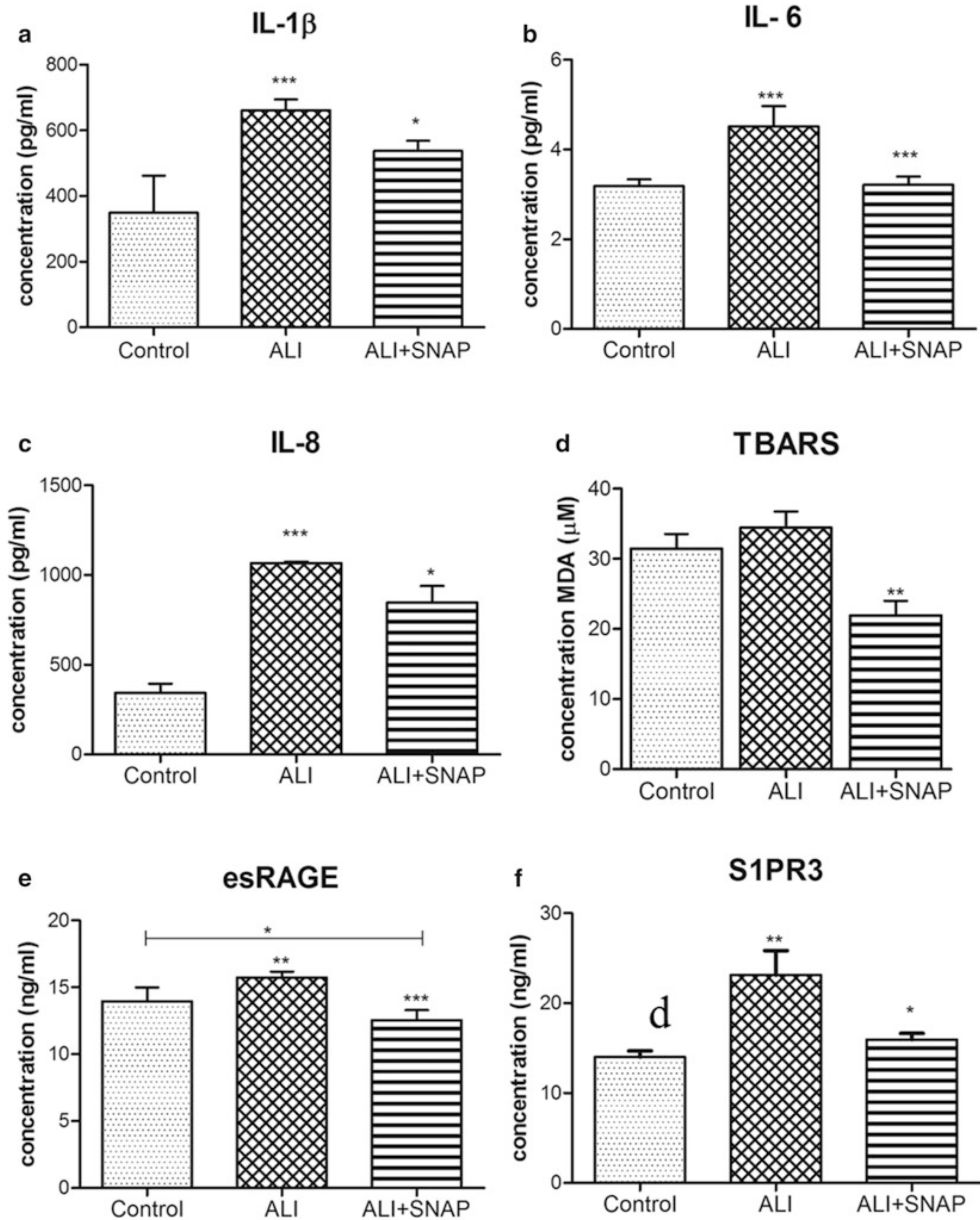

Fig. 2 Markers of inflammation: IL-1β, IL-6, and IL-8 (**a**, **b**, and **c**, respectively), oxidation: TBARS (**d**), epithelial injury: esRAGE (**e**), and endothelial injury: S1PR3 (**f**) injury in lung homogenates. *IL* interleukin, *TBARS* thiobarbituric acid-reactive substances, *esRAGE* endogenous soluble receptor for advanced glycation end-products, and *S1PR3* sphingosine-1-phosphate receptor 3; *p $<$ 0.05; **p $<$ 0.01; and ***p $<$ 0.001 for ALI *vs.* control, ALI *vs.* ALI + SNAP, and control *vs.* ALI + SNAP

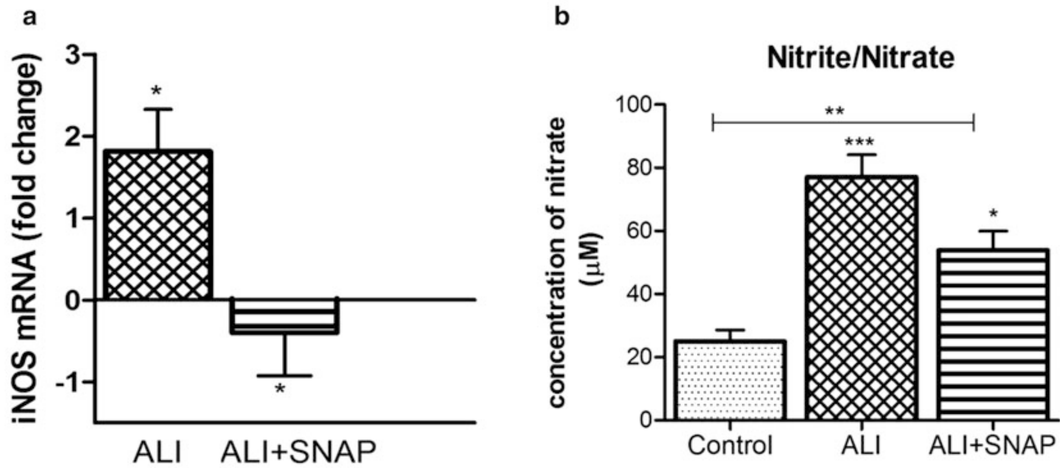

Fig. 3 Changes in the NO pathway: iNOS mRNA expression in lung tissue (**a**) and plasma concentration of nitrite/nitrate (**b**); $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ for ALI *vs*. control, ALI *vs*. ALI + SNAP, and control *vs*. ALI + SNAP

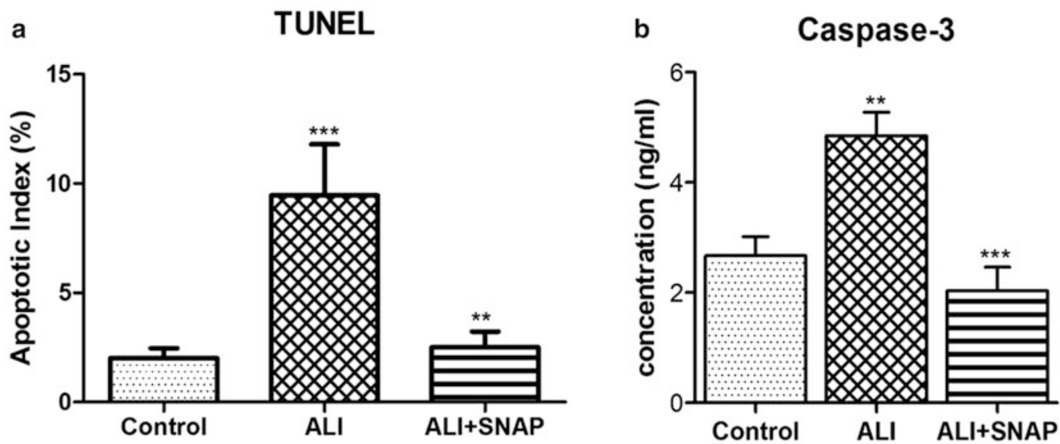

Fig. 4 Apoptotic index of lung cells, expressed as a ratio of number of TUNEL-positive cells/number of DAPI-stained cells (**a**); concentration of caspase-3 in lung tissue (**b**); $**p < 0.01$ and $***p < 0.001$ for ALI *vs*. control, ALI *vs*. ALI + SNAP, and control *vs*. ALI + SNAP

with that in the untreated ALI animals ($p < 0.001$; Fig. 4b).

4 Discussion

Acute lung injury (ALI) may be detected several hours after initial insult. It is characterized by increased vascular permeability, alveolar flooding with protein-rich fluid, diffuse alveolar damage with alveolar haemorrhage, and a massive neutrophil infiltration (Matthay and Zemans 2011; Lu et al. 2005). Activated lung and endothelial cells and leukocytes produce various substances potentially dangerous for the lung cells, causing inflammatory and oxidative/nitrosative changes and facilitating apoptosis. Considering the role of inflammation in ALI, the goal of this study was to evaluate how the treatment with the intratracheal soluble NO

donor SNAP would affect lung injury, inflammation, apoptosis, and the NO pathway in the acute phase of experimentally induced ALI. We found that repetitive saline lung lavage induced obvious migration of neutrophils into the alveolar space and caused their activation, as verified by the increased concentrations of pro-inflammatory cytokines, markers of epithelial and endothelial injury, expression of iNOS and NO metabolites, and the extent of cell apoptosis. The SNAP treatment effectively alleviated all of the inflammatory indices above outlined.

In ALI, dysfunction of the alveolar-capillary barrier enhances the transendothelial diapedesis of leukocytes into lung tissue. In the present study, a higher number of neutrophils was observed in BALF already within 5 h after induction of injury. Activation of neutrophils at the site of injury enhanced the pro-inflammatory cytokines IL-1β, IL-6, and IL-8 in lung tissue. These cytokines are sensitive biomarkers of lung injury also in patients (Bhargava and Wendt 2012). A higher concentration of IL-1β in lung tissue signalizes an injury that may lead to severe and progressive pulmonary fibrosis. The cytokines of IL-1 family stimulate induction of other pro-inflammatory cytokines, e.g., IL-6 and TNF-α, which act in concert with IL-1 to perpetuate inflammation (Kolb et al. 2001).

Beside inflammation, lung injury is characterized by massive damage to epithelial and endothelial cells and formation of lung edema. Injury to alveolar epithelial cells type I is expressed by increased production of endogenous soluble receptor for advanced glycation end products (esRAGE) which is responsible for the propagation of inflammatory response *via* nuclear factor-kappa B (NF-kB), leading to increases in pro-inflammatory cytokines, ROS, and proteases (Uchida et al. 2006). On the other hand, injury to endothelial cells elevates the concentration of sphingosine-1-phosphate receptor 3 (S1PR3) which has been identified as a direct enhancer of vascular permeability both *in vivo* and *in vitro* (Singleton et al. 2006). In line with that, a decrease in S1PR3 is associated with attenuated vascular hyperpermeability *in vivo* (Sun et al. 2012). Endothelial microparticles are complex vesicular structures shed by activated or apoptotic endothelial cells. These microparticles contain enzymes, transcription factors, and mRNA. Endothelial cells release the microparticles after activation by a variety of inflammatory stimuli, including cytokines (Szotowski et al. 2007). The present findings demonstrate that markers of both epithelial and endothelial damage clearly increased in our model of lung injury compared with healthy animals, which underscores the gravity of injury.

In the context of complex changes in acute lung injury, application of exogenous NO seems a rational therapeutic approach. NO may function as an anti-inflammatory mediator. When it is released from NO donors it decreases cytokine-induced endothelial cell activation, inhibits endothelial-leukocyte interactions, and attenuates vascular inflammation (Tsao et al. 1997). On the other hand, NO produced by iNOS in excess can mediate lung injury (Lang et al. 2002). iNOS is not constitutively expressed in healthy tissue and its concentration is regulated mainly at the transcriptional and translational levels. Transcription of iNOS is regulated by various signaling pathways, including the NF-kB pathway. In injury, activated macrophages generate high concentrations of ROS. NO reacting with superoxide anions produces peroxynitrite, which is a highly oxidative species capable of nitrating tyrosine residues of numerous proteins. That leads to the formation of nitrotyrosine, which results in protein inactivation and DNA degradation, fostering cell apoptosis.

In the present study, high expression of iNOS was observed in the lung-injured animals, but treatment with SNAP effectively downregulated iNOS expression. Downregulation of iNOS is one of the mechanisms responsible for anti-inflammation and cell protection (Yang et al. 2004). NO, at a high level, can inhibit iNOS expression in a feedback way in macrophages and can terminate the inflammatory process. The mechanisms underlying this regulation have recently been found to involve S-nitrosylation of the inflammasome protein NLRP3 (Mishra et al. 2013). NO is a feeble, unstable molecule which is rapidly decomposed into nitrites and nitrates; the metabolites measurable in the plasma (Kelm 1999). The present study

demonstrates that plasma concentration of nitrites/nitrates decreased in response to SNAP treatment, which confirms the presence of a lower level of NO.

Apoptosis of cells is induced through two pathways. The intrinsic pathway is activated by mitochondrial ROS. The extrinsic pathway, on the other hand, depends on the action of inflammatory molecules, such as TNF-*α*. TNF-α, activating production of ROS *via* NADPH oxidase, also contributes to the intrinsic pathway (Rossi and Gaidano 2003). Either pathway leads to activation of the initiator caspase-8 or caspase-9, and finally to activation of the effector caspase-3; the latter being responsible for the execution of cell death (Lu et al. 2005). The present findings demonstrate that the concentration of caspase-3 in lung tissue and the apoptotic index in lung tissue specimens increased within first hours after induction of lung injury, indicating the activation of pro-apoptotic processes. NO may have both pro- and anti-apoptotic effects depending on its level. A low level of NO inhibits TNF-α-induced apoptosis, whereas a high level induces apoptosis. S-nitrosylation of caspases by NO may be one of the mechanisms mediating the NO-induced anti-apoptotic effect. Exposure of purified recombinant caspase-3 to NO or NO donor directly inhibits caspase-3-like activity through protein S-nitrosylation (Kim et al. 1997), which may rescue the cell from a suicidal death.

5 Conclusions

In the rabbit model of acute lung injury, intratracheal administration of a soluble NO donor (S-nitroso-N-acetylpenicillamine, SNAP) reduces the migration of polymorphonuclear neutrophils into the lung and their consequent activation, mitigates inflammation, and inhibits iNOS expression and pro-apoptotic pathway.

Acknowledgements The authors thank D. Kuliskova, Z. Remisova, M. Petraskova, and M. Hutko for technical assistance. In addition, we would like to thank for support by grants APVV-0435-11, APVV-15-0075, VEGA 1/0305/14, BioMed (ITMS 26220220187), and UK/28/2015.

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2016) 26: 25–34
DOI 10.1007/5584_2016_24
© Springer International Publishing Switzerland 2016
Published online: 22 June 2016

Combination Therapy with Budesonide and Salmeterol in Experimental Allergic Inflammation

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Abstract

The aim of this study was to determinate bronchodilator, antitussive, and ciliomodulatory activity of inhaled combination therapy with budesonide and salmeterol, and to correlate the results with the anti-inflammatory effect. The experiments were performed using two models of allergic inflammation (21 and 28 days long sensitization with ovalbumine) in guinea pigs. The animals were treated daily by aerosols of budesonide (1 mM), salmeterol (0.17 mM), and a half-dose combination of the two drugs. Antitussive and bronchodilator activities were evaluated *in vivo*. The ciliary beat frequency (CBF) was assessed *in vitro* in tracheal brushed samples, and inflammatory cytokines (IL-4, IL-5, IL-13, GM-CSF, and TNF-α) were determined in bronchoalveolar lavage fluid (BALF). We found that the combination therapy significantly decreased the number of cough efforts, airway reactivity, and the level of inflammatory cytokines in both models of allergic asthma. Three weeks long sensitization led to an increase in CBF and all three therapeutic approaches have shown a ciliostimulatory effect in order: salmeterol < budesonid < combination therapy. Four weeks long ovalbumine sensitization, on the other hand, decreased the CBF, increased IL-5, and decreased IL-13. In this case, only the combination therapy was able to stimulate the CBF. We conclude that a half-dose combination therapy of budesonide and salmeterol shows comparable antitussive, bronchodilator, and the anti-inflammatory effect to a full dose therapy with budesonide alone, but had a more pronounced stimulatory effect on the CBF.

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Keywords
Airway reactivity • Ciliary beat frequency • Cough • Inhaled corticosteroids • Th2 cytokines

1 Introduction

Allergic asthma is a chronic inflammatory disease of conducting airways in which many inflammatory cells (mast cells, macrophages, T-lymphocytes, eosinophils, and neutrophils) and epithelial cells play a role. These cells release a multitude of mediators such as cytokines, chemokines, and growth factors resulting in chronic sustained inflammation that affects the airway function. In allergic asthma, bronchial smooth muscles become more responsive and contract in response to usually harmless particles (Halwani et al. 2011). Many inflammatory mediators have a deleterious effect on the airway epithelium. They stimulate the production of pathological mucus, induce goblet cell hyperplasia, cause epithelial shedding, and alter the ciliary movement. All these changes disrupt the normal function of mucociliary clearance and lead to mucus retention (Erle and Sheppard 2014; Rogers 2004). Resulting mucus airway obstruction significantly contributes to airway responsiveness, clinically manifested by cough, wheezing, and chest tightness (Barnes 2011a).

Since there is no cure for asthma, attenuation of ongoing inflammation is the main target in asthma treatment, achieved mainly by regular administration of inhaled corticosteroids (ICS). Although several new anti-inflammatory drugs, designated against specific inflammatory molecules, are currently in clinical studies, taking into the consideration the complexity of the disease, it is unlikely that blocking a single mediator could provide a better anti-inflammatory spectrum than currently used ICS (Durham et al. 2015). Their regular administration is associated with improvements in symptoms and lung function, and reduces disease exacerbations. The efficacy of ICS demonstrates the dose-response relationship, but their higher doses, more absorbed through the lungs, increase risk of side effects. Thus, one of the goals of asthma treatment is to optimize the use of ICS to increase treatment potency while maintaining side effects at bay (Chung et al. 2009). In this regard, long acting β_2-agonists (LABA), used in asthma due to their bronchodilator activity, have been shown to possess steroid-sparing effect. Adding LABA to ICS therapy seems more effective than simply doubling the dose of ICS. In addition to have a positive effect on asthma symptoms control, ICS may have an additive effect on airway inflammation (Tamm et al. 2012). In the present study, we set out to gain insight into the interaction between LABA and ICS, by examining their effects on several defense mechanisms in animal models of experimentally induced allergic inflammation. We focused attention on a half-dose combination therapy with inhaled budesonide and salmeterol, monitoring the anti-tussive, bronchodilator, and ciliomodulatory effects of such a combination. We further correlated these effects with the level of inflammatory mediators, interleukins (IL) IL-4, IL-5, IL-13, tumor necrosis factor (TNF-α), and Granulocyte-macrophage colony-stimulating factor (GM-CSF) in the bronchoalveolar lavage fluid (BALF).

2 Methods

The study was approved by the Institutional Ethics Committee of Jessenius Faculty of Medicine in Martin, Slovakia (permit IRB 00005636) and all experimental procedures were carried out according the Slovakian and European Community regulations for the use of laboratory animals and guidelines on animal welfare (EU decision

No. 1249/2013). Healthy, adult male TRIK-strain guinea pigs were purchased from the Department of Experimental Pharmacology of the Slovak Academy of Sciences in Dobrá Voda, Slovakia, an accredited breeding facility. The animals were housed under the controlled conditions with access to food and water *ad libitum*.

Drugs and other substances used in the experiment were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Cytokine concentration was determined with a multiplex kit, Bio-Plex Pro Human cytokine Th1/Th2 immunoassay, purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.1 Experimental Groups

The experiments were performed using two models of experimentally induced allergic inflammation, where animals were submitted to 3- or 4-week-long ovalbumine (OVA) sensitization. The animals were randomly divided into several experimental groups, each consisting of eight guinea pigs. In the control group, animals received saline instead of OVA and drug treatments. In the negative control groups, animals were exposed to OVA during 21 and 28 days according to sensitization scheme. There were three therapeutic groups, in which the animals were treated during the ongoing sensitization with aerosols of budesonide (1 mM) or salmeterol (0.17 mM), or their half-dose combination, applied on a daily basis.

Drug solutions were aerosolized by a PARI jet nebulizer (output 5 $1 \cdot s^{-1}$, particles mass median diameter 1.2 μm; Paul Ritzau, Pati-Werk GmbH, Starnberg, Germany) and delivered to the head chamber of a double body plethysmograph (HSE type 855; Hugo Sachs Electronic, March, Germany), where the animals were placed.

Antitussive and bronchodilator activities of inhaled drugs were assessed 24 h after the last exposure, under *in vivo* laboratory conditions. After sacrificing the animals, ciliary beat frequency (CBF) was assessed in tracheal brushed-out ciliary samples and the level of inflammatory mediators (IL-4, IL-5, IL-13, TNF-α, and GM-CSF) were determined in BALF.

2.2 Experimentally Induced Allergic Airway Inflammation

The animals were sensitized to ovalbumin (OVA), applied as a 1 % solution, intraperitoneally, subcutaneously, or by inhalation. For intraperitoneal and subcutaneous administration, OVA was injected together with aluminium hydroxide $[Al(OH)_3]$ (0.5 ml saline solution of 5 mg of OVA in conjunction with 100 g of $Al(OH)_3$ per animal). Three weeks long sensitization was conducted according to the method described by Franova et al. (2013). During the 4 weeks long sensitization, animals were injected with OVA intraperitoneally on Days 1 and 4 and subcutaneously on Days 1, 12, and 20. On Days 15, 18, 20, 22, 24, and 27 of sensitization, animals were exposed to OVA aerosol for 1–3 min.

2.3 Evaluation of Airway Smooth Muscle Reactivity *in Vivo*

Airway smooth muscle reactivity was evaluated in a double chamber plethysmograph (HSE type 855, Hugo Sachs Electronic, March, Germany), consisting of nasal and thoracic chamber. Airway reactivity was assessed from changes in thoracic and nasal airflow induced by 30 s inhalation of the bronchoconstrictor mediator histamine (10^{-6} M). From the resulting phase shift between nasal and thoracic respiratory flows, specific airway resistance (sRaw) was calculated according to Pennock et al.'s (1979) method using the HSE Pulmodyn Pennock respiratory software. sRaw was used as a measure of bronchodilator effects of inhaled drugs.

2.4 Evaluation of Cough Reflex *in Vivo*

Conscious guinea pigs were individually placed in a double chamber plethysmograph. The cough reflex was provoked by inhalation of aerosol of citric acid (0.3 M) for 3 min. A sudden enhancement of expiratory flow during coughing was detected by a pneumotachograph head connected to the nasal chamber of the plethysmograph. PC-recorded changes in expiratory airflow, which were simultaneously accompanied by characteristic cough sound and movement, were regarded as cough efforts. The sound and movement typical for cough reflex were evaluated by two trained observers and verified with video recordings.

2.5 Evaluation of Ciliary Beat Frequency (CBF) in Vitro

After sacrificing the animal, a small window was dissected in a precisely cleaned area of the upper part of trachea to expose the epithelium for a brushing collection of ciliated cells. The brushing method is little invasive, relatively simple, and reliable technique to obtain ciliated epithelium and is an accepted method for studying ciliary function. The material acquired was suspended in a drop of warm saline solution of $36.5 \pm 0.5°$ placed on a microscope slide and was covered. Undamaged strips of ciliated epithelium, with the presence of beating cilia, were selected using an inverted phase contrast microscope (Zeiss Aixo vert. A1; carl Zeiss AG; Göttingen, Germany) and the beating was recorded by a high speed video camera (Basler A504kc; Adept Turnkey Pty Ltd, Brookvale, Australia) with the frame rate of 256–512 frames per sec. The recorded video sequences of beating regions, approximately 10 sequences per sample, were analyzed with Labwiew™ software to identify ciliary regions of interest (ROI). For every ROI, a median of CBF was calculated and used as an evaluation parameter. A final value of CBF, expressed in Hz, was an average of ten median values obtained from each specimen.

2.6 Evaluation of Cytokines in Bronchoalveolar Lavage Fluid (BALF)

BALF was collected by washing out the right bronchus with warm saline (37 °C). The volume of saline introduced to right bronchus was calculated according to animal's body weight of animal (10 ml/kg). The level of IL-4, IL-5, IL-13, TNF-α, and GM-CSF cytokines was determined in the supernatant that was centrifuged for 2 min at $377 \times g$. For the determination and quantification of cytokines we used a commercial Th1/Th2 panel Human Cytokine kit (Bio-Rad; Hercules, California, US) containing all required reagents and antibodies. A simultaneous detection of different molecules is based on fluorescently dyed magnetic beads, having a distinct color code, which are covalently coupled to antibodies directed against the desired biomarkers. Magnetic beads were first incubated with standards, samples, or controls, followed by incubation with capture antibody, and finally with the fluorescent reporter streptavidin-phycoerythrin conjugate. Between each incubation, a series of washes, using Bio-Plex Pro wash station (Bio-Rad, Hercules, California, USA), was performed to remove unbounded proteins. The reaction data were acquired with the Bio-Plex® 200 array system (Bio-Rad, Hercules, California, USA) in which the beads flow in single file through a region illuminated by two lasers. One laser (635 nm excitation) illuminates the fluorescent dye within each bead to provide the analyte identification and the second one excites streptavidin-phycoerythrin conjugate generating a signal used for the analyte quantification. A high-speed digital processor manages data output and Bio-Plex Manager™ 6.0 software presents results as a concentration in pg/ml.

2.7 Statistical Analysis

Data are represented as means ± SE. Statistical analysis was performed using one-way analysis of variance ANOVA. A p-value < 0.05 was taken as a threshold defining the statistical significance.

3 Results

3.1 Cough Reflex

Changes in the cough reflex were evaluated as a change in the number of cough efforts induced by citric acid inhalation. Three weeks long OVA sensitization led to a significant increase of cough. The increase was attenuated after the extension of OVA sensitization up to 4 weeks. In both models of sensitization, all three therapeutic approaches suppressed cough, with the greatest suppression observed in the group receiving a half-dose combination of budesonide and salmeterol (Fig. 1).

3.2 Airway Reactivity – Specific Airway Resistance (sRaw)

Comparing the effects on sRaw of histamine inhalation in two different models of experimental allergic inflammation, the shorter OVA sensitization caused a greater increase in the reactivity of bronchial smooth muscle. However, a significant increase in sRaw was observed in both sensitization models. Also, in both models, chronic treatment with budesonide, salmeterol, or their half-dose combination showed a significant protective effect against bronchoconstriction provoked by histamine inhalation (Fig. 2).

3.3 Ciliary Beat Frequency (CBF)

Three weeks long OVA sensitization caused a slight increase in CBF. In this experimental model, chronic administration of drugs increased CBF in the following increasing order: salmeterol < budesonid < combination of both drugs. By contrast, 4 weeks long sensitization caused a significant suppression of CBF and only a half-dose combination treatment was capable of increasing CBF (Fig. 3).

3.4 Inflammatory Cytokines in Bronchoalveolar Fluid (BALF)

In both models of OVA-induced allergic inflammation, the cytokines IL-4, IL-5, IL-13, TNF-α, and GM-CSF were increased in BALF. The longer 4-week-sensitization was characterized by a marked increase in the level of IL-5 and a drop of

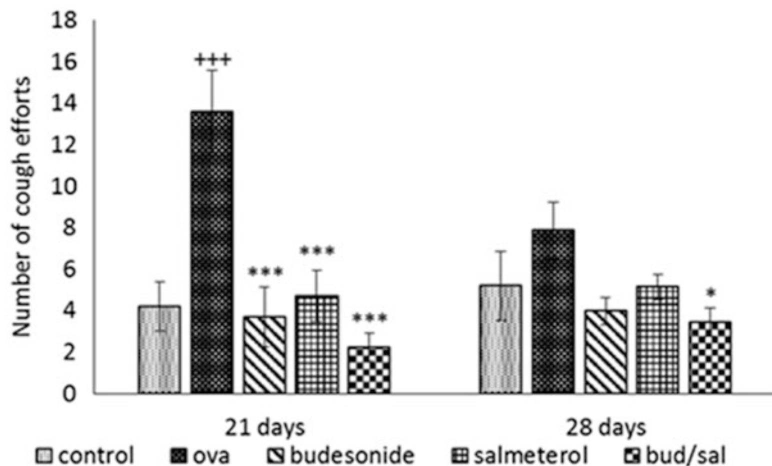

Fig. 1 Changes in number of cough efforts after chronic administration of inhaled budesonide, salmeterol, or their half-dose combination (bud/sal) in experimental allergic inflammation induced by 21 or 28 days long ovalbumin (OVA) sensitization in guinea pigs; +++$p < 0.001$ *vs*. control; ***$p < 0.001$ and *$p < 0.5$ *vs*. OVA

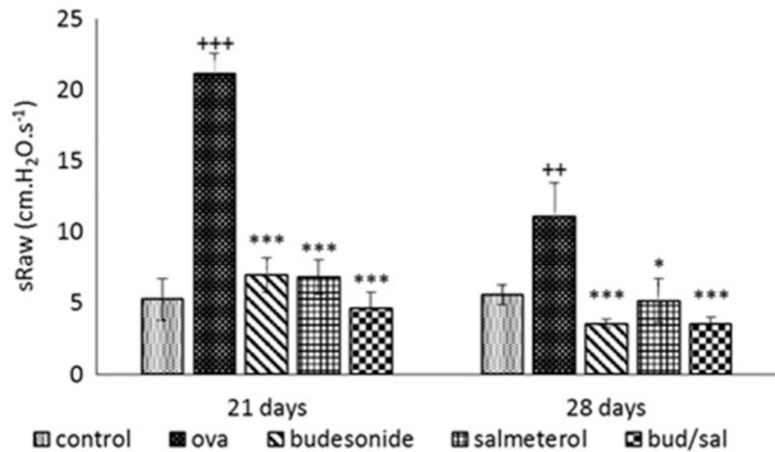

Fig. 2 Changes in specific airway resistance (sRaw) after chronic administration of inhaled budesonide, salmeterol, or their half-dose combination (bud/sal) in experimental allergic inflammation induced by 21 or 28 days long ovalbumin (OVA) sensitization in guinea pigs; +++$p < 0.01$ and ++$p < 0.001$ *vs.* control; ***$p < 0.5$ and *$p < 0.001$ *vs.* OVA

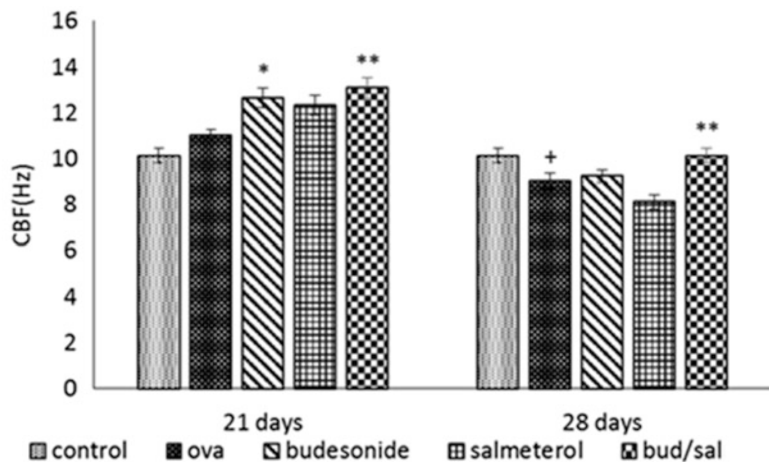

Fig. 3 Changes in ciliary beat frequency (CBF) after chronic administration of inhaled budesonide, salmeterol, or their half-dose combination (bud/sal) in experimental allergic inflammation induced by 21 or 28 days long ovalbumin (OVA) sensitization in guinea pigs; *$p < 0.5$ and **$p < 0.01$ *vs.* OVA; +$p < 0.05$ *vs.* control

IL-13 compared with the shorter 3-week-sensitization. Salmeterol had no effect on the expression of inflammatory markers. Budesonide and a half dose budesonide/salmeterol treatment had a comparable suppressive effect on all cytokines assessed (Fig. 4).

4 Discussion

Chronic combination therapy with ICS and LABA is the recommended approach in the treatment of moderate and severe asthma (GINA 2015). Many studies demonstrated a clinically beneficial interaction of the two drugs, leading to a better control of asthma symptoms, fewer exacerbations, and improved lung function (Koopmans et al. 2006). The goal of the present study was to evaluate the bronchodilator, antitussive, and ciliomodulatory effect of a half-dose combination of budesonide and salmeterol in the context of their anti-inflammatory efficacy. These effects were assessed in two experimental models of allergic inflammation, both characterized by a significant increase in the Th2 cytokines IL-4, IL-5, and IL-13, which are operational in shaping the inflammatory process (Holgate 2011). The major differences in the expression of these cytokines in the two inflammatory models was an augmentation of IL-5 and a reduction of IL-13 in the longer 4 weeks compared with the shorter 3 weeks model.

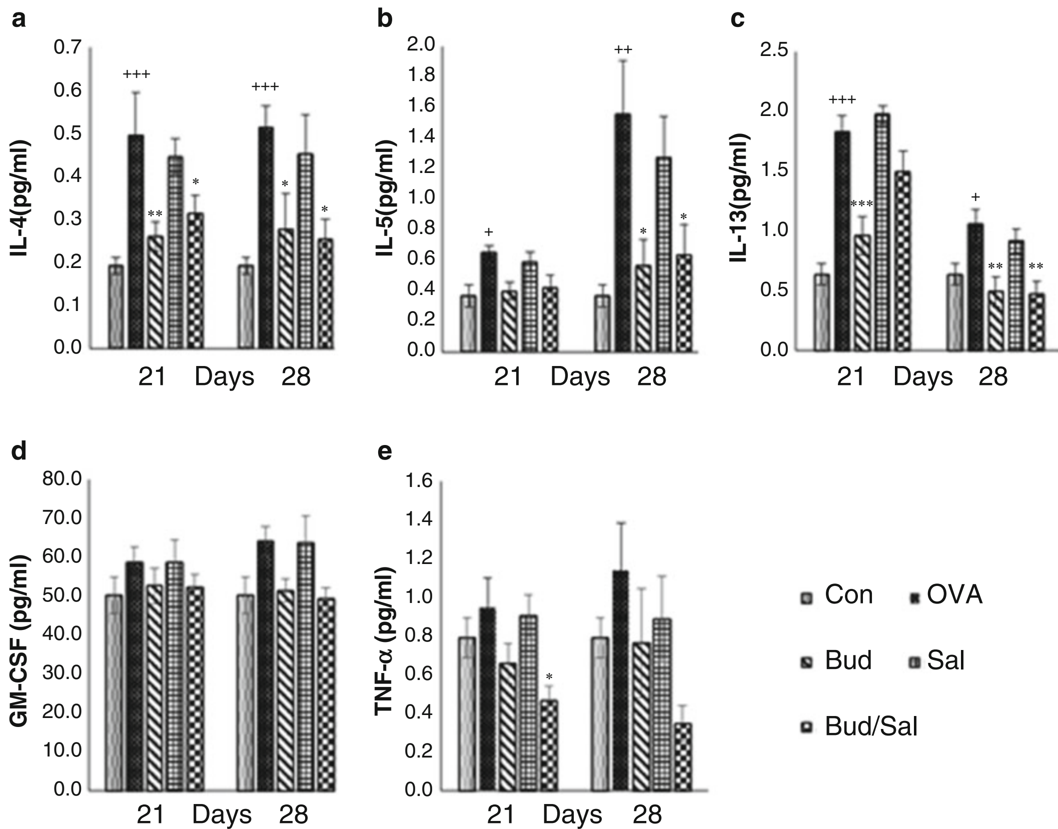

Fig. 4 Changes in the cytokines IL-4 (**a**), IL-5 (**b**), IL-13 (**c**), GM-CSF (**d**), and TNF-α (**e**) after chronic administration of inhaled budesonide (Bud), salmeterol (Sal), or their half-dose combination (Bud/Sal) in experimental allergic inflammation induced by 21 or 28 days long ovalbumin (OVA) sensitization in guinea pigs; $+p < 0.05$, $++p < 0.01$, and $+++p < 0.001$ *vs.* control (Con); $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ *vs.* OVA

Since IL-13 has been identified as a potent bronchoconstrictor and main inducer of airway hyperreactivity (Mattes et al. 2001), its reduction may be associated with the observed decrease in specific airway resistance after 4 weeks long sensitization. Mechanical changes of the airways induced by bronchoconstriction stimulate mechanoreceptors involved in mediation of cough reflex (Mazzone 2005). Thus, a decrease in airway reactivity in the 4 weeks model of allergic asthma may explain the attenuation of the cough reflex. Another possible explanation for less cough may also be the destruction of C-fibers due to persistent inflammation and subsequent depletion of tachykinins, potent cough triggers. Some studies have confirmed that inflammatory changes in airways may actuall decrease cough reflex sensitivity (Franova et al. 2013).

IL-13 in addition to inducing bronchoconstriction may also reduce ciliary movement (Gomperts et al. 2007; Laoukili et al. 2001). In the present study we failed to observe a cilioinhibitory effect when IL-13 concentration was the most. On the contrary, decreased CBF was recorded after 4 weeks OVA sensitization, when IL-13 concentration was not as high. However, in this condition we noted a substantial increase in IL-5 that, along with GM-CSF, acts as a promoter and activator of eosinophils. These cells release toxic substances that have marked cilioinhibitory effect (Thomas et al. 2010). IL-5, rather than IL-13, may plausibly inhibit CBF through the activation of eosinophils. Moreover,

according to the study of Svartengren et al. (1989), mucociliary clearance may be either increased or decreased in asthma. These authors submit that there may be a principal irritation caused by inflammation, which actually leads to an increase in mucociliary clearance, at least in the early stages of asthma. Taking into consideration that CBF is one of the key factors regulating the rate of mucociliary clearance (Braiman and Priel 2008), the present findings of increased CBF in the 3 weeks model and decreased CBF in the four weeks model of allergic asthma are in line with the reasoning above outlined.

Since inflammation plays a key role in the asthma pathogenesis, its suppression is one of the main goals in asthma therapy (Chung et al. 2009). In this regard, ICS represent the drugs of choice whose effects are mediated through the activation of anti-inflammatory (transactivation) genes and transrepression of pro-inflammatory genes (Strehl and Buttgereit 2013). In the present study, budesonide, a representative member of ICS, in monotherapy, significantly reduced the bronchoconstriction of histamine challenge, suppressed the cough reflex in both models of allergic asthma, and led to a slight increase in CBF.

Some anti-inflammatory activity has also been demonstrated in several *in vitro* studies with β_2-agonists. Airway β_2-adrenoreceptors, beside of being widely expressed in airway smooth muscles, have been identified in epithelial cells and in many pro-inflammatory and immune cells, including mast cells, lymphocytes, macrophages, eosinophils, and neutrophils. β_2-agonists suppress the pro-inflammatory activity of the cells above mentioned, mainly by activation of protein kinase A (PKA). Activated PKA phosphorylates and inactivates several proteins involved in the abrupt transient increase in cytosolic calcium, which is necessary for immune cell activation (Theron et al. 2013). A clear anti-inflammatory effect of β_2-agonists observed *in vitro* has not been confirmed *in vivo* due likely to rapid receptor desensitization. As the inflammatory and immune cells express β_2-receptors at a lower density than airway smooth muscles do, these cells are more susceptible to the development of tolerance (Barnes 1999). Although salmeterol did not suppress inflammatory markers in the present study, its protective effect against inhaled histamine-induced bronchoconstriction persisted. It is also well known that β_2-agonists possess ciliostimulatory activity (Wohlsen et al. 2010). In the three weeks model of allergic inflammation salmeterol stimulated CBF, but the stimulation was smaller than that after budesonide alone. In the 4 weeks model, characterized by a marked increase in IL-5, salmeterol's ability to stimulate CBF was abolished.

To avoid the development of receptor tolerance, LABA should be administrated in combination with ICS in chronic use. Glucocorticoids, in the process of transactivation, not only increase the transcription of β_2-receptors, but also improve the receptor coupling leading to better receptor responsiveness after stimulation (Sin and Man 2006). That may be important for the non-bronchodilator effects of β_2-agonists, such as down-regulation of calcium in inflammatory cells. (Theron et al. 2013; Barnes 2011a). On the other hand, β_2-agonists can enhance the anti-inflammatory activity of ICS *via* interaction with the glucocorticoid signal transduction. LABA activate glucocorticoid receptors and enhance transcription of anti-inflammatory mediators (Tamm et al. 2012). In the present study, half-dose combination therapy with budesonide and salmeterol demonstrated a comparable suppressive effect on IL-4, IL-5, IL-13, and GM-CSF as monotherapy with budesonide in a full dose. However, regarding TNF-α, the suppressive effect of half-dose combination therapy was greater than that observed after budesonide alone. This cytokine is released from a variety of cells, including macrophages and epithelial cells, and may be important in amplifying the allergic inflammatory response in severe asthma (Barnes 2011b). Glucocorticoids and LABA alter the expression of TNF-α by differential mechanisms. The former increase the expression of proteins that degrade TNF-α messenger RNA.

The latter directly suppress the synthesis of TNF-α *via* cAMP-dependent activation of guanine nucleotide exchange protein (Epac1). A combination of these two effects, along with increased density of β_2-receptors on target cells and enhancement of transcription activity of budesonide may be responsible for a substantial decrease in TNF-α.

In the present study, combination therapy also demonstrated a beneficial effect on defense mechanisms investigated. It remarkably suppressed the bronchoconstriction of histamine challenge and the number of cough efforts induced by citric acid inhalation. In comparison with monotherapy, half-dose combination therapy stronger enhanced ciliary movement. The enhancement was present only with the combination therapy in the presence of high levels of IL-5 in the 4 weeks model of allergic inflammation. That could be a result of mutual potentiation of anti-inflammatory effect of either drug, along with increased expression of β_2-receptors on the surface of epithelial cells. In addition, enhanced mucociliary clearance accelerates the expulsion of noxious particles (Braiman and Priel 2008) and protects epithelial cells from activation and consequent production of inflammatory mediators (Holgate 2011), which contributes to decreased airway reactivity.

In conclusion, half-dose combination therapy consisting of budesonide and salmeterol demonstrates comparable antitussive, bronchodilator, and anti-inflammatory effects as monotherapy with budesonide. The major advantage of combination therapy seems its enhancing the ciliary movement.

Acknowledgements This work was supported by the Slovak Research and Development Agency under the contract APVV 0305–12; CERK II- Project co-financed from EU sources; grant VEGA 1/0165/14; grant MZ 2012/35-UKMA-12; and opportunities for career growth in research and development in the medical sciences 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 (2016) 26: 35–42
DOI 10.1007/5584_2016_29
© Springer International Publishing Switzerland 2016
Published online: 23 June 2016

Monoclonal Antibodies for the Management of Severe Asthma

Renata Rubinsztajn and Ryszarda Chazan

Abstract

Asthma is a heterogeneous inflammatory disease. Most patients respond to current standard of care, i.e., bronchodilators, inhaled glucocorticosteroids and other anti-inflammatory drugs, but in some adequate asthma control cannot be achieved with standard treatments. These difficult-to-treat patients would be the target population for new biological therapies. At present, omalizumab is the only biological agent approved for the treatment of early-onset, severe IgE-dependent asthma. It is safe, effective, and well tolerated. Also, discovery of asthma subtypes suggests new treatments. Half of patients with severe asthma have T-helper type 2 (Th-2) inflammation and they are expected to benefit from monoclonal antibody-based treatments. The efficacy of the investigational monoclonal antibody mepolizumab which targets IL-5 has been well documented in late onset non-atopic asthma with persistent eosinophilic airway inflammation. Anti-IL-4 and IL-13 agents (dupilumab, lebrikizumab, and tralokinumab) which block different Th-2 inflammatory pathways and agents targeting the Th-17 inflammatory pathway in severe refractory asthma are under development. In clinical trials, these drugs reduce disease activity and improve lung function, asthma symptoms, and quality of life. However, studies on larger groups of patients are needed to confirm their safety and efficacy.

Keywords

Airway inflammation • Asthma • Interleukins • Monoclonal antibodies • Omalizumab • Pulmonary function • Therapy

1 Introduction

Asthma is a chronic inflammatory disease which affects growing numbers of people worldwide. Several biological pathways have been shown to

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play a role in asthma pathogenesis. Exploring the mechanisms involved in inflammation and identifying asthma phenotypes could be a step toward individualized treatment. The pathways leading to inflammation in asthma, especially severe asthma, are only partly understood. Endobronchial tissue gene expression analyzes revealed three major clusters of the disease: Th2 high; Th17 high; Th2/Th17 low (Newcomb and Peebles 2013). Many patients with severe asthma require regular treatment with oral glucocorticosteroids in addition to high-dose inhaled therapy. The long-term use of systemic glucocorticosteroids can be associated with serious and often irreversible adverse effects. It is recognized that different asthma phenotypes respond differently to asthma treatments. Allergic Th-2 driven immunoglobulin E (IgE)-dependent asthma is the most common sub-phenotype. A search for new drugs which would alter the natural history of the disease is underway with the focus on novel biological candidates for use in the treatment of severe refractory asthma.

2 Omalizumab

A relationship between risk of asthma and serum IgE level has been observed. In 2007, approximately 60 % asthma patients in the US were found to have evidence of atopy (Arbes et al. 2007). Omalizumab is the first biological targeted therapy approved for the treatment of IgE-dependent asthma and it is recommended by GINA guidelines as step 5 treatment of severe asthma (GINA Report 2015). Omalizumab (XOLAIR) is a recombinant DNA-derived humanized IgG 1_K monoclonal antibody that selectively binds free IgE in the blood, thereby inhibiting IgE binding to high-affinity IgE receptors (FcεRI) on mast cells and basophils, which mitigates the allergic response. Omalizumab also inhibits binding of IgE to FcεRI on dendritic cells and to FcεRII on lymphocytes T and B, eosinophils, and on other cells (Sarinho and Cruz 2006). This binding reduces the expression of receptors on the effector cells. In MacGlashan et al.'s (1997) study, expression of FcεRI receptors decreased from 220,000 to 8300 *per* basophile after 3 months' omalizumab treatment. Omalizumab is indicated for the treatment of adults and adolescents (12 years of age and older), with moderate-to-severe persistent asthma, who have a total IgE between 30 and 1500 IU/ml and a positive skin test or *in vitro* reactivity to an aeroallergen, when the symptoms are inadequately controlled with a high dose of inhaled corticosteroids.

2.1 Clinical Efficacy

Clinical efficacy of omalizumab in allergic asthma is well documented based on studies in different populations. In the INNOVATE (Investigation of Omalizumab in Severe Asthma Treatment) trial of add-on omalizumab *vs.* placebo, conducted in patients aged 12–75 years with severe asthma, omalizumab significantly reduced the rate of severe asthma exacerbations (0.24 *vs.* 0.48) and emergency visits (0.24 *vs.* 0.43), and significantly improved asthma-related quality of life, morning peak expiratory flow, and the asthma symptoms score (Humbert et al. 2005). In a study by Novelli et al. (2015) in 306 patients under omalizumab treatment for a median of 32 months, good asthma control (according to GINA guidelines) was achieved in 25.2 %, partial control in 47.1 %, and poor control in 24.5 % of patients. The authors found that co-morbidities such as obesity, gastroesophageal reflux disease, aspirin intolerance, and chronic sinusitis or mental disorders could be responsible for an inadequate response to the treatment. In another multicenter, prospective study conducted in the US and Canada, in patients aged 12–75 years, with inadequately controlled asthma despite treatment with a high-dose inhaled corticosteroids (ICS) plus long-acting β2-agonists (LABAs), with or without other controllers, omalizumab for 48 weeks reduced the number of exacerbations and mean daily albuterol puffs, decreased asthma symptoms, and improved results in the Asthma

Quality of Life Questionnaire (AQLQ [S]) compared with placebo (Hanania et al. 2011). In Mexican patients with uncontrolled asthma, three years of omalizumab treatment, improved the result of the asthma control test (ACT) (12.4 *vs.* 20.5 points), increased forced expiratory volume in 1 s of predicted (FEV_1%) (from 66.3 to 88.45 %), and reduced the mean beclomethasone dose (from 1750 to 766 μg/day), emergency room visits, hospitalization, and intensive care therapy (López Tiro et al. 2015). In German adult patients with uncontrolled asthma, six months' treatment with omalizumab had marked beneficial effects on daily and nocturnal symptoms, and the number of exacerbations, unscheduled heath care contacts, and hospitalizations (Korn et al. 2009). In the PERSIST study, the physician-rated global evaluation of treatment effectiveness (GETE) score after 16 and 52 weeks' therapy was good and very good in 82 % and 72 % of patients, respectively. The treatment improved the quality of life and reduced the number of exacerbations compared with the baseline level (Brusselle et al. 2009). The longitudinal EXCELS study has shown that patients on omalizumab *vs.* non-omalizumab therapy experience similar, significant reductions in asthma-related work, school, and activity impairment, regardless of asthma severity (Zazzali et al. 2015). The authors conclude that omalizumab improves asthma control, which becomes evident within 6 months of therapy and persists for five years Omalizumab therapy has reduced the number of emergency visits, hospitalizations, and the dose of systemic and inhaled corticosteroids in asthma patients in other studies as well (Grimaldi-Bensouda et al. 2013; Lafeuille et al. 2012). In a review on safety and tolerability of omalizumab by Corren et al. (2009), which encompassed 15 randomized trials and 7500 patients, severe adverse events have been reported in 10.8 % of patients treated with omalizumab *vs.* 12.6 % in controls and serious adverse events in 4.2 % of patients treated with omalizumab *vs.* 3.8 % in controls. In the placebo-controlled studies, 1.3 % of omalizumab-treated patients and 1.5 % of placebo-treated discontinued therapy. On the other hand, in the standard treatment-controlled studies, more patients treated with omalizumab (3.1 %) than those on standard treatment (0.3 %) discontinued therapy. The most frequently reported adverse events are cold symptoms, sinusitis, cough, sore throat, headache, stomach problems, muscle pain, fever, and insomnia. Anaphylactic reaction occurs in 0.14 % of patients on omalizumab *vs.* 0.07 % in placebo groups. There is no evidence of increased risk of malignant neoplasia or thrombocytopenia by omalizumab (Corren et al. 2009). The numbers of major congenital anomalies, prematurity, low birth weight, and small size for gestational age due to omalizumab therapy noted in the EXPECT registry are consistent with the findings in other asthma populations (Namazy et al. 2015). According to a meta-analysis of 25 trials that evaluated omalizumab as add-on therapy in patients with moderate-to-severe asthma out of control with inhaled corticosteroids, the following observations have been made (Normansell et al. 2014):

- reduction in asthma exacerbation episodes (odds ratio (OR) 0.55, 95 % confidence intervals (CI) 0.42–0.60), ten studies;
- reduction in the number of hospitalizations (OR 0.16, 95 % CI 0.06–0.42);
- significantly more patients are able to completely withdraw ICS (OR 2.50, 95 % CI 2.00–3.13);
- small but statistically significant reduction in a daily dose of ICS, (weighted mean difference – 118 μg) beclomethasone dipropionate equivalent per day, (95 % CI 154–84);
- no difference in the number of patients who are able to withdraw from oral corticosteroid therapy (OR 1.18, 95 % CI 0.53–2.63);
- small but statistically significant reduction in rescue beta2-agonist medication (mean difference (MD) 0.39 puffs per day, 95 % CI 0.55–0.24, nine studies, 3524 participants). This benefit is present in both moderate-to-severe (MD 0.58, 95 % CI 0.84–0.31) and severe (MD 0.30, 95 % CI 0.49–0.10) asthma subgroups on a background ICS therapy.

However, no appreciable benefit is apparent in patients with severe asthma receiving a background therapy of inhaled plus oral corticosteroids.

- significantly fewer serious adverse events (OR 0.72, 95 % CI 0.57–0.91; 15 studies, 5713 patients), but more injection site reactions (9.1 % of omalizumab-treated patients *vs*. 5.6 % of placebo-treated patients).

A meta-analysis performed by Lai et al. (2015) has shown that omalizumab, used for as long as 52 weeks, is well tolerated but lacks a mitigating effect on asthma exacerbations. Omalizumab treatment is associated with higher costs than conventional therapy, but the cost actually pays off in case of severe allergic asthma.

3 Other Anti-IgE Antibodies

Ligelizumab is a humanized anti-IgE antibody that has a 50-fold higher affinity for IgE than omalizumab. This agent suppresses free IgE more than omalizumab does in atopic patients. It may benefit patients unable to receive, or sub-optimally treated with, omalizumab (Arm et al. 2014).

Quilizumab is another humanized monoclonal antibody targeting the M1 prime epitope of human membrane IgE. In allergic rhinitis and mild asthma, quilizumab reduces total and allergen-specific serum IgE; the effect persists for at least 6 months after treatment cessation. In asthma patients, subjected to allergen challenge, quilizumab inhibits generation of new IgE, and reduces allergen-induced early and late airway responses and sputum eosinophila (Gauvreau et al. 2014).

3.1 Mepolizumab

Persistent eosinophilia, the most important factor of the late-onset phenototype of severe refractory asthma, is associated with fixed airway limitation and it correlates with disease severity and recurrent asthma exacerbation. Targeting the eosinophils with anti-IL-5 agents is effective therapy for patients with eosinophilic asthma.

Mepolizumab is a humanized monoclonal antibody of IgG1k type which targets human IL-5 and prevents its interaction with the α-chain of IL-5 receptor (Abonia and Putnam 2011). This agent reduces peripheral blood and sputum eosinophilia; the response also observed after allergen challenge in patients with allergic asthma (Leckie et al. 2000). Treatment with mebolizumab *vs*. placebo in asthmatic patients with eosinophilia and a history of severe asthma exacerbations has been associated with fewer severe exacerbations, improved score of Asthma Quality of Life Questionnaire, and lower eosinophil counts in the blood and sputum (Haldar et al. 2009). Mepolizumab enables a dose reduction of prednisone in patients who have asthma-related eosinophilia despite prednisone treatment (Nair et al. 2009). In a study by Ortega et al. (2014), intravenous and subcutaneous mepolizumab has been compared with placebo effects in 576 patients. Mepolizumab significantly reduced the rate of asthma exacerbations; the reduction was by 47 % with intravenous route and 53 % with subcutaneous route of administration. Emergency visits and hospitalizations also were reduced by 32 % and 61 %, respectively. These positive effects were accompanied by increased mean forced expired volume in 1 s (FEV1), improved control of asthma symptoms, and better quality of life. In another study, mepolizumab had a significant glucocorticoid sparing effect, reduced exacerbations and improved asthma symptoms in 135 patients requiring daily oral glucocorticoid therapy to achieve asthma control (Bel et al. 2014). The DREAM study has demonstrated that mepolizumab reduces the risk of exacerbation in asthma patients with eosinophilia, with the safety profile akin to that of placebo (Pavord et al. 2012). In a meta-analysis by Powell et al. (2015), including eight studies and 1707 participants, mepolizumab has improved the health-related quality of life and reduced asthma exacerbations in patients suffering from severe eosinophilic asthma. The

authors conclude that longer and larger studies with licensed treatment regimens are required to establish mepolizumab's role in asthma therapy (Powell et al. 2015).

3.2 Reslizumab

Reslizumab is a humanized anti-IL-5 monoclonal antibody that disrupts eosinophil maturation and promotes programed cell death. The results of two multicenter, double-blind, parallel-group, randomized, placebo-controlled phase 3 trials which enrolled patients with uncontrolled asthma aged 12–75 years show that reslizumab significantly reduces asthma exacerbations (Castro et al. 2015).

3.3 Benralizumab

Benralizumab targets eosinophils by binding IL-5 receptor α, inducing apoptosis through antibody-dependent cell-mediated cytotoxicity. Single-dose intravenous and multiple-dose subcutaneous benralizumab reduce eosinophil counts in airway mucosa/submucosa, sputum, bone marrow, and peripheral blood (Laviolette et al. 2013). One dose of benralizumab added to the usual management reduces the rate and severity of exacerbations during 12 weeks' observation in patients presented to the emergency unit with acute asthma (Nowak et al. 2015).

3.4 Anrukinzumab/Lebrikizumab/Tralokinumab

Interleukin-13 is involved in several aspects of allergic inflammation such as IgE synthesis, proliferation of bronchial fibroblasts, and recruitment of eosinophils and basophils (Gallelli et al. 2013). Anrukinzumab significantly inhibits allergen-induced late asthmatic response within 14, but not 35, days after its administration (Gauvreau et al. 2011).

Lebrikizumab is a humanized monoclonal antibody that binds to soluble IL-13 with high affinity and blocks signaling through the active IL-4Rα/IL-13Rα1 heterodimer (Ultsch et al. 2013). This agent has been used in asthmatic patients with inadequately controlled disease despite glucocorticosteroid treatment and improved lung function in a subset of patients with a high serum periostin level before onset of treatment (Corren et al. 2011). Lebrikizumab also has reduced the number of exacerbations and improved lung function; the results being again more pronounced in patients with a high periostin level (Hanania et al. 2015).

Tralokinumab is a human IgG4 anti IL-13 monoclonal antibody which is effective in a subset of asthma patients characterized by the highest sputum IL-13 levels (Antohe et al. 2013). In phase 2b study, tralokinumab regimens had an acceptable safety and tolerability profile, but did not significantly reduce asthma exacerbation rates in patients with severe uncontrolled asthma. An improvement in FEV_1 with tralokinumab given every 2 weeks and the results of *post-hoc* subgroup analyzes suggest a positive treatment effect in patients with higher than the population baseline median serum periostatin and dipeptidyl peptidase-4 (DPP-4) concentrations (Brightling et al. 2015).

3.5 Daclizumab

Daclizumab a humanized IgG1 monoclonal antibody against IL-2R chain of activated lymphocytes that improves asthma score and lung function (Busse et al. 2008).

3.6 Dupilumab

Dupilumab is a fully human monoclonal antibody binding to the α subunit of IL-4 receptor. This agent reduces the number of exacerbations by 82 % in patients with persistent moderate-to-severe eosinophilic asthma, increases FEV_1, and improves asthma control despite discontinuation of LABAs and inhaled glucocorticoids. Dupilumab reduces the concentration of

biomarkers associated with Th2-driven inflammation (Wenzel et al. 2013).

3.7 T helper (Th) cytokines

The expression of bronchial tissue genes stratifies asthmatics into Th2 high and Th17 aggregations which are distinctly regulated by IL-13 and IL-17. Neutralization of either interleukin increases the number of Th17 cells and promotes neutrophilic lung inflammation. In contrast, neutralization of both IL-13 and IL-17 has a protective effect on eosinophilia and inflammation. Thus, combination treatment targeting both pathways seems to optimize therapeutic efficacy (Choy et al. 2015). The pro-inflammatory role of Th17 cells is supported by other investigations. Park and Lee (2010) have found that Th17 play a role in airway hyperresponsiveness by recruiting both eosinophils and neutrophils. In sputum of asthmatic patients, expression of IL-17 mRNA correlates with the number of neutrophils (Bullens et al. 2006). Anti-IL-17 agents demonstrate a clinical efficacy in psoriasis (Brown et al. 2015).

3.8 Brodalumab

Brodalumab is a human, anti-IL-17 receptor A (IL-17RA) monoclonal antibody (IgG2). It binds with high affinity to human IL-17RA and blocks the biological activity of the 17A, 17 F, 17A/F, and 17E heterodimers. In a randomized, placebo-controlled study brodalumab in a range of trial doses of 140, 210, or 280 mg has improved the score in the Asthma Control Questionnaire (ACQ) in a subset of patients displaying a high reversibility of obstruction (post-bronchodilator FEV1 improvement $\geq$20 %) and only in the 210 mg subgroup (Busse et al. 2013).

4 Other Candidate Anti-Inflammatory Drugs with Potential Therapeutic Effect in Asthma

In a mouse study, a neutrophil elastase inhibitor has shown a mitigating effect on airway hyperresponsiveness in response to inhaled methacholine, which indicates potential usefulness of this line of agents as modulators of the immune/inflammatory response in both neutrophil- and eosinophil-dominant phases of response to secondary allergen challenge (Koga et al. 2013). In another experimental study in mice, anti-proteinase-activated receptor 2 (PAR_2) antibody has inhibited ovalbumin and cockroach extract-induced airway hyperresponsiveness and airway inflammation, which also might be of potential interest for asthma therapy (Asaduzzaman et al. 2015). IL-33 and its receptor have been found to play a role in the development of asthma and allergic airway inflammation. In a mouse model, vaccination against IL-33 has inhibited house dust mite-induced development of airway hyperresponsiveness and decreased the production of inflammatory cytokines (Lei et al. 2015).

Asthma is a heterogeneous disease underlain by different molecular processes. Most of the studies outlined in this review article have been performed without previous phenotyping of patients; the procedure that is currently an essential precondition for identifying potential responders to targeted therapy, as similar disease symptoms may be due to different pathogenetic mechanisms. Advances in the understanding of asthma pathobiology and in the development of anti-cytokine agents have a great research potential which, when translated into clinical medicine, may cause a turnaround in the global scenario of optimal asthma treatment.

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 (2016) 26: 43–52
DOI 10.1007/5584_2016_30
© Springer International Publishing Switzerland 2016
Published online: 23 June 2016

Cough and Arabinogalactan Polysaccharide from the Bark of *Terminalia Arjuna*

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Abstract

In this work we investigated the antitussive activity of the medicinal tree *Terminalia arjuna*. We used the stem bark for extraction and preparation of water extracted isolate and its two fractions: acetone-soluble (TA-S) and acetone precipitated (TA-P) fraction. The presence of a pectic arabinogalactan was confirmed in TA-P fraction by chromatographic and spectroscopic analysis. The antitussive activity of samples was assessed after oral administration in a dose of 50 mg.kg^{-1} in healthy guinea pigs, in which cough was elicited by inhalation of citric acid (0.3 mol/L) in body plethysmograph. The water extracted isolate showed a significant ability to decrease the number of cough efforts by 64.2 %; the antitussive activity on par with that of codeine phosphate. The TA-P fraction showed the antitussive activity of 54.8 %. In contrast, TA-S fraction had only a mild antitussive activity. No changes in *in vivo* airway resistance were noted. We conclude that arabinogalactan is an essential component of *Terminalia arjuna* that underlies its antitussive action.

Keywords

Antitussive activity • Arabinogalactan • Codeine phosphate • Cough • Plethysmography

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1 Introduction

Cough is a protective reflex which helps keep patent airways. Although cough is vital for maintaining health, the occurrence of cough is often associated with respiratory diseases. Suitable treatment of cough remains an unmet clinical challenge (Dicpinigaitis et al. 2014). Cough can seriously impair quality of life; leading to

vomiting, exhaustion, depression, insomnia, and rib fractures (Irwin et al. 2006). The range of active substances for effective treatment of cough is limited, particularly that the use of most effective of them, opioid antitussives, is disadvantaged by adverse effects (Dicpinigaitis 2004). Thus, a search continues for new, particularly natural, substances with the ability to suppress cough. Natural substances from herbs are the basis of traditional medicines which have been time proven for cough treatment. Such compounds are considered safer than synthetic drugs (WHO 2001).

Terminalia arjuna (Roxb.) Wt. and Arn (Arjuna; family Combretaceae) is a deciduous tree growing abundantly in the Indian subcontinent. The stem bark of *T. arjuna* contains glycosides, large amounts of flavonoids (arjunone, arjuolone, and leteilin), gallic acid, ellagic acid, and phytosterols. It also is rich in minerals, such as calcium, magnesium, zinc, copper, and in carbohydrates (Kumar 2014). The bark, leaves, and fruits are traditionally used in natural medicine. The powder of stem bark was used for treatment of angina pectoris already in ancient times and its cardioprotective activity is the primary reason for regained interest in this plant (Dwivedi 2007). There are, however, other medicinal applications of *T. arjuna* bark extracts such as its being an astringent, aphrodisiac, tonic, or used in therapy of fractures, ulcers, diabetes, tumors, inflammation, skin disorders (Kumar 2014; Paarakh 2010). Some of these activities have already been experimentally approved, e.g., antioxidant and anticancer action (Jain et al. 2009), or anti-inflammatory, immunomodulatory, and antinociceptive action (Halder et al. 2009). The effect of this plant on cough and respiratory system has not yet been investigated.

Polysaccharides are known to be bioactive (Ghosh et al. 2013; Paulsen 2002) having, among others, antitussive activity (Nosáľová et al. 2014; Nosáľová et al. 2013a). However, effects of *Terminalia arjuna* on cough have not yet been studied. In the present study we seek to determine the antitussive activity of water extracted isolate from the stem bark of *T. arjuna*. We hypothesized that any such activity could have to do with the presence of arabinogalactan, a polysaccharide, in the extract. To this end, we investigated two fractions prepared from the isolate, namely the acetone soluble (TA-S) and acetone precipitated (TA-P) fractions.

2 Methods

The study protocol was approved by the institutional Ethics Committee of the Jessenius Faculty of Medicine in Martin, Slovakia (approval no. IRB 00005636). The experiments were consistent with the Slovak and European Community regulations for the use and care of laboratory animals. All possible efforts were undertaken to minimize the suffering of laboratory animals.

The bark of *T. arjuna* was collected from the green garden of medicinal plants of the University of Burdwan in West Bengal, India. Chemical reagents were of analytical grade and were purchased from Sigma-Aldrich (Bratislava, Slovakia) unless otherwise specified. Distilled water was used in all isolation and extraction reactions and other chemical procedures. Codeine phosphate was obtained from Slovakofarma (Hlohovec, Slovakia).

2.1 Isolation and Chemical Characterization of Arabinogalactan

The cut-off of dialysis tube was 12 kDa. Dialysis was performed in double distilled water with continuous stirring, and toluene was added to inhibit microbial growth. Evaporations were performed under reduced pressure at temperature below 50 °C (N-1100 Rotary Evaporator; Eyela, Tokyo, Japan) and concentrated solutions were freeze-dried on a Cool Safe 55-F freeze drier (Scanvac; Lynge, Denmark). Neutral sugar and uronic acid contents were determined colorimetrically using phenol-sulfuric acid (Dubois et al. 1956) and *m*-phenylphenol (el Rayah Ahmed and Labavitch 1977), respectively. Neutral sugars were

quantified after acid hydrolysis (2 M trifluoroacetic acid, 2 h at 100 °C) of TA-P as its alditol acetate derivatives (Blakeney et al. 1983) by gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS) at 70 eV, with inositol as an internal standard, using a Shimadzu QP 5050A GC-MS apparatus (Shimadzu; Tokyo, Japan). The GC–MS conditions were as previously described (Mazumder et al. 2004). Acid-liberated monosaccharides were also analyzed by thin-layered chromatography on a Kieselgel 60 F plate (Merck; Darmstadt, Germany) using nBuOH/$(CH_3)_2CO$/pyridine/H_2O (2/2/1/1 v/v) as an eluent. The detection of monosaccharides was done with sulfosalicylic acid reagent (Ray et al. 1984). UV-VIS spectra were recorded on a UV-2450 spectrophotometer (Shimadzu; Tokyo, Japan). Fourier Transform InfraRed (FT-IR) spectrum of thin films of freeze-dried powder and KBr mixtures was recorded on a Perkin Elmer RX 1 spectrophotometer (Bruker Optics; Kalsruhe, Germany). ^{1}H NMR spectrum was recorded on a Bruker 400 spectrometer (BrukerBiospin AG; Fallanden, Switzerland) in ^2H_2O. All determinations were done at least in duplicate.

2.2 Preparation of Water-Extracted Isolate and Isolation of Acetone Soluble (TA-S) and Precipitated Polymeric (TA-P) Fractions

The bark of *T. arjuna* was air dried and ground by a mixer grinder. Then, 300 g of ground sample was extracted with distilled water (3× 3.0 L, pH 6.5, 14 h) at 4–8 °C. After removing the insoluble residue by centrifugation (10,000 *g*, 15 min), the supernatant was filtered (G 2), evaporated to a small volume, and lyophilized to yield the water extracted isolate (18 g). A part (12 g) of the molecular isolate was then dissolved in water and the solution diluted with acetone (3 × volume). Freeze drying of the solution obtained following the centrifugation yielded an acetone soluble (TA-S, 5.6 g) fraction. The precipitate obtained was dissolved in water, dialyzed and the retained material lyophilized to yield the polymeric fraction named as TA-P (1.4 g).

2.3 Assessment of Chemically-Induced Cough Reflex *in Vivo*

In this part of the study we used 30 healthy adult male guinea pigs (TRIK strain) weighing 250–300 g. The animals were supplied by the Department of Experimental Pharmacology, Slovak Academy of Science, Dobrá Voda in Slovakia. The animals were quarantined for one week before onset of experiments, stayed in cages of 4 in a standard air conditioned system on a 12/12 light cycle schedule, and were provided with food and water *ad libitum*.

The animals were divided into five groups, each consisting of six guinea pigs. Group 1: animals received a vehicle (water for injection) – 1 mL/kg; Group 2 received codeine phosphate – 10 mg/kg, Group 3 received the water extracted isolate – 50 mg/kg, Group 4 received TA-S – 50 mg/kg, and Group 5 received TA-P – 50 mg/kg. In addition, codeine phosphate was used as positive control and water for injection as negative control in comparison with the receiving the extracted isolates of *T. arjuna* bark. All solutions were given *via* the oral route.

Conscious guinea pigs were individually placed in a double chamber body plethysmograph box (HSE type 855; Hugo Sachs Elektronik, Germany) and restricted so that the head protruded into the sealed head chamber. The animals had a 60 min acclimatization session in the body box before the experiment to minimize the stress of a new environment during the experiment. The cough reflex was induced by inhalation of 0.3 mol/L aerosol of citric acid, 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 to the head chamber. We assessed the intensity of the cough response by counting the number of cough efforts during three minutes

long 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, which enabled to differentiate cough from sneezes and other movements. The cough response was evaluated by two independent examiners.

The cough response was assessed at baseline before administration of any agent (denoted by *N* value in graphs) and subsequently 30, 60, 120, and 300 min after administration of an agent. The minimum time elapsing between two consecutive cough responses was two hours to prevent the adaptation of cough receptors to the irritating stimulus (Morice et al. 2007).

2.4 Specific Airway Resistance *in Vivo*

Airway smooth muscle reactivity *in vivo* was expressed as specific airway resistance (sRaw), calculated according to the method described by Pennock et al. (1979). In the method, sRaw is proportional to the phase difference between nasal and thoracic respiratory airflows recorded in the head and thoracic chambers of the plethysmograph. A greater sRaw corresponds to a greater degree of bronchoconstriction. sRaw was recorded at baseline before administration of any agent (*N* value in graphs) and then 30, 60, 120, and 300 min after administration of an agent. The measurement of airway smooth muscle reactivity was made following the cough test after replacing the air in the nasal chamber for fresh air.

2.5 Statistical Analysis

Data are presented as means ± SE and statistical difference between means were compared with one-way ANOVA followed by the Bonferoni *post-hoc* test. A p-value <0.05 defined statistically significant differences. Data elaboration was performed with a commercial GraphPad Prism package (ver. 5.02).

3 Results and Discussion

3.1 Isolation and Chemical Characterization of Polymeric TA-P Fraction Extracted From *T. Arjuna* Bark

In the Indian Ayurvedic system of medicine, decoction of the bark of *T. arjuna* water is traditionally used for the preparation of an herbal remedy for several aliments, including common cold. Therefore, in the present study we investigated water-extracted and freeze-dried isolate from the *T. arjuna* bark. The isolate, containing 6 % (w/w) of the starting bark amount, was made up in 46 % (w/w) from carbohydrates. A 4 % part of it was further separated by acetone precipitation into two fractions: a soluble TA-S fraction and an insoluble TA-P polymeric fraction. The latter, containing 0.47 % of the starting bark weight, was made up in 28 % (w/w) from carbohydrates. An analysis of sugar composition of the TA-P fraction indicated the presence of rhamnose (Rha) (19 %), arabinose (Ara) (39 %), xylose (Xyl) (16 %), and galactose (Gal) (26 %). The uronide content of this fraction was 1.5 % (w/w). Thin-layered chromatographic analysis of the monosaccharides present in the hydrolysate indicates, *inter alia*, the presence of uronic acid with an R*f*-value similar to that of galacturonic acid (GalA). GC analysis of trimethylsilyl derivatives of methyl glycosides confirmed the identity of GalA residue. The FT-IR spectrum of the polymeric TA-P fraction bears resemblance to that of the arabinogalactan type of polysaccharide. The spectrum consists of (i) a broad band around 3358 cm^{-1} originated from stretching vibrations of OH groups in sugar moieties, (ii) a band at 1609 cm^{-1} attributed to carboxylate O–C–O asymmetric stretching, and (iii) a band at 1408 cm^{-1} related to the carbonyl stretching of the carboxylate anion (Fig. 1). Moreover, the shape of the band at 1072 cm^{-1} indicates the presence of an arabinogalactan polysaccharide.

Signals in the ^{1}H NMR spectrum (Fig. 2) of the TA-P fraction were assigned according to the

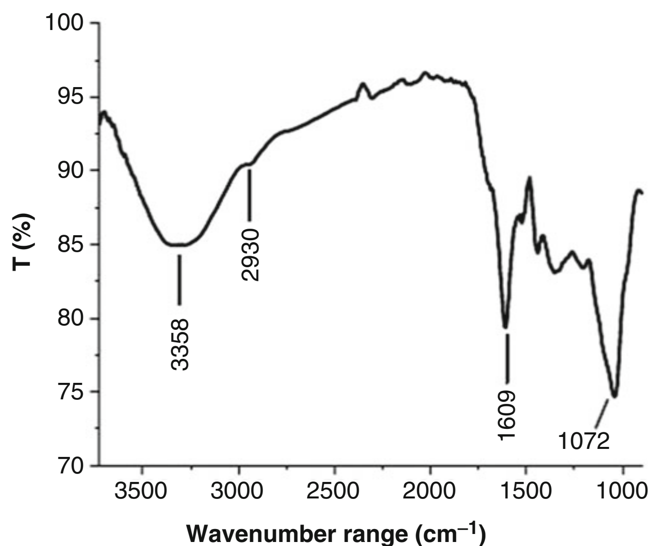

Fig. 1 Fourier Transform InfraRed (FT-IR) spectrum of the polymeric acetone precipitated TA-P fraction isolated from *T. arjuna* bark

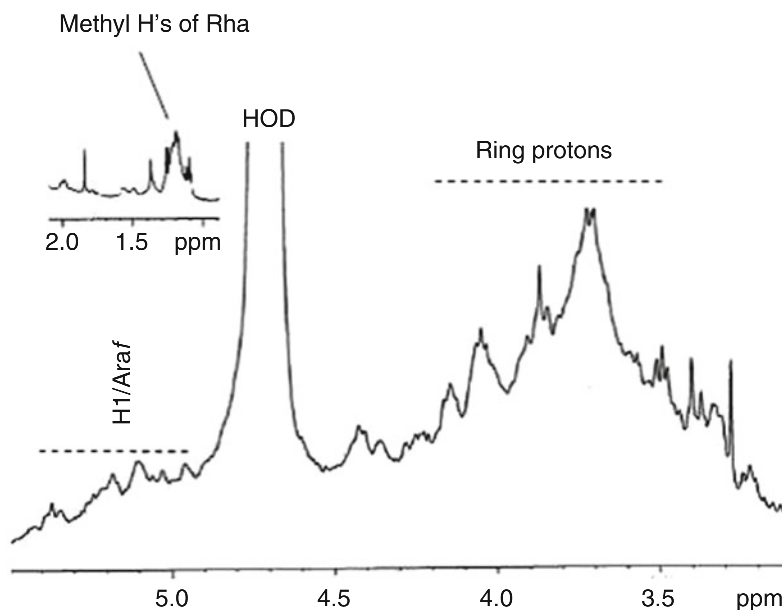

Fig. 2 The proton NMR spectrum at 400 MHz of the polymeric acetone precipitated fraction (TA-P) isolated from *T. arjuna* bark. The spectrum was recorded at 25 °C in D_2O solution. *HOD* signal of a deuterated NMR solvent

sugar composition and data in the literature (Ghosh et al. 2013; Cipriani et al. 2006). The signal in a range of δ 4.91–5.34 ppm was assigned to the anomeric protons (H1) of α-Ara residues. This spectrum also contained signals characteristic of ring protons (H2 – H5) between 3.20–4.35 ppm and of methyl protons H-6; a major one at about 1.19 ppm and a minor envelope of signals at about 1.87 ppm originated from Rha residues.

3.2 Structural Features of Acetone Soluble Fraction (TA-S)

The TA-S fraction, amounting to 1.9 % of the starting bark weight, contained 38 % of carbohydrates. Incidentally, phytochemical fractions, such as triterpenoid, flavonoid, tannin, etc., extracted from this plant have been reported to be responsible for various pharmacological activities (Jain et al. 2009). It is thus likely that

mainly those less hydrophilic from these bioactive compounds can be present in the TA-S fraction. Taken together, it is evident that the water extracted isolate from the stem bark of *T. arjuna* is a mixture containing both the arabinogalactan as well as the acetone soluble phytochemicals above outlined.

3.3 Antitussive Activity of Arabinogalactan Polysaccharide Isolated from *T. arjuna*

The fact that *T. arjuna* is used in the traditional Indian medicine for alleviating cough and other respiratory problems, along with the knowledge that its stem bark is rich, among other phytochemicals, in carbohydrates, led us to the presumption that the plant's polysaccharides could have to do with the cough suppressive activity. The more so that we have already demonstrated antitussive activity of a polysaccharide arabinogalactan protein isolated from another member of the same *Combretaceae* family – the tree *T. chebula* (Nosáľová et al. 2013b). Therefore, in the present study we set out to compare the influence of the water extracted isolate from the bark of *T. arjuna* on cough reflex *in vivo*, employing negative and positive controls.

The negative control was a group of animals in which the vehicle only was administered. Fig. 3 shows that the number of cough efforts (NE) recorded at baseline (N values) and at the time intervals after vehicle administration did not vary significantly. The positive control was represented by another group of animals in which codeine phosphate, in a dose of 10 mg/kg, was the agent administered. Codeine significantly suppressed cough starting off at 30 min from administration. After 60 min, a decrease in NE was evidently pronounced and persisted until the end of measurement.

Peroral administration of the water extracted isolate from the bark of *T. arjuna* in a dose of 50 mg/kg also caused a significant decrease in NE after 30 min. After 60 min, a decrease in NE surpassed that caused by codeine and persisted until the end of measurement, too (Fig. 3). These findings are consistent with those previously demonstrated that water extractable

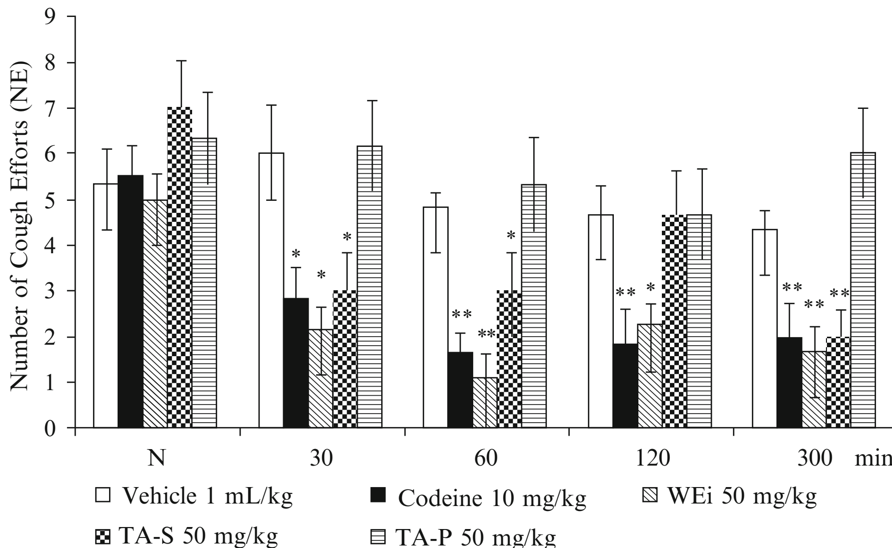

Fig. 3 Influence on the number of citric acid-induced cough efforts (NE) in guinea pigs of perorally administered vehicle, codeine phosphate, crude water extracted isolate (WEi) from the bark of *T. arjuna*, acetone soluble TA-S fraction of the water extract, and acetone precipitated TA-P fraction of the water extract. N – initial values of the number of cough efforts before administration of any agent (Data are means ± SE; *p $<$ 0.05, **p $<$ 0.01 (ANOVA))

polysaccharides have antitussive activity (Nosálová et al. 2013a).

The acetone insoluble TA-P fraction, in a dose 50 mg/kg, in which the arabinogalactan polysaccharide was identified, also significantly suppressed cough. This suppression was similar to that evoked by the crude water extracted isolate, but somehow lower. The TA-P fraction was effective in suppressing cough especially 30 and 60 min after administration; the effect abated at 120 min to bounce back up at 300 min (Fig. 3).

The acetone soluble TA-S fraction yielded different results concerning the influence on cough *in vivo*. This fraction failed to suppress cough significantly at any time point investigated. A slight suppressive tendency appeared only at 120 min after its administration when NE decreased to 4.7 ± 0.8 from the baseline figure of 6.3 ± 1.9 ($p > 0.05$).

For the sake of clear demonstration of antitussive activities of the isolates from *T. arjuna*, they are compared against the 10 mg/kg codeine standard in the Table 1. The crude water extracted isolate reached a slightly higher antitussive activity than was that of codeine. The activity of TA-P and TA-S fractions was substantially different. The acetone precipitated TA-P activity amounted to about 85 % of that of the crude water extracted isolate. In contrast, the acetone soluble TA-S activity corresponded to just about 20 % of the crude isolate.

3.4 Influence of Extracts from *T. arjuna* on Specific Airways Resistance *in Vivo*

The exact relationship between cough and bronchoconstriction remains debatable. Some authors argue that bronchoconstriction triggers cough and thus bronchodilating agents suppress it (Ohkura et al. 2009). In contrast, others argue that although the mechanisms of cough and bronchoconstriction are closely connected, they are in fact two independent defense phenomena of airways (Freund-Michel et al. 2010).

The present study failed to demonstrate a significant effect of the crude water extract from *T. arjuna* and fractions prepared from it on airway reactivity (Fig. 4). Specific airway resistance

Table 1 Percentage of total antitussive activity of crude water polysaccharide extract (WE_i,) acetone precipitated (TA-P) fraction of water extract and acetone soluble (TA-S) fraction of water extract from the bark of *T. arjuna*, compared against the antitussive activity of codeine phosphate; all given perorally in guinea pigs

Agent	Codeine phosphate	WEi	TA-P	TA-S
Total antitussive activity (%)	62.1	64.2	54.8	12.5

Doses: *codeine* 10 mg/kg, *WE_i* 50 mg/kg, *TA-P* 50 mg/kg, *TA-S* 50 mg/kg. Expression of antitussive activity represents an average relative decrease in the number of cough efforts summed up in all time intervals investigated as compared to the baseline number of coughs (N)

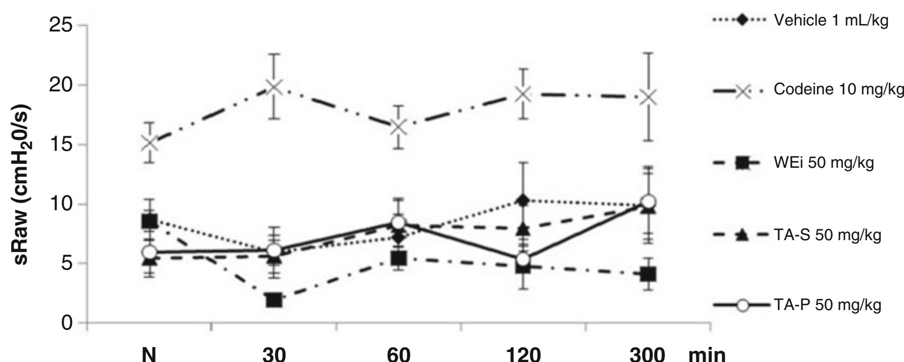

Fig. 4 Specific airway resistance (sRaw) in guinea pigs recorded at baseline (N) and after peroral administration (30, 60, 120, and 300 min) of vehicle, codeine phosphate, crude water extracted isolate (WEi) from the bark of *T. arjuna*, acetone soluble TA-S fraction of the water extract, and acetone precipitated Ta-P fraction of the water extract (*N* initial values of sRaw before administration of any agent. Data are means ± SE)

varied only slightly at the time intervals measured; the effect being not different from that of vehicle. Therefore, we submit that bronchodilation was not part of the antitussive activity of polysaccharides from *T. arjuna*.

4 Summary and Conclusions

In the present study, antitussive activity and chemical features of a water extracted crude isolate and its fractions obtained from the stem bark of *Terminalia arjuna* were investigated. We revealed a high content of carbohydrates in the isolate. The extract showed a significant ability to suppress chemically-induced cough *in vivo*, as assessed from a lower number of cough efforts recorded, compared with baseline. The antitussive activity of the extract surpassed that of codeine, the archetype standard for cough comparison. These results support our previous findings showing that some naturally occurring polysaccharides exhibit considerable antitussive activity (Nosáľová et al. 2013a). We presume that physico-chemical properties of polysaccharides are essential for antitussive activity. Binding a high amount of water into the polysaccharide structures may help keep the epithelial cells of the pharyngeal and epipharyngeal areas of airways rehydrated. That would also enable to create a thin biofilm layer in these areas, which would dampen cough receptor sensitivity. The present findings also oppose the notion that the antitussive activity of arabinogalactan from *T. arjuna* could have to do with bronchodilation.

In an attempt to identify the molecule responsible for antitussive activity we prepared two fractions from the water extracted isolate using the addition of acetone. That provided us with the separation of acetone soluble and insoluble constituents of the extract. The precipitation with acetone is commonly used for sorting out hydrophilic, usually high-molecular weight macromolecules. Indeed, structural analysis of the acetone precipitated TA-P fraction confirmed the presence of pectic arabinogalactan (28 % w/w), with a highly branched structure, and a small amount of uronic acid (1.5 % w/w). Similar types of polysaccharides from different medicinal plants appear actively acting antitussive substances (Nosáľová et al. 2013a).

In the present study, the acetone precipitated TA-P fraction showed a distinct ability to suppress cough. The antitussive activity of TA-P was only slightly lower than that of the crude water extract. Therefore, it is a rational reasoning that pectic arabinogalactan represents an important bioactive part of the water extract from *T. arjuna*, which is to the greatest extent responsible for antitussive activity. Interestingly, the antitussive effect of this arabinogalactan was evident along all of the time intervals studied except the 120 min time mark after administration; the time at which the antitussive activity of the crude water extracted isolate was not aborted. The assumption arose that there were some other constituents in the crude isolate, which may partake in antitussive activity. One of those other constituents seems the acetone soluble TA-S fraction which expressed the strongest, yet statistically insignificant, suppressive effect on cough just at 120 min after its administration. Overall, antitussive activity of TA-S made up only one fifth of the activity of the crude isolate.

It is noteworthy that these two fractions tend to act additively on cough suppression. Although the activity of the whole crude isolate (64.2 %) was not exactly the same as the sum of the activities of both fractions (TA-P + TA-S = 67.3 %), it reached approximately 95 % of this value, which is considerably high. To wrap it up, we can say that we have confirmed that the presence of arabinogalactan polysaccharide in the water extract from the *T. arjuna* bark is essential for its cough suppressive action. However, we have also shown that polysaccharides exhibit their antitussive activity more significantly when they are administered with other constituents. The issue of advantage of chemical complexity of herbs in comparison with conventional drugs is regarded as a pillar in explaining the activity of natural medicine. Additive effects and synergy are important elements in herbal pharmacology in the context of natural chemical complexity of such agents. An action of a chemically miscellaneous mixture is the same as (additive effect), of even

greater (synergy) than, the arithmetical sum of actions of its components (Bone and Mills 2013).

In conclusion, phytochemicals extracted from *Terminalia arjuna* have distinct antitussive activity that is for the most part due to the content of arabinogalactan polysaccharides. Polysaccharides seem a perspective pharmacological target for developing new effective and safe antitussives. Thus, it seems of therapeutic importance to clarify the exact mechanisms of antitussive action of polysaccharides in alternative study designs.

Acknowledgements Supported by grants: UK/148/2015, MZ 2012/35-UKMA-12, APVV-0305-12 and BioMed Martin ITMS 26220220187.

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 (2016) 26: 53–62
DOI 10.1007/5584_2016_31
© Springer International Publishing Switzerland 2016
Published online: 23 June 2016

Bronchodilator and Anti-Inflammatory Action of Theophylline in a Model of Ovalbumin-Induced Allergic Inflammation

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Abstract

Phosphodiesterases (PDEs) represent a super-family of 11 enzymes hydrolyzing cyclic nucleotides into inactive 5′ monophosphates. Inhibition of PDEs leads to a variety of cellular effects, including airway smooth muscle relaxation, inhibition of cellular inflammation, and immune responses. In this study we focused on theophylline, a known non-selective inhibitor of PDEs. Theophylline has been used for decades in the treatment of chronic inflammatory airway diseases. It has a narrow therapeutic window and belongs to the drugs whose plasma concentration should be monitored. Therefore, the main goal of this study was to evaluate the plasma theophylline concentration and to determine its relevance to pharmacological effects after single and longer term (7 days) administration of theophylline at different doses (5, 10, 20, and 50 mg/kg) in guinea pigs. Airway hyperresponsiveness was assessed by repeated exposure to ovalbumin. Theophylline reduced specific airway resistance in response to histamine nebulization, measured in a double chamber body plethysmograph. A decrease in tracheal smooth muscle contractility after cumulative doses of histamine and acetylcholine was confirmed *in vitro*. A greater efficacy of theophylline after seven days long treatment indicates the predominance of its anti-inflammatory activity, which may be involved in the bronchodilating action.

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Keywords
Airway reactivity • Allergic inflammation • Bronchial responsiveness • Guinea pigs • Organ bath • Phosphodiesterase inhibitors • Smooth muscle • Theophylline • Xanthine derivatives

1 Introduction

Theophylline (dimethylxanthine) is considered a main representative of methylxantines used in pharmacotherapy of chronic obstructive diseases associated with inflammation. Due to its relatively low price and availability, theophylline belongs to widely prescribed drugs for the treatment of chronic obstructive pulmonary disease and bronchial asthma. Theophylline occurs in trace amounts naturally in tea leaves and cocoa beans. Originally, it was extracted from tea and later in 1895 it was synthesized chemically. Theophylline was initially used as a diuretic, but due to its potent bronchodilating action, it has been used as a reliever for bronchial asthma since 1922 (Barnes 2013). At therapeutic concentrations, theophylline is a weak nonselective inhibitor of phosphodiesterase isoenzymes (PDEs). It relaxes airway smooth muscles by inhibition of mainly PDE3 activity, but relatively high concentrations of theophylline are necessary for maximal relaxation. Its inhibitory effect on mediator release from alveolar macrophages is mediated by inhibition of PDE4 activity (Ford et al. 2010).

The development of newer anti-asthma medications, especially inhaled steroids, has resulted in a decline in the use of theophylline in the treatment of bronchial asthma. Theophylline is now relegated to the position of a third-line treatment as an additional bronchodilator, indicated only for patients with relatively severe asthma, who are not controlled with high doses of inhaled steroids (Sheffer 1992). Some authors suggest that theophylline may be obsolete, although others emphasize its special beneficial effects, ensuring an important position in asthma management (Barnes and Pauwels 1994). Beside bronchodilation, theophylline has several other anti-asthma activities that may be even more important in the long-term management of asthma. The ‘non-bronchodilating’ effects of theophylline are present already at lower plasma concentrations, which reduces risk of adverse effects; a major limitation of theophylline use in clinical practice. Therefore, the role of theophylline in the management of bronchial asthma should be reassessed.

In the present study we focused on demonstrating the relationship between plasma concentration of theophylline, reached after single or repeated doses, and the pharmacological effects, i.e. *in vivo* and *in vitro* airway reactivity and eosinophilia in blood and bronchoalveolar lavage fluid (BALF).

2 Methods

The study protocol was approved by a local Ethics Committee of the Jessenius School of Medicine in Martin, Slovakia. Experimental procedures were carried out according the Slovakian and European Community regulations for the use of laboratory animals and guidelines on animal welfare (EU decision No. 1249/2013).

Male, adult guinea pigs of TRIK-strain, weighing 250–350 g, purchased from the Department of Experimental Pharmacology of the Slovak Academy of Sciences in Dobrá Voda, Slovakia, were used for the study. The guinea pigs were kept in an animal house and had adequate food and water *ad libitum*. The animals were randomly divided into six groups, each consisting of eight guinea pigs. One of the groups was left without sensitization and served as a control group. The remaining five groups were sensitized with ovalbumine (OVA) for the investigation of airway responsiveness in response to

repeated exposures to OVA antigen. The guinea pigs in one of the sensitized groups were given vehicle only and served as an OVA-sensitized control group. The remaining four sensitized groups were treated with theophylline at the doses of 5, 10, 20, or 50 mg/kg, given i.p. for one day (single doses) or seven days (seven dose repeats) from the 14th day of sensitization. All reagents used in this study were purchased from Sigma-Aldrich (Darmstadt, Germany).

2.1 Ovalbumin-Induced Airway Hyperresponsiveness

Sensitization of animals by OVA, which causes airway reactivity changes on the immunological basis, was performed during 14 days (Franova et al. 2007; Mokry et al. 2009). The allergen (1 % OVA dissolved in *aqua pro injectione*) causes tissue injury, with subsequent structural changes accompanied by inflammation (Tagaya and Tamaoki 2007). OVA was administered intraperitoneally (0.5 mL) and subcutaneously (0.5 mL) on the first day of sensitization, followed by intraperitoneal injection on the third day (1.0 mL). The sensitization was followed by inhalation challenge with 1 % OVA dissolved in saline on the 14th and 21st day. *In vivo* airway reactivity to histamine, a mediator of bronchospasm, was assessed five hours after the OVA challenge and *in vitro* responses to histamine and acetylcholine were investigated after sacrificing the animal. In the theophylline-treated groups, theophylline was injected the last time 30 min before the *in vivo* assessment on the last day. Only the animals with a minimum of 20 % increase in specific airway resistance after OVA challenge on the 14th day were included in further testing.

2.2 *In vivo* Airway Reactivity Assessment

Specific airway resistance (sRaw), a marker of airway reactivity in *in vivo*, was measured in a double chamber whole body plethysmograph (HSE type 855; Hugo Sachs Electronic, March, Germany) according to a method of Pennock et al. (1979). The method is based on the measurement of a time delay between the pneumotachometer signals from the thoracic and nasal chambers. sRaw, calculated from phase displacement between the two chambers, was evaluated within 2 min after inhalation of histamine at a concentration of 10^{-6} mol/L in saline on the 14th and 21st day of sensitization 5 h after administration of OVA. For comparison, reactivity after nebulization of saline was used. Between the investigative tests, fresh air was insufflated into the nasal chamber.

sRaw results were evaluated using HSE Pulmodyn Pennock software. To avoid a potential bias in sRaw interpretation caused by mucosal edema or changes in epithelial secretions, we also analyzed tracheal and lung tissue specimens in *in vitro* condition.

2.3 *In vitro* Airway Reactivity Assessment

After OVA challenge and eventual treatment, the animals were killed by an overdose of anesthetics and exsanguination, and trachea and lungs were immediately excised. Tracheal smooth muscle strips (approximately 15 mm in length) were cut off. Lung tissue strips (2 × 2 × 15 mm) were cut off the upper lobe margin of the left lung. The strips were mounted between two hooks and placed in a tissue bath chamber containing Krebs-Henseleit buffer (NaCl 110.00 mmol/L, KCl 4.80 mmol/L, $CaCl_2$ 2.35 mmol/L, $MgSO_4$ 1.20 mmol/L, KH_2PO_4 1.20 mmol/L, $NaHCO_3$ 25.00 mmol/L, and glucose 10.00 mmol/L in glass-distilled water). The chamber was maintained at 36.5 ± 0.5 °C and aerated continuously with a mixture of 95 % O_2 and 5 % CO_2 to maintain pH 7.5 ± 0.1. One of the hooks was connected to a force transducer and an amplifier, and tension changes were recorded on-line (Experimetria, Budapest, Hungary). Tissue strips were initially set to 4 g

of tension for 30 min (loading phase). Then, tension was readjusted to a baseline value of 2 g for another 30 min (adaptation phase). During both periods, tissue strips were washed at 10 min intervals with pre-warmed Krebs-Henseleit buffer. After the adaptation phase, the contractile response to cumulative doses of histamine and acetylcholine ($10^{-8} - 10^{-3}$ mol/L) was taken as a marker of *in vitro* airway smooth muscle reactivity. Data of tracheal and lung tissue reactivity are shown in grams (g) of the smooth muscle tension (Mokry et al. 2008).

2.4 Determination of Plasma Concentration of Theophylline

Deproteinization of samples was performed by mixing 250 μL of plasma with a double amount of methanol. The mixture was vortexed, centrifuged for 5 min at 14,000 rpm, and filtered. The plasma concentration of theophylline was measured by a high performance liquid chromatography (HPLC) method originally prepared for these experiments. A C8 column with reverse phases, and the eluent methanol:water (25:75) were used. Temperature of the column was kept at 40 °C and the flow at 1 mL/min during the analytical procedure. The volume of feed was 20 μL. The detection was performed using a UV spectrometer.

2.5 Evaluation of Eosinophils Count

Samples of blood were taken from the heart immediately after anesthesia was reached during the exsanguination phase. Bronchoalveolar lavage (BAL) of the right lung was performed twice using pre-heated saline (37 °C) at a dose of 0.01 mL/g body weight. Total white blood cells (WBC) count in blood was determined in Bürker's chamber after staining by Türck's solution. Differential leukocyte count in blood and BAL fluid was evaluated microscopically after panchromatic staining by the May-Grünwald-Giemsa staining and relative counts of eosinophils were determined (in %).

2.6 Statistical Analysis

Data are given as means ± SE. For statistical analysis, one-way ANOVA was used. A p-value <0.05 defined statistical significance.

3 Results

sRaw after nebulization of histamine in OVA-sensitized animals was used as a marker of *in vivo* airway reactivity. OVA-sensitization was increasing sRaw; the increase reached significance on the 21st day of sensitization (Fig. 1).

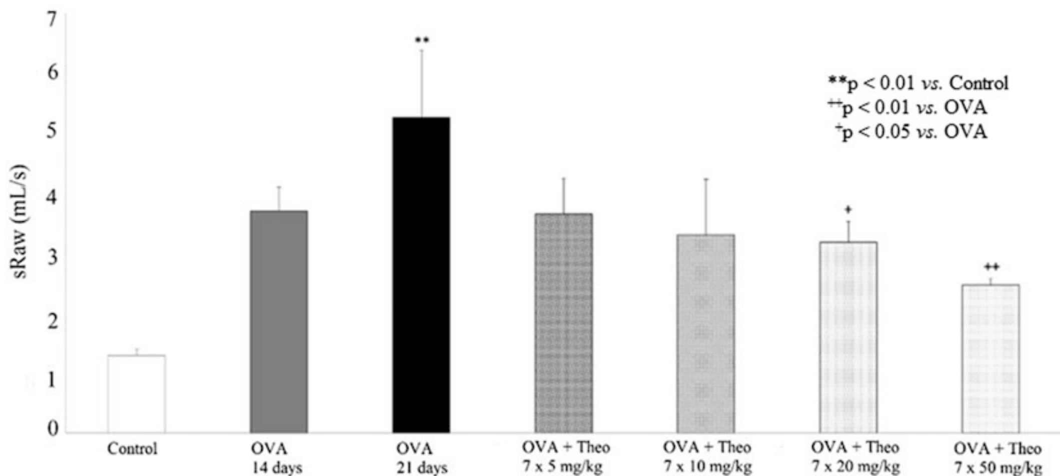

Fig. 1 Changes of specific airway resistance (sRAW) after 7 days' therapy with incremental doses of theophylline (Theo) administered between the 14th and 21st day of ovalbumin (OVA) sensitization in separate experiments

Theophylline administered between the 14th and 21st day of sensitization at the doses of 5, 10, 20, or 50 mg/kg decreased sRaw in response to histamine challenge. The suppression of *in vivo* airway reactivity was evidently significant after the two highest doses of theophylline.

Increases in sRaw caused by OVA sensitization and the subsequent bronchoconstricting challenges on the 21st day were confirmed also in *in vitro* conditions. Both tracheal and lung tissue strips displayed significantly increased reactivity in response to cumulative doses of acetylcholine and histamine compared with tissue strips taken from control naïve animals (Fig. 2). Seven days long treatment with theophylline at the doses of 5 and 10 mg/kg tended to decrease the contractility of airway smooth muscle compared with the sensitized non-treated animals. A significant decrease in contractility was recorded only in tracheal tissue strips after cumulative doses of histamine (Fig. 2b).

Theophylline treatment at the larger doses of 20 and 50 mg/kg led to significant decreases in airway smooth muscle reactivity in both tracheal and lung tissue strips in OVA-sensitized animals. The decreases reached significance predominantly at the lower concentrations of histamine and acetylcholine of 10^{-8} and 10^{-7} mol/L (Fig. 3a and b). Furthermore, theophylline at a dose of 20 mg/kg given for seven days caused a pronounced suppression of *in vitro* airway reactivity compared with a single dose of theophylline (Fig. 3c and d)

OVA sensitization led to a significant increase in the differential count of eosinophils in both blood and BALF. Both single (Table 1) and repeated doses of theophylline (Table 2) suppressed the eosinophils count significantly,

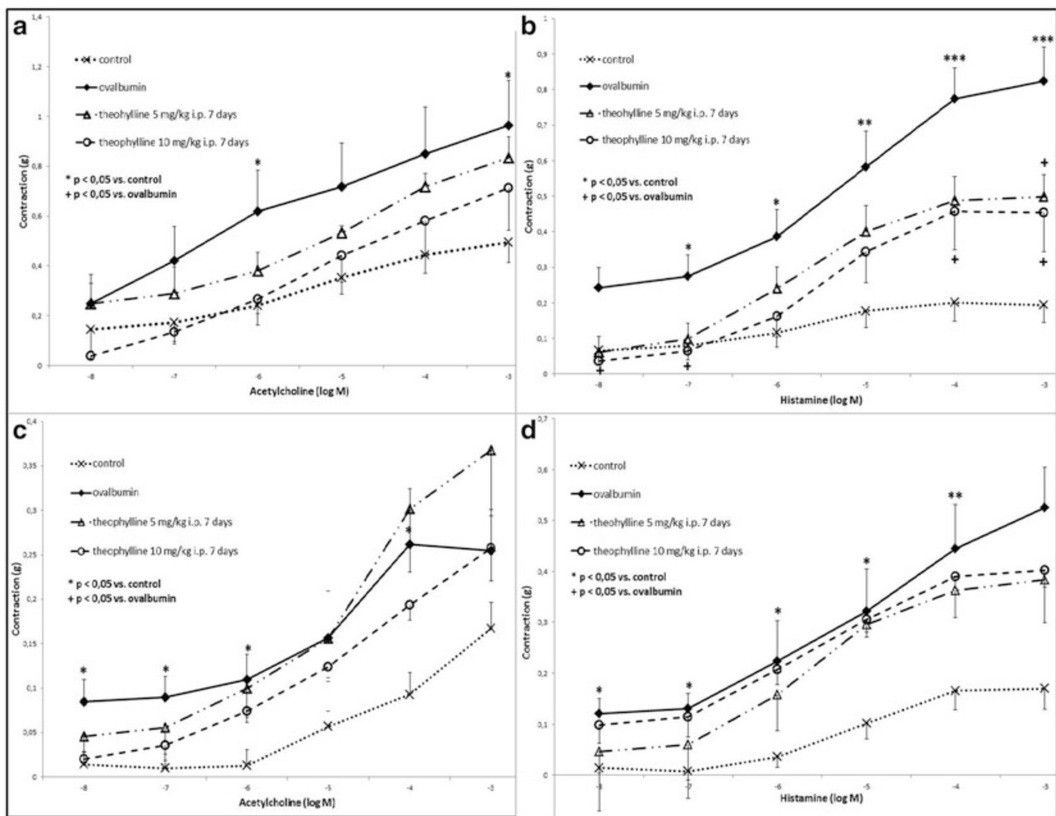

Fig. 2 Changes of tracheal (**a**, **b**) and lung (**c**, **d**) tissue reactivity to cumulative doses of histamine and acetylcholine after 7-day-treatment with theophylline at the doses of 5 mg/kg and 10 mg/kg

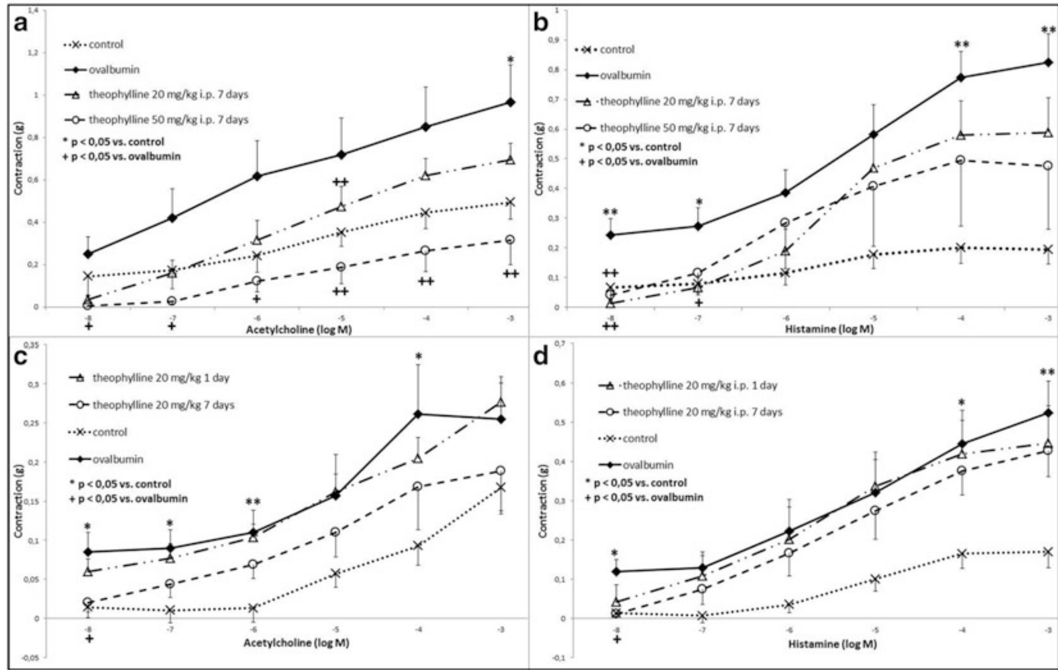

Fig. 3 Changes of tracheal (**a**, **b**) and lung (**c**, **d**) tissue reactivity to cumulative doses of histamine or acetylcholine after 1-day- or 7-day-treatment with theophylline at the doses of 20 mg/kg and 50 mg/kg

Table 1 Absolute number of leukocytes and relative number of eosinophils in blood and BAL after single doses of theophylline

1 dose	Control	OVA	OVA + Theo 5 mg/kg	OVA + Theo 10 mg/kg	OVA + Theo 20 mg/kg	OVA + Theo 50 mg/kg
Blood leukocytes ($\times 10^9$/L)	2.1	3.4	3.4	1.9	2.8	3.3
Blood eosinophiles (%)	0.7	2.2^*	1.4	1.4	1.9	0.8^+
BAL eosinophils (%)	1.2	32.6^*	12.4^+	18.0	14.4^+	8.3^+

OVA ovalbumin, *Theo* theophylline, *BAL* bronchoalveolar lavage fluid
*p < 0.05 *vs.* control, +p < 0.05 *vs.* OVA

Table 2 Absolute number of leukocytes and relative number of eosinophils in blood and BAL after 7-day-long theophylline treatment

7 doses	Control	OVA	OVA + Theo 5 mg/kg	OVA + Theo 10 mg/kg	OVA + Theo 20 mg/kg	OVA + Theo 50 mg/kg
Blood leukocytes ($\times 10^9$/L)	2.1	3.4	2.8	3.7	4.1	3.5
Blood eosinophiles (%)	0.7	2.2^*	0.5^+	0.5^+	1.1^+	1.2^+
BAL eosinophils (%)	1.2	32.6^*	17.2^+	23.1	10.4^+	9.4^+

OVA ovalbumin, *Theo* theophylline, *BAL* bronchoalveolar lavage fluid
*p < 0.05 *vs.* control, +p < 0.05 *vs.* OVA

with the most prominent effect at the highest theophylline dose of 50 mg/kg. However, we observed a potent effect on eosinophils count in blood also at the lowest repeated doses of theophylline of 5 mg/kg.

Theophylline at the dose of 5 mg/kg and 10 mg/kg did not reach the recommended therapeutic plasma threshold for human medicine. There were no significant differences between the concentrations reached after single and

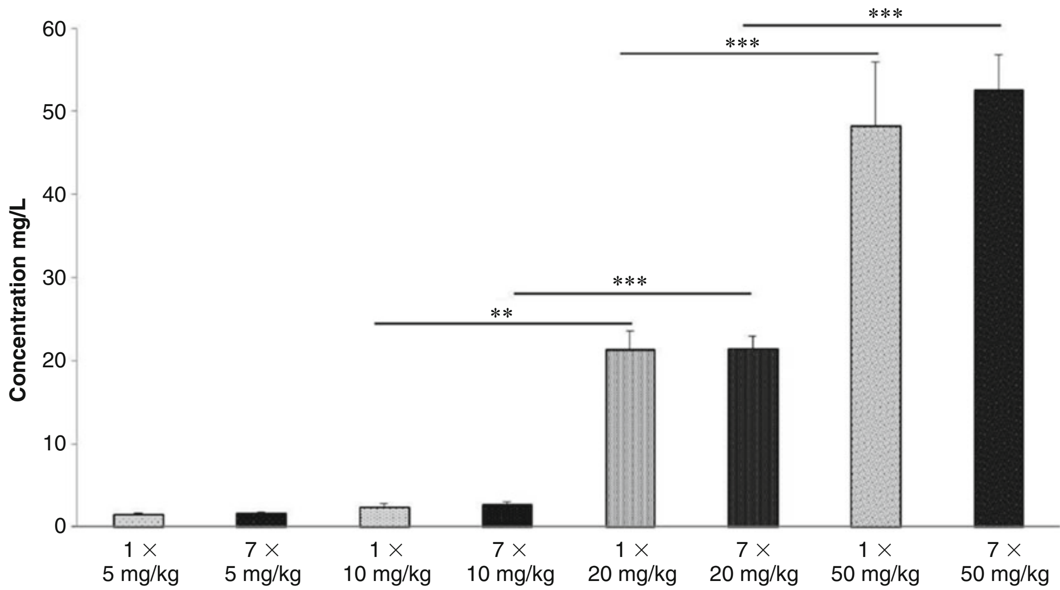

Fig. 4 Plasma concentration of theophylline administered at the doses of 5 mg/kg, 10 mg/kg, 20 mg/kg, and 50 mg/kg; **$p < 0.01$, ***$p < 0.001$

seven repeats of theophylline, irrespective of the dose used (Fig. 4). After the 50 mg/kg dose of theophylline its concentration reached over 50 mg/L in plasma, which was significantly greater than that after the 20 mg/kg dose, which in turn was significantly greater than that reached after the below therapeutic threshold dose of 10 mg/kg.

4 Discussion

Theophylline has been the main representative of non-selective inhibitors of PDEs used in the treatment of chronic obstructive disease and bronchial asthma for several decades. Except for its potent bronchodilating action, other effects of theophylline, including anti-inflammatory and immunomodulatory, have been described. These effects may be observed at plasma concentrations lower than those required for bronchodilation, thereby decreasing the risk of adverse effects which have been the main reason for the regress in theophylline application (Spears et al. 2009). The bronchodilating effect of theophylline is associated with inhibition of PDEs which hydrolyze cyclic nucleotides in the cell, thereby leading to an increase in intracellular cyclic 3′,5′ adenosine monophosphate (cAMP) and cyclic 3′,5′ guanosine monophosphate (cGMP) concentrations (Zhou et al. 2006). The other molecular mechanisms suggested to participate in the pharmacological effect of theophylline include activation of histone-deacetylase (HDAC-2), antagonisms of adenosine receptors, or blockade of calcium channels (Mokry and Nosalova 2011).

The present study confirms that theophylline strongly influences airway hyperresponsiveness in ovalbumin-sensitized and challenged guinea pigs. In both *in vivo* and *in vitro* conditions, theophylline was more effective at higher intraperitoneal doses (20 and 50 mg/kg). The differences between the responses of tracheal and lung smooth muscles are most likely based on a different smooth muscle origin of either tissue, with the presence of around half vascular and half airway smooth muscle in lung tissue (Mokry et al. 2009).

Currently there are several families of PDEs confirmed in the literature (Chung 2006). The differences among single isoenzymes of PDE

are in their organ distribution and function (Mokry et al. 2009; Mokry and Mokra 2013). As mentioned previously, theophylline is a non-selective PDE inhibitor. However, it is unclear what part of the anti-asthma effect of theophylline is dependent on the inhibition of PDE activity. The degree of inhibition is usually insufficient to demonstrate clinically relevant pharmacological effects at theophylline concentrations which are therapeutically relevant (Barnes 2013). In an older study it has been found that PDE activity in human lung extracts is inhibited by some 5–20 % in the therapeutic range of theophylline (Bergstrand 1980). However, this modest inhibition may, in the presence of endogenous activators of adenylyl cyclase, be sufficient to cause a substantial increase in intracellular cyclic nucleotide levels (Polosa and Blackburn 2009). Although adenosine has only a small effect in normal human airway smooth muscle *in vitro,* it causes a constriction of asthmatic airways *in vivo* and bronchoconstriction in asthmatic subjects when given by inhalation (Voduc et al. 2012). The mechanism of bronchoconstriction is indirect and involves release of histamine and leukotrienes from airway mast cells (Björk et al. 1992). This can partly explain our results, where a significant decrease of specific airway resistance as a marker of *in vivo* airway reactivity was accompanied by a relevant *in vitro* airway reactivity suppression only after higher doses of theophylline (20 and 50 mg/kg), which actually led to its plasma concentrations higher than the upper therapeutic range of 15 mg/L. The bronchoconstricting effect of adenosine is prevented by therapeutic concentrations of theophylline (Mann and Holgate 1985). This confirms the fact that theophylline is able to antagonize the effects of adenosine at therapeutic concentrations, but it does not necessarily indicate that this is important for its anti-asthma effect (Polosa and Blackburn 2009). Adenosine antagonism may be responsible for some of the side-effects of theophylline, such as gastro-esophageal reflux, gastric hypersecretion, central nervous system stimulation, cardiac arrhythmias, and diuresis (Barnes and Pauwels 1994).

There is increasing evidence that theophylline has several anti-inflammatory effects at concentrations that are therapeutically relevant. In the present study, a strong suppressive effect on the number of eosinophils in blood at lower plasma concentrations was found after longer-term theophylline administration. This indicates an anti-inflammatory activity of theophylline. Similar results have been reported by Tarayre et al. (1992), who found that theophylline decreases influx of inflammatory cells into the airways of sensitized guinea pigs and rats 24 h after exposure to an aerosolized antigen. In guinea-pigs, treatment with theophylline 5 min and 5 h after aerosol exposure significantly inhibits the eosinophilic infiltration of the airways. In rats, a similar treatment with theophylline inhibits influx of both eosinophils and neutrophils. In another guinea pig model of allergen-induced bronchial hyperresponsiveness, Andersson et al. (1985) have demonstrated that intravenous injection of a low dose of theophylline immediately before the antigen challenge significantly inhibits both the immediate bronchoconstriction and the late reaction. The late reaction in the guinea pig is characterized by influx of neutrophils, eosinophils, and lymphocytes. Pretreatment with theophylline significantly reduces the neutrophilic airway inflammation. Intravenous injection of theophylline 90 min after the antigen-induced immediate bronchoconstriction also has an inhibitory effect on the late reaction (Andersson et al. 1985). In the present model of ovalbumin-induced airway inflammation we confirmed a suppressive effect on eosinophil accumulation in both blood and BALF, which demonstrates a beneficial influence of theophylline on eosinophilic inflammation, typically present in asthmatic patients.

As already mentioned, theophylline at higher plasma concentrations demonstrates numerous adverse effects. Therefore, monitoring its plasma concentration is essential, especially at the beginning of therapy or when adverse effects occur. One of the aims of this study was to evaluate the plasma concentration reached after various doses and dosing regimens of theophylline. The HPLC method used could be considered as being

sufficiently reliable, as the results of plasma samples were relatively uniform in the respective groups of animals.

The recommended therapeutic plasma level of theophylline in humans is in a range of 8–15 mg/L (Barnes 2013). The lower doses used in this study (5 and 10 mg/kg) failed to reach this range. Despite that, we observed several effects in treated animals, which were due likely to the anti-inflammatory and not direct smooth muscle relaxing effect of theophylline. The higher doses used of 20 and 50 mg/kg resulted in reaching the therapeutic range and beyond after both single and repeated doses. As differences between the single and repeated administration regimens were inappreciable concerning the plasma level, we demonstrate that theophylline had no tendency to accumulate in the body and it was fully metabolized. The study was not designed to confirm or exclude side effects of therapy. However, we failed to observe any adverse effects of theophylline, even after the highest dose used in the present study.

5 Conclusions

Theophylline, a non-selective PDE inhibitor, causes a decrease of *in vivo* and *in vitro* airway reactivity and suppresses the accumulation of eosinophils in both plasma and bronchoalveolar lavage fluid in ovalbumin-sensitized and challenged guinea pigs; the effect present at plasma concentrations that are below the lower therapeutic cut-off level. A greater efficacy of longer-term theophylline administration indicates the predominance of its anti-inflammatory action, which may indirectly be involved in theophylline's bronchodilating effect.

Acknowledgments This work was supported by the Slovak Research and Development Agency under the contract No. APVV-0305-12, by grant MZ 2012/35-UKMA-12, grant VEGA 1/0260/14, CEKR 2, and by project UK/131/2015.

This work was supported by the project "Biomedical Center Martin", ITMS code: 26220220187, the project is 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 (2016) 26: 63–73
DOI 10.1007/5584_2016_35
© Springer International Publishing Switzerland 2016
Published online: 30 June 2016

Importance of Social Relationships in Patients with Chronic Respiratory Diseases

Donata Kurpas, Katarzyna Szwamel, and Bozena Mroczek

Abstract

The literature lacks reports on the role of the social relationships domain (SRD) of quality of life (QoL) in shaping care for patients with chronic respiratory diseases in primary care. In this study we examined a group of 582 patients with chronic respiratory diseases and chronic non-respiratory diseases recruited from 199 primary care centers. In the patients with chronic respiratory diseases, higher SRD correlated with more frequent patient visits due to medical issue, fewer district nurse interventions over the past 12 months, less frequent hospitalizations over the past 3 years, and fewer chronic diseases. In these patients, a high SRD was most effectively created by high QoL in the Psychological, Environmental, and Physical domains, and the satisfaction with QoL. Programs for preventing a decline in SRD should include patients with low scores in the Psychological, Environmental, and Physical domains, those who show no improvement in mental or somatic well-being in the past 12 months, those with a low level of positive mental attitudes, unhealthy eating habits, and with low levels of met needs. Such programs should include older widows and widowers without permanent relationships, with only primary education, living far from a primary care center, and those whose visits were not due to a medical issue.

Keywords

Chronic disease • Pulmonary disease • Quality of life • Social isolation • Primary health care • Social support • Social skills • Social distance • Social behavior • Health care system

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1 Introduction

A burden caused by chronic respiratory diseases (CRD) is increasing across the globe, with asthma and chronic obstructive pulmonary diseases (COPD) among the main causes of mortality and morbidity (Ehteshami-Afshar et al. 2016). The analysis of mortality rates in the US in the period of 1969–2013 indicates that the death rate due to COPD increased by 100.6 %, while that from all causes decreased by 42.9 % (Ma et al. 2015). Disability-adjusted life years (DALYs), a measurement of disease burden, increased for both sexes from 537.6 million in 1990 to 764.8 million in 2013 due to population growth and aging (Murray 2015).

Disease does not exist in isolation from health, psychological, and social problems, and the symptom presentation is multifactorial (Starfield et al. 2009). Higher degrees of social integration are associated with lower risks of physiological dysregulation in a dose-response manner in both early and later life. Conversely, a lack of social connections is associated with vastly elevated risk in specific life stages. For instance, social isolation increases risk of inflammation by as much as physical inactivity in adolescence, and the effect of social isolation on hypertension exceeds clinical risk of such factors like diabetes in old age. The physiological impact of the structural and functional dimensions of social relationships emerges in adolescence and midlife, and persists into old age (Yang et al. 2016).

The Global Initiative for Asthma defines clinical manifestations of asthma to include symptoms, sleep disturbances, limitations of daily activity, impairment of lung function, and use of rescue medications (GINA 2015). Dyspnea is a cardinal symptom in patients with COPD, which, together with fatigue, limits patients' daily domestic activities, physical activity, and health status (Nakken et al. 2015). CRD symptoms negatively affect patients' activities, professional lives, intimate lives, and hobbies (Kupryś-Lipińska and Kuna 2014; Wong et al. 2014), leading to loneliness and social isolation (Schroedl et al. 2014), often accompanied by depression and anxiety (Panagioti et al. 2014). The isolation of CRD patients may also result from insufficient knowledge and false beliefs concerning the disease and treatment. This points to the need for informative support for this group (Wong et al. 2014). A lack of support, difficult personal, family, or financial situations, helplessness about symptoms and effects of the disease, and negative perception of one's life situation are psychosocial reasons behind mental disorders in some of these patients (Andrysz and Merecz 2012). All that leads to worse health outcomes, longer hospitalization, impaired health-related quality of life (QoL), and increased mortality risk in patients with asthma and COPD (Panagioti et al. 2014; Ross et al. 2013).

Studies show that CRD patients also suffer from concomitant conditions such as cardiovascular diseases, osteoporosis, denutrition, obesity, aging, anemia, sleeping disorders, diabetes, metabolic syndrome, cancer (Brinchault et al. 2015), hypertension, gastroesophageal reflux, arthritis, Alzheimer's disease, allergic rhinitis, and glaucoma (Ross et al. 2013). Social isolation may intensify existing symptoms and generate new health problems. In humans, deficits in social relationships, social isolation or low social support, can lead to chronic activation of immune, neuroendocrine, and metabolic systems that lie in the pathways to cardiovascular, neoplastic, and other common aging-related diseases (Yang et al. 2016). Socially isolated individuals are at increased risk of the development of cardiovascular disease, infectious illness, and cognitive deterioration. Social isolation has been associated with elevated blood pressure, C-reactive protein, fibrinogen, and with heightened inflammatory and metabolic responses to stress (Steptoe et al. 2013).

In COPD patients, positive social support is associated with fewer hospitalizations and acute disease exacerbations, better health status, and better health-promoting and disease-managing behaviors, such as quitting cessation and performing physical exercise. As indicated,

CRD patients require social support, but negative experience in this area (e.g., unsympathetic support, failures to help) are associated with increased anxiety (Dinicola et al. 2013). Thus, the role of the patients' environment, family, friends, and physicians, is to give support and demonstrate areas in which they can be active, which helps ascertain their feeling of being in control (Andrysz and Merecz 2012).

The literature lacks reports on the role played by the social relationship domain (SRD) of QoL in shaping care for patients with chronic respiratory diseases in primary care. Such information is of key importance for a tailored holistic healthcare model in modern medicine. Therefore, in the present we seek to determine the components that shape SRD in patients with chronic respiratory diseases and to identify the most important differences in these components between patients with chronic respiratory diseases patients and those with other chronic diseases.

2 Methods

The research followed the Declaration of Helsinki and was approved by the Bioethical Commission of the Medical University in Wrocław (approval no. KB-422/2014). The main inclusion criteria were age (at least 18 years old) and diagnosis of at least one chronic respiratory disease.

The study group consisted of 582 adult patients with chronic diseases: 291 with chronic respiratory disease (CRD) and 291 with chronic non-respiratory disease (CnRD); the latter constituted a control group for CRD. The CnRD patients had a sociodemographic structure akin to that in the CRD group. The sociodemographic structure was described by five two-category variables: gender, age (younger/older than median), marital status (married/unmarried), place of residence (urban/rural), and education (primary or vocational/secondary or higher). The median age of the patients of both groups was 65 (min–max: 18–92 years). Participants were recruited from the patients of 199 general practices between June 2014 and April 2015.

QoL was assessed using the Polish version of the WHO Quality of Life Instrument Short Form (WHOQOL-BREF), which measures QoL in four domains: Physical, Psychological, Social relationships (SRD), and Environmental. Answers to all questions, including two questions on satisfaction with QoL and health, are given on a five-point Likert-type scale. The reliability of the Polish version of the WHOQOL-BREF questionnaire, measured using Cronbach's α coefficient, proved acceptable for the parts that evaluate each domain (coefficients ranging from 0.81 to 0.69) and for the questionnaire as a whole (coefficient 0.90).

The authors also used Juczyński's Health Behavior Inventory, consisting of 24 statements measuring four categories of prohealth behavior: healthy eating habits, preventive behavior, positive mental attitudes, and health practices. Respondents mark the frequency of the healthy behavior and the correct healthy activity: 1: almost never; 2: rarely; 3: from time to time; 4: often; and 5: almost always. The sum of results from all four scales gives the score for the general health behavior (range 24–120); the higher the score, the healthier the behavior. The inventory's internal consistency, measured using Cronbach's α, equals 0.85.

The patient's adaptation to life with disease was assessed using the Acceptance of Illness Scale, which contains eight statements on the negative consequences of the health state, each statement being rated on a five-point Likert-type scale: 1 denotes poor adaptation to a disease and 5 its full acceptance. The score for illness acceptance is the total of all points and can range from 8 to 40. Low scores (0–29) indicate a lack of acceptance and adaptation to the disease and intense feeling of mental discomfort. High scores (35–40) indicate acceptance of the illness, manifesting as a lack of negative emotions associated with the disease. Cronbach's α was 0.85 for the Polish version and 0.82 for the original version.

The levels of met and unmet needs were assessed using the Modified Short Camberwell

Needs Assessment. This questionnaire focuses on 22 problem areas for patients with chronic somatic diseases (without severe mental disorders); 0 denoted unmet needs and 1 satisfied needs. Next, 24 questions present 22 needs and inquire whether they are met (1) or unmet (0). In this way, the number of satisfied needs (M) of the total (N) needs could be established and the Camberwell index calculated as M/N. The internal consistency of the assessment is $\alpha = 0.96$.

The somatic index was calculated for each patient. Somatic symptoms reported by patients were assigned values from 1 (occurring once a year) to 7 (constant symptoms). The index was calculated by adding the values assigned and dividing by 49 (the highest possible score for somatic symptom frequency).

The service index was calculated by summing the services received and dividing by the number of types of services received during visits to doctors over the past 12 months.

2.1 Statistical Elaboration

The Shapiro–Wilk test showed none of the 52 variables to be normally distributed. Medians and variability ranges (extremes) were calculated for measurable (quantitative) variables; for qualitative variables, the frequency (percentage) was determined. Analysis of qualitative variables was based on contingency tables and the chi-squared test or on Fisher's exact test for count data. The numerical variables were grouped into several ranges. The relationship between SRD and other variables was analyzed by Cramér's *V* coefficient and Spearman's rho rank correlation coefficient. The *V* coefficient was calculated using categorized variables, and the R correlation coefficient using source variables (alternatively, non-numerical variables are coded with numbers; for example, 'no' is replaced by '0' and 'yes' by '1'). A significant linear relationship (*r* significantly different from '0') was confirmed by the significant strength of the relationship (the V coefficient being significantly different from '0'). Where there was no linear relationship, the strength of the relationship was insignificant. Therefore, 26 variables significantly correlating with SRD were selected for hierarchical cluster analysis, whose aim was to divide the initial group of variables into clusters of variables that correlate similarly with SRD. In the cluster analysis, a feature by which the objects will be classified needs to be chosen. We selected the variable correlation degree with SRD, measured wtih the *r* correlation coefficient. The absolute value of the difference between correlation coefficients was taken as a measure of a distance between variables. Thus, the distance is close to '0' between variables with similar correlation coefficients and higher between variables with different correlation coefficients. The division into classes was performed using Ward's method. Variables were divided into four clusters (Fig. 1).

To analyze differences between the CRD and CnRD groups, a test of significance for the difference between two correlation coefficients, based on independent groups, was applied. The contribution of some variables to higher SRD scores was determined using the odds ratio. Patient groups with above-median and below-median variable values were created. A 95 % confidence interval was set for each odds ratio. The critical level of significance *p* was set as 0.05 for all tests. R 3.1.3 (for Mac OS X 10.10.5) statistical software was used for all analysis.

3 Results

The majority of patients had 65 and more years of age (153, 52.6 %), were from urban areas (163, 56.0 %), and were females (150, 51.5 %). Detailed sociodemographic data on the patients and their chronic diseases are shown in Table 1. The variables chosen for CRD patients are shown in Table 2.

3.1 Significant Correlations

Patients with low QoL in SRD more often were of advanced age ($r = -0.33$, $p < 0.001$),

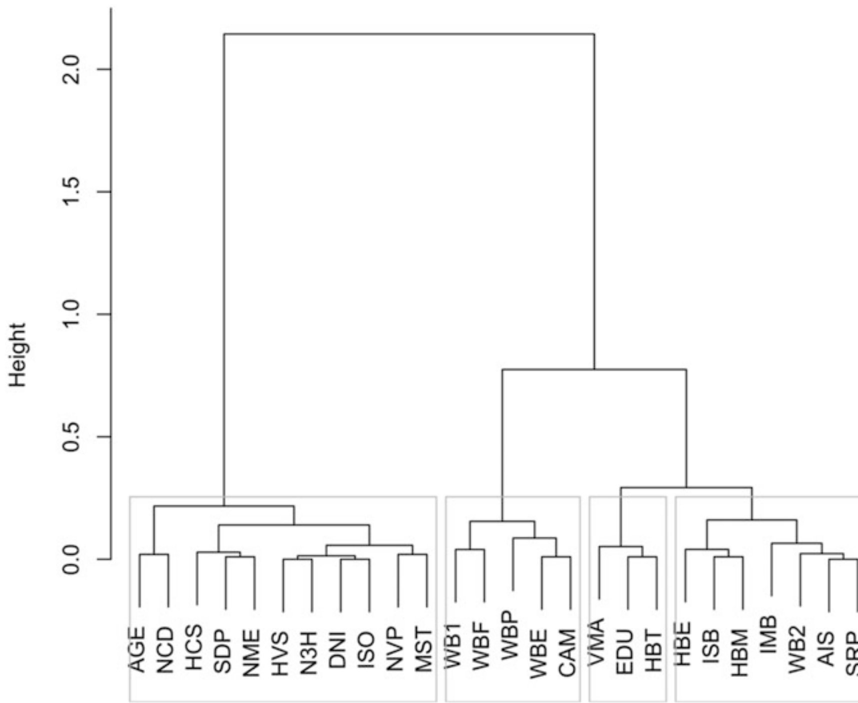

Fig. 1 Hierarchical cluster analysis for social relationships domain (SRD) of quality of life (QoL) in patients with chronic respiratory diseases (CRD)
AGE age, *AIS* level of illness acceptance, *CAM* Camberwell index, *DNI* number of district nurse interventions in past 12 months, *EDU* education, *HBE* level of healthy eating habits, *HBM* level of positive mental attitudes, *HBT* pro-health behaviors (total), *HCS* health care services index, *HVS* number of home visits in past 12 months, *IMB* improvement in mental well-being in past 12 months, *ISB* improvement in somatic well-being in past 12 months, *ISO* somatic index, *MST* marital status, *N3H* number of hospitalizations in past 3 years, *NCD* number of chronic diseases, *NME* number of medications, *NVP* number of visits to primary care center in past 12 months, *SDP* patient's subjective assessment of distance from primary care center, *SRP* stable relationship/without a partner, *VMA* today's visit to the doctor concerned medical issue, *WB1* satisfaction with QoL, *WB2* satisfaction with quality of health status, *WBE* QoL in Environmental domain, *WBF* QoL in Physical domain, *WBP* QoL in Psychological domain

widowed ($r = -0.19$, $p < 0.001$), lacked a permanent relationship ($r = 0.34$, $p < 0.001$), had only primary education ($r = 0.19$, $p < 0.001$), lived, in their opinion, far from primary care centers ($r = -0.15$ $p = 0.030$), and lower number of visits to the family physician were due to medical issue ($r = 0.15$, $p = 0.044$).

Patients with low QoL in SRD more often had low illness acceptance ($r = 0.34$, $p < 0.001$), were dissatisfied with QoL ($r = 0.50$, $p < 0.001$), were dissatisfied with health ($r = 0.36$, $p < 0.001$), had low QoL in the Physical ($r = 0.54$, $p < 0.001$), Psychological ($r = 0.67$, $p < 0.001$), and Environmental domain ($r = 0.59$, $p < 0.001$), had lower levels of healthy behaviors ($r = 0.20$, $p = 0.009$), including proper healthy eating habits ($r = 0.25$, $p < 0.001$) and positive mental attitudes ($r = 0.28$, $p < 0.001$), had a low level of satisfied needs (low Camberwell index) ($r = 0.60$ $p < 0.001$), and had a high somatic index ($r = -0.24$, $p < 0.001$).

Low QoL in SRD was accompanied by more frequent visits to a family doctor in the past 12 months ($r = -0.21$, $p = 0.010$), more home visits ($r = -0.23$, $p < 0.001$), more district nurse interventions ($r = -0.24$, $p < 0.001$), more hospitalizations in the past 3 years ($r = -0.23$, $p = 0.001$), a lack of improvement in somatic ($r = 0.29$, $p < 0.001$) and mental

Table 1 Sociodemographic data and ICD-10 diagnoses[a] of patients[b] with chronic respiratory diseases (CRD) and chronic non-respiratory diseases (CnRD)

		CRD, n = 291		CnRD, n = 291	
		n	%	n	%
Gender	Women	150	51.5	150	51.5
	Men	141	48.5	141	48.5
Age	24 and below	8	2.7	10	3.4
	25–44	29	10.0	29	10.0
	45–64	101	34.7	101	34.7
	65–84	136	46.7	140	48.1
	85 and above	17	5.8	11	3.8
Place of residence	Rural area	128	44.0	128	44.0
	Urban population				
	Below 5000	28	9.6	36	12.4
	5000–10,000	14	4.8	18	6.2
	10,000–50,000	55	18.9	49	16.8
	50,000–100,000	22	7.6	28	9.6
	100,000–200,000	21	7.2	18	6.2
	Over 200,000	23	7.9	14	4.8
Education	Primary	77	26.5	55	18.9
	Vocational	84	28.9	106	36.4
	Secondary	70	24.1	71	24.4
	Post-secondary	31	10.7	32	11.0
	Higher	29	10.0	27	0.3
Marital status	Single	33	11.3	39	13.4
	Married	186	63.9	186	63.9
	Separated	3	1.0	3	1.0
	Divorced	8	2.7	6	2.1
	Widowed	61	21.0	57	19.6
Diagnosis	J45 Bronchial asthma	112	38.5	–	–
	J44 Other chronic obstructive pulmonary diseases	96	33.0	–	–
	J42 Unspecified chronic bronchitis	39	13.4	–	–
	J43 Pulmonary emphysema	37	12.7	–	–
	J41 Chronic simple and mucous purulent bronchitis	37	12.7	–	–
	J47 Bronchiectasis	13	4.5	–	–
Most common coexisting diseases (top 5)	I10 Primary hypertension	111	38.1	114	39.2
	M47 Spondylosis	96	33.0	52	17.9
	I70 Atherosclerosis	56	19.2	65	22.3
	E10 Insulin-dependent diabetes mellitus			50	17.2
	E11 Non-insulin-dependent diabetes mellitus			43	14.8
	I11 Hypertensive heart disease	33	11.3		
	M15 Osteoarthritis of multiple joints	32	11.0		
Comorbidity	1 Chronic disease	53	18.2	78	29.8
	2 Chronic diseases	52	17.9	73	27.9
	3 Chronic diseases	51	17.5	43	16.4
	4 Chronic diseases	54	18.6	26	9.9
	5 Chronic diseases	28	9.6	19	7.3
	>5 Chronic diseases	53	18.7	23	8.8

[a]Some patients were diagnosed as having at least two pathological entities
[b]The figures in column *n* do not sum to 291 due to gaps left in the questionnaires by patients

Table 2 Variables for patients with chronic respiratory diseases (CRD)

Variables	Median	Min–Max
Number of chronic diseases	3.0	1.0–15.0
Body mass index (BMI) (kg/m^2)	2.3	15.6–41.0
Somatic index	0.4	0.0–1.0
Camberwell index	0.8	0.2–1.0
Disease acceptance level (points)	26.0	8.0–40.0
WHOQOL-BREF (points)		
Satisfaction with QoL	4.0	1.0–5.0
Satisfaction with health state	3.0	1.0–5.0
Social Relationships Domain	14.1	4.0–20.0
Environmental Domain	13.5	7.5–19.5
Physical Domain	13.1	4.0–19.4
Psychological Domain	12.7	4.0–19.3
HBI (points)		
Total pro-health behaviors	86.0	31.0–116.0
Preventive behaviors	3.8	1.5–5.0
Positive mental attitudes	3.7	1.5–5.0
Health practices	3.6	1.5–5.0
Healthy eating habits	3.3	1.0–5.0

WHOQOL-BREF World Health Organization QoL Instrument Short Form, *HBI* Health Behavior Inventory

well-being ($r = 0.40$, $p < 0.001$); a high index of health care services ($r = -0.17$, $p = 0.010$), a greater number of medications taken ($r = -0.14$, $p = 0.007$), and a greater number of chronic diseases ($r = -0.31$, $p < 0.001$). Table 3 shows statistically significant differences between CRD and non-CRD patients depending on their QoL SRD and other variables.

3.2 Results of Hierarchical Cluster Analysis

Hierarchical cluster analysis is shown in Fig. 1 and Table 4.

3.3 Odds Ratios for Chosen Variables

In the CRD patients with:

- more frequent visits due to medical problems, the odds of a higher SRD score (above the median of 14.7) are 1.95 (95 % CI 1.05–3.61) times greater;
- a lower number of visits of a district nurse in the past 12 months (equal to the median of 0), the odds of a higher QoL level in SRD (above the median of 14.7) are 2.52 (95 % CI 1.49–4.27) times greater;
- less frequent (below the median of 1) hospitalizations in the 3-year period, the odds of higher QoL in SRD (above the median of 14.7) are 2.04 (95 % CI 1.24–3.34) times greater;
- a lower number of chronic diseases (below or equal to the median of 3), the odds of a higher QoL in SRD (above the median of 14.7) are 1.97 (95 % CI 1.21–3.24) times higher.

4 Discussion

Social relationships are central to human well-being and are critically involved in the maintenance of health. Social isolation is one measurable reflection of insufficient social contact. Elderly people run the greatest risk of this, due to decreasing economic resources, mobility impairment, and the death of contemporaries; all of which conspires to limit social contact (Steptoe et al. 2013). In the present study, we show that low SRD more often occurred in patients of advanced age, who were widowed, and who lacked a permanent relationship. Moreover, we show that in patients with chronic respiratory diseases, higher QoL in SRD was more common among younger patients, as opposed to patients with chronic non-respiratory where no such relationship was observable. Ng et al. (2015) have found that living alone, irrespective of age, sex, socioeconomic, marital, and health status, is associated with increased overall mortality among older Singaporeans. The impact on mortality of living alone appears stronger among men and those who were single, divorced, or widowed, rather than married. Older persons who live alone are recognized to be a vulnerable group that requires special attention.

Table 3 Results of significance test for differences between two correlation coefficients based on independent groups of chronic respiratory diseases (CRD) and chronic non-respiratory diseases (CnRD) patients

Variables	CRD			CnRD			r_{CRD} *vs.* r_{CnRD}
	n	*r*	p	n	*r*	p	p
Age	291	−0.33	<0.001	291	−0.11	0.055	0.005
Number of chronic diseases	291	−0.31	<0.001	270	−0.09	0.162	0.007
Visits to doctor due to medical issue	193	0.15	0.044	272	−0.06	0.351	0.026
Number of district nurse interventions in the past 12 months	291	−0.24	<0.001	291	0.02	0.734	0.002
Number of hospitalizations in the past 3 years	291	−0.23	<0.001	291	0.00	0.961	0.005
Average waiting time at doctor’s office (min)	200	0.05	0.445	279	−0.17	0.004	0.018
Number of private medical service fees in the past 12 months	47	0.12	0.407	51	−0.33	0.017	0.026

r_{CRD} correlation coefficients in the group of CRD patients, r_{CnRD} correlation coefficients in the group of CnRD patients

Table 4 Hierarchical cluster analysis for patients with chronic respiratory diseases (CRD)

Variables	*r*	p
Strongest positive correlation with SRD		
QoL in Psychological domain	0.67	<0.001
Camberwell index	0.60	<0.001
QoL in Environmental domain	0.59	<0.001
QoL in Physical domain	0.54	<0.001
Satisfaction with QoL	0.50	<0.001
Weaker positive correlation with SRD		
Improvement in mental well-being in the past 12 months	0.40	<0.001
Satisfaction with quality of health status	0.36	<0.001
Level of illness acceptance	0.34	<0.001
Being in a permanent relationship	0.34	<0.001
Positive mental attitudes	0.28	<0.001
Improvement in somatic well-being	0.29	<0.001
Healthy eating habits	0.25	<0.001
Weakest positive correlation with SRD		
Level of healthy behaviors	0.20	0.009
Education	0.19	<0.001
Visits to doctort due to medical issue	0.15	0.044
Negative correlation with SRD		
Age	–0.33	<0.001
Number of chronic diseases	–0.30	<0.001
Number of district nurse interventions in the past 12 months	–0.24	<0.001
Somatic index	–0.24	<0.001
Number of home visits in the past 12 months	–0.23	<0.001
Number of hospitalizations in the past 3 years	–0.23	0.001
Number of visits to a primary care center in the past 12 months	–0.21	0.010
Marital status	–0.19	<0.001
Health care services index	–0.17	0.010
Patient’s subjective assessment of distance from primary care center	–0.15	0.030
Number of medications	–0.14	0.007

r correlation coefficients, *p* level of significance, *SRD* social relationships domain, *QoL* quality of life

Numerous studies have shown that elderly people living alone in the community are characterized by difficult living situations, limited resources, and a lack of support (Haslbeck et al. 2012). Likewise, Steptoe et al. (2013) have found that both social isolation and loneliness are good predictors of mortality over a 7 years long follow-up in a national sample of older patients.

A study Ross et al. (2013) has indicated that living alone is associated with an increased number of unscheduled visits to the primary care physician or asthma specialist, and older age is associated with more hospitalizations. That suggests that a more integrative, nontraditional approach is needed in older adults with asthma, treating not only the physical aspects of asthma, but also its psychological and social aspects. The present findings are in line with that reasoning as we show that higher SRD scores correlated with less frequent hospitalizations in CRD, but not CnRD, patients. Additionally, in CRD patients hospitalized less frequently over the past 3 years, the odds of a higher SRD score were more than twice as great. Interestingly, we show that SRD was not associated in CRD patients with the waiting time at the doctor's office or the expense of private medical care. However, greater SRD was significantly associated with a lower expenditure for private treatment and a shorter waiting time in CnRD patients.

The present study shows that CRD with low SRD scores patients paid visits to the doctor's office more often and they did so for nonmedical issues as well. That may reflect the search for emotional or informative support by CRD patients. In this group, higher SRD scores correlated with more frequent visits for medical reasons, while in the CnRD group, no significant relationship was seen between these variables. Further, in CRD patients with a higher number of visits for medical problems, the odds of a better SRD score were nearly twice as high. In these patients, higher SRD scores also correlated with a smaller number of visits from a district nurse in the past 12 months, while there was no significant association between these variables in CnRD patients.

Age of a patient seems the main determinant of a type of social support required. As reported by Strzelecka et al. (2015), there is a fundamental difference in the demand for emotional support in various age groups: patients aged 30–44 indicate a much lower demand for emotional support than those aged over 45. The main reason why patients over 65 visit a primary care doctor is to receive emotional support; younger patients principally expect information on the disease and treatment. Similar results have been reported by Kemicer-Chmielewska et al. (2015) who show that most respondents (68.4 %, 182) seek emotional support from doctors; the most common reasons for which were to hear the doctor's explanation of psychological problems (61.3 %, 163) and to get reassurance in a difficult moment (63.9 %, 170). Meanwhile, a study by Kurpas et al. (2013) in chronically ill patients shows that only 69.9 % of family doctors explain the results of tests, 68.1 % explain treatment methods, 67.7 % explain the cause of symptoms, 50.0 % ask about the symptoms of a disease, 42.9 % help patients cope with health-related fears and concerns, 27.3 % show interest in the patient's material situation, 24.0 % show interest in the patient's personal situation (specifically the source of social support), and 23.2 % show interest in other members of the patient's family. As reported by Sarver et al. (2002), when primary health care does not satisfy patients' needs, visits to Emergency Departments are more frequent, even for nonurgent cases. Those data are consistent with the findings of the present study.

The hierarchical cluster analysis in the present study shows that SRD had the strongest positive correlation with the Psychological Domain of QoL. A study of Medinas-Amorós et al. (2012) conducted among 80 patients with COPD, indicates a significant relationship between the level of stress, as measured by the Hospital Stress Rating Scale (HSRS), and social support. The average stress level among patients without social support is higher than among those with support. Further, lack of positive social interaction entails higher HSRS scores. Lee et al. (2013) have suggested that better psychological outcomes for COPD patients are associated

with greater personal resources, and some of these relationships are mediated by coping strategies. A problem-oriented coping partially or fully mediates the relationship of the knowledge of a disease and perceived social support depression and anxiety.

The findings of the present study show that patients with low SRD scores more often had lower levels of healthy behaviors, including proper healthy eating habits and positive mental attitudes. Holm et al. (2010) have indicated an association between social relationships, psychological distress, and unhealthy behaviors. They show that unsupportive family relationships are associated with psychological distress, which may be accompanied by increased cigarette smoking. Shankar et al. (2011) have shown that loneliness and social isolation may independently affect health through the effect on healthy behaviors. In addition, social isolation may also affect health through biological processes associated with the development of cardiovascular disease. Social isolation brings higher risk of cardiovascular (Barth et al. 2010) and infectious diseases (Cohen et al. 1997). This is confirmed by the findings of the present study, showing that CRD patients with fewer chronic diseases had almost twice as high a chance of better SRD scores. Among the CRD patients, higher SRD scores correlated with fewer chronic diseases, whereas no such relationship existed in CnRD patients.

Both social isolation and loneliness are associated with greater risk of being inactive, smoking, and reporting multiple health-risk behaviors. Social isolation is also positively associated with blood pressure, C-reactive protein, and fibrinogen levels (Shankar et al. 2011). Satisfactory relationships affect physiological responses to stress, decreasing activation of the autonomic nervous system and hypothalamic–pituitary–adrenal axis associated with increases in blood pressure, obesity, hypercortisolemia, and dyslipidemia (Makaya et al. 2009).

The present study has some limitations. Participation in the study was voluntary. Thus, enrollment into the study may have been somewhat biased toward participants who were potentially satisfied with primary health care and in good health. Future research should analyze the relationship between patients' heterogenic multimorbidity and SRD. Such data might help identify unsatisfied social needs of patients and their family members. Also, family problems were not taken into consideration in our study. Given our findings, such an approach might be essential for treatment efficacy (Kupryś-Lipińska and Kuna 2014).

5 Conclusions

Among patients with chronic respiratory diseases, high quality of life in the social relationship domain is correlated with lower level of health care utilization and is most effectively produced by high quality of life in the Psychological, Environmental, and Physical domains and by satisfaction with quality of life. Satisfaction with the quality of health state and acceptance of illness are also of great importance. Programs for preventing a decline in the social relationship domain should include patients with low scores in the variables above outlined, patients who do not show improvement in mental and somatic well-being during the preceding 12 months, those lacking positive mental attitudes and healthy eating habits, and those with a low level of their needs being met. Additionally, such programs should include older widowed people without permanent relationships, those with only primary education, living far from primary care centers, and those who pay visits to doctors for nonmedical issues.

Conflicts of Interest The authors have no financial or other relations that might lead to a conflict of interest.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2016) 26: 75–82
DOI 10.1007/5584_2016_39
© Springer International Publishing Switzerland 2016
Published online: 23 June 2016

The Renin-Angiotensin-Aldosterone System in Smokers and Non-Smokers of the Ludwigshafen Risk and Cardiovascular Health (LURIC) Study

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Abstract

High concentrations of renin and aldosterone are risk factors for cardiovascular diseases (CVD) which are the leading cause of morbidity and mortality worldwide. Enhanced activation of the renin-angiotensin-aldosterone system (RAAS) by cigarette smoking has been reported. The aim of our study was to analyze the effect of cigarette smoking on parameters of the RAAS in active smokers (AS) and life-time non-smokers (NS) of the Ludwigshafen Risk and Cardiovascular Health (LURIC) Study as well as the utility of RAAS parameter for risk prediction. We determined the concentration of aldosterone, renin, angiotensin-I and angiotensin-II in participants of the LURIC study. Smoking status was assessed by a questionnaire and the measurement of plasma cotinine concentration. Parameters were log transformed before entering analyses, where appropriate. We used a multivariate Cox regression analysis to assess the effect of parameters on mortality. From the 3316 LURIC participants 777 were AS and 1178 NS. Within a median observation period of 10 years 221 (28.4 %) AS and 302 (25.6 %) NS died. After adjustment for age, gender, and the use of anti-hypertensive medication, only angiotensin-I was significantly different in AS compared to NS with an estimated

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marginal mean (95 % CI) of 1607 (1541–1673) ng/L and 1719 (1667–1772) ng/L, respectively. For both NS and AS renin and angiotensin-II were directly associated with mortality in the multivariate Cox regression analysis. Angiotensin-I was only associated with increased risk for mortality in NS (HR (95 % CI) of 0.69 (0.53–0.89)). We conclude that increased renin and angiotensin-II are independent predictors of mortality in AS and NS, while angiotensin-I was associated with reduced risk of death in NS only.

Keywords

Aldosterone • Angiotensin • Cigarette smoking • Mortality • Renin • Smokers

1 Introduction

It has long been known that blood pressure, heart rate, and plasma catecholamine levels increase during smoking and hemodynamic effects might contribute to the addictive properties of tobacco consumption (Omvik 1996; Rabinowitz et al. 1979; Cellina et al. 1975). These effects are specifically associated with nicotine while the other components of cigarette smoke seem to be of minor importance (Tachmes et al. 1978). Nicotine also activates the renin-angiotensin-aldosterone system (RAAS) by increasing activities of aldosterone and angiotensin-converting enzyme (ACE) (Ferrari et al. 2007). The RAAS system was discovered more than a century ago and represents one of the most important hormonal systems and is considered to be an integral part of the cardiovascular disease continuum. It controls cardiovascular, renal, and adrenal functions including the regulation of blood pressure and fluid volume as well as sodium and potassium balance (Ferrario and Strawn 2006). Further, aldosterone and angiotensin are described to induce vascular and myocardial remodeling. This abnormal activity of the RAAS has been linked to the development of cardiovascular diseases such as hypertension, atherosclerosis, ventricular hypertrophy, or renal disease and also events like myocardial infarction, stroke as well as onset and progression of congestive heart failure (Tomaschitz et al. 2011; Ferrario and Strawn 2006).

In animal models it has been shown that chronic cigarette exposure leads to lung parenchyma and airway inflammation and induces the reconstruction of small pulmonary vessels and small airways, resulting in pulmonary arterial hypertension (Wang et al. 2014) the processes in which the activation of the RAAS is also involved (de Man et al. 2012). In rats it has been demonstrated that acute and chronic cigarette smoke exposure can promote the conversion of Ang-I to Ang-II in the lung (Yu et al. 1992). The notion of an activated RAAS system by smoking is further supported by studies performed in monozygotic twins which showed the presence of a higher plasma renin activity (PRA) in the smoking twin (Laustiola et al. 1988). Also, the mean aldosterone concentration at rest was 23 % and during exercise 40 % higher in smokers than in non-smokers. Unfortunately, data from patients with increased cardiovascular risk, which are most prone to the adverse effects of RAAS dysregulation, are sparse. Therefore, the aim of our study was to analyze the effect of cigarette smoking on parameters of the RAAS in active smokers (AS) and life-time non-smokers (NS) of the Ludwigshafen Risk and Cardiovascular Health (LURIC) Study as well as the utility of RAAS parameter for individual risk prediction.

2 Methods

2.1 Study Population

The LUdwigshafen RIsk and Cardiovascular Health (LURIC) study is an ongoing prospective study of 3316 patients of German ancestry who had an indication for coronary angiography and were recruited between June 1997 and May 2001 at the Ludwigshafen Cardiac Center (Winkelmann et al. 2001). All patients were clinically stable (except for acute coronary syndromes). The study was approved by the 'Landesärztekammer' Ethics Committee of the Rheinland-Pfalz state in Germany. All patients gave written consent at study entry. Information on the vital status was obtained from local registries. Death certificates were obtained in 97 % of dead participants. Among the 3316 LURIC participants there were 777 AS and 1178 NS who were included into this study. Former smokers were excluded. Of the persons studied, 523 deaths (26.8 %) occurred during a median follow-up of 9.9 (9.4–10.8) years. Smoking status was assessed based on a questionnaire and verified by measurement of serum cotinine concentration.

2.2 Laboratory Procedures

Fasting blood samples were taken by venipuncture in the early morning prior to angiography. Aliquots were frozen at −80 °C. Cholesterol and triglycerides were measured with enzymatic reagents from WAKO (Neuss, Germany) on an Olympus AU640 analyzer (Center Valley, PA). Aldosterone and renin were determined by RIA using the Active™ aldosterone kit and the Active™ renin kit (Diagnostic Systems Laboratories Deutschland GmbH; Sinsheim, Germany) on a Berthold Multi-crystal counter LB2014. Angiotensin I and II were measured by RIA (LKB Valle 1277, γ Master, Uppsala, Sweden).

2.3 Statistical Analyses

All continuous variables were checked for normality and those showing a skewed distribution were logarithmically transformed to get a normal distribution. Extreme outliers, of more than four standard deviations (SD) off the mean, were excluded. Continuous variables were compared between groups by Student's *t*-test. Estimated marginal means adjusted for age, gender, and anti-hypertensive medication were calculated using ANOVA. Associations between categorical variables were examined by chi-squared testing. To examine the relationship of RAAS factors with mortality, we calculated hazard ratios and 95 % confidence intervals (95 % CI) using the Cox proportional hazards model. Multivariable adjustment was carried out as indicated. The functional form of covariates was analyzed by calculating Martingale residuals and the proportional hazard assumption was checked by examination of scaled Schoenfeld residuals. IBM SPSS Statistics v. 22.0 (IBM Corporation; San Jose, CA) and R statistical software v. 3.2.3 (http://www.r-project.org) were used for all analyses. Hazard ratio plots were drawn using the R-package 'rms' with RAAS factors modeled as restricted cubic splines with three knots.

3 Results

3.1 Differences in Biomarker Levels Between Smokers and Non-Smokers

Among the 3316 LURIC participants, there were 777 AS and 1178 NS. AS were, on average, younger as compared to NS and were more commonly males (Table 1). AS had lower LDL-C and HDL-C but higher concentrations of oxidized LDL and triglycerides than those in NS. hsCRP was higher in AS and they had a higher mean estimated glomerular filtration rate

Table 1 Selected anthropometric data of study patients at study begin

	Never smoker	Active smoker	p
n	1178	777	–
Age (year)	65.3 ± 10.1	56.2 ± 10.3	<0.001
Male sex (%)	45.4	77.9	<0.001
BMI (kg/m^2)	27.4 ± 4.2	27.0 ± 4.2	0.833
LDL-C (mg/dl)	119.1 ± 36.4	117.5 ± 32.1	0.012
oxidized LDL-C (U/l)	73.5 ± 26.7	78.4 ± 25.4	<0.001
HDL-C (mg/dl)	41.2 ± 11.1	36.2 ± 10.2	0.002
Triglycerides (mg/dl)	136 (102 – 192)	154 (112 – 218)	<0.001
Galectin-3 (ng/ml)	15.8 ± 6.29	15.5 ± 7.29	0.496
hsC-reactive protein (mg/l)	2.72 (1.17 – 7.04)	4.93 (1.84 – 10.30)	<0.001
eGFR (ml/min/1.73 m^2)	78.7 ± 19.1	88.2 ± 20.1	<0.001
Aldosterone (pg/mL)	77.5 (47.3 – 123)	76.0 (48.0 – 124)	0.701
Renin (pg/mL)	10.2 (5.39 – 21.0)	12.0 (5.99 – 26.3)	<0.001
Angiotensin-I (ng/L)	1579 (1226 – 2053)	1402 (1078 – 1780)	<0.001
Angiotensin-II (ng/L)	19.0 (13.0 – 34.0)	20.0 (12.0 – 35.0)	0.877
Coronary artery disease (%)	68.1	80.1	<0.001
Diabetes mellitus (%)	38.3	36.0	0.314
Hypertension (%)	76.6	63.3	<0.001
Antihypertensive therapy (%)	86.2	85.3	0.604
Lipid lowering therapy (%)	42.4	52.8	<0.001

Data are means ± SD or median and 25–75th percentile. Antihypertensive medication included ACE inhibitors, angiotensin II receptor antagonists, diuretics, beta blocker, and calcium channel blockers. *BMI* body mass index, *eGFR* estimated glomerular filtration rate, *HDL-C* high density lipoprotein cholesterol, *LDL-C* low density lipoprotein cholesterol

(eGFR). The percentages of patients suffering from coronary artery disease and hypertension were higher in the AS group as compared to lifetime non-smokers. Regarding the components of the RAAS, we found a higher renin concentration in AS, while angiotensin-I (Ang-I) was lower in unadjusted analyses. There were no significant differences in the concentration of aldosterone or angiotensin-II (Ang-II). However, after adjustment for age, gender, and the use of anti-hypertensive medication (ACE inhibitors, Ang-II receptor blockers diuretics, calcium antagonists, and beta blockers) only Ang-I was significantly different in AS compared to NS with an estimated marginal mean (95 % CI) of 1607 (1541–1673) ng/mL and 1719 (1667–1772) ng/mL, respectively (Table 2). Additional adjustment for kidney function (eGFR), heart failure (NYHA stages), BMI, diabetes mellitus, and hypertension did not materially alter the results.

3.2 Cox Regression Analysis in Smokers and Non-Smokers

Within a medium follow-up time of 10 years, 221 (28.4 %) of AS and 302 (25.6 %) of NS died. For both AS and NS, renin and Ang-II were directly associated with mortality (Fig. 1). For Ang-I, we found an association only for NS with a HR (95 % CI) of 0.69 (0.53–0.89) per increase of one unit log transformed Ang-I in a model adjusted for age, gender, and anti-hypertensive medication. Further adjustment for cardiovascular risk factors did not alter the results substantially. These differences were also seen when plotting the concentration of RAAS factors against their log relative hazard (Fig. 2). The association was similar in AS and NS for renin, aldosterone, and Ang-II, while Ang-I was associated with an almost linear risk reduction in NS only. In AS, the association between Ang-I and mortality seemed to be

Table 2 Estimated marginal means (95 % CI) of the renin-angiotensin-aldosterone system (RAAS) adjusted for age, gender, and the use of anti-hypertensive medication

	Never smoker	Active smoker	p
Aldosterone (pg/mL)	98.8 (93.9–104.0)	98.0 (91.8–104.0)	0.856
Renin (pg/mL)	32.7 (23.4–42.0)	32.0 (20.2–43.7)	0.928
Angiotensin-I (ng/L)	1719 (1667–1772)	1607 (1541–1673)	0.014
Angiotensin-II (ng/L)	31.3 (27.5–35.0)	34.3 (29.5–39.0)	0.358

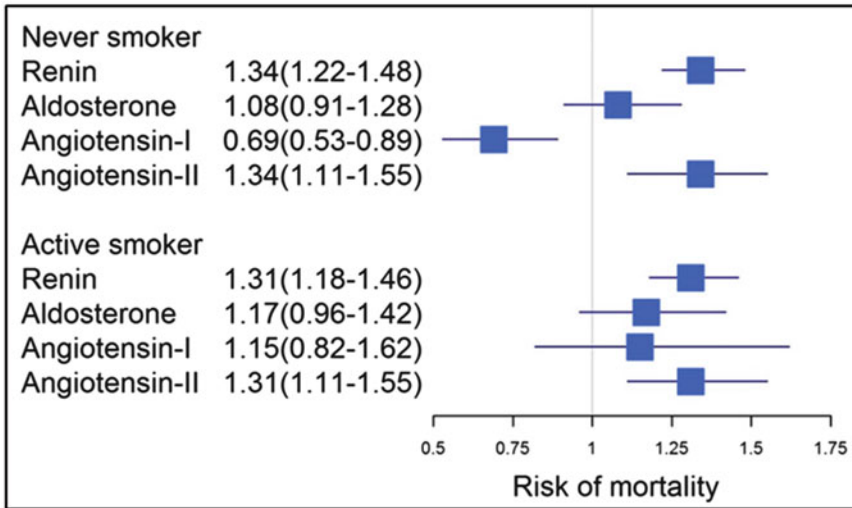

Fig. 1 Risk of all-cause mortality per 1 unit increase in logarithmically transformed renin, aldosterone, Ang-I, and Ang-II adjusted by age, gender, and the use of anti-hypertensive medication and stratified by smoking status (hazard ratio and 95 % confidence interval)

slightly U-shaped, but the confidence intervals in the very low and very high concentration ranges were quite large. We also performed a sensitivity analysis excluding all patients who received ACE inhibitors or presented with an acute coronary syndrome at study entry, which left us with 399 NS and 233 AS. That analysis did not introduce any appreciable changes compared with the bulk data above outlined; therefore detailed results are not shown.

4 Discussion

Our main findings are that that there was no difference in the concentration of renin, aldosterone, and Ang-II between AS and NS and that increased renin and Ang-II are independent predictors of mortality in both AS and NS. Ang-I was only associated with reduced risk of death in NS. This finding contradicts previous evidence and might be related to the complex neurohumoral response to smoking. The relationship between smoking and blood pressure seems somewhat paradoxical: Smoking induces an immediate rise in blood pressure but, on the other hand, larger epidemiological studies have not found a consistent association between elevated blood pressure among smokers compared to non-smokers (Green et al. 1986). A hypothetical sequence how smoking leads to increased blood pressure and renal damage has been sympathoadrenal activation. This would lead to an increase in circulating catecholamines that, in turn, increase the expression of renin *via* α1-adrenergic stimulation (Orth 2002). Although

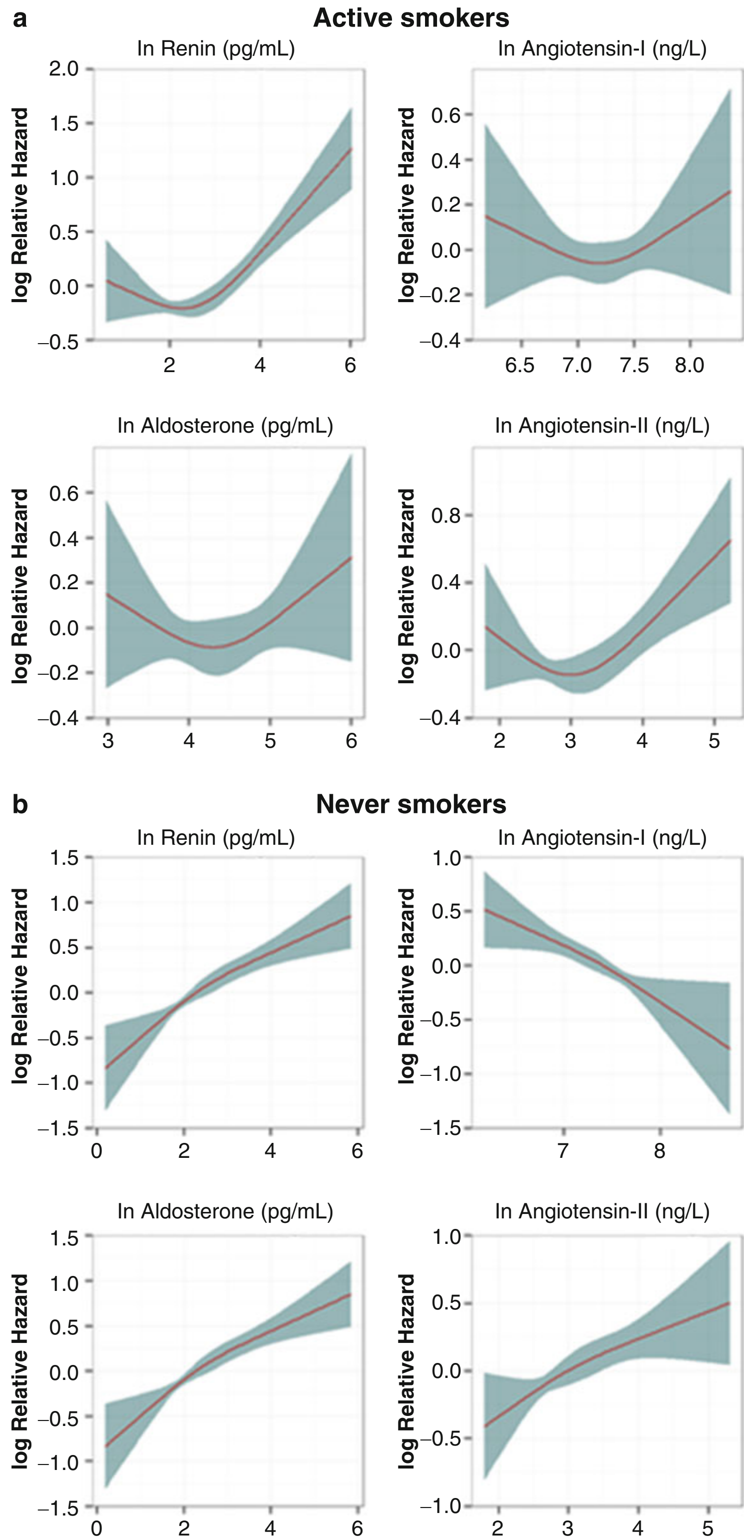

Fig. 2 Relationship between the renin-angiotensin-aldosterone system (RAAS) components and all-cause mortality in active smokers (**a**) and never smokers (**b**). Logarithmically transformed renin, aldosterone, Ang-I and Ang-II were modeled as restricted cubic spline in Cox regression analysis and their concentration was plotted against the respective log relative hazard (*red line*) with 95 % confidence intervals (*shaded*)

this is a plausible mechanism and also supported by experimental data, it has been observed that the immediate smoking-induced rise in blood pressure can occur before any noticeable increase in circulating catecholamines pointing to the possible existence of other mediating pathways or a neural-behavioural component. One of these could be a direct effect of renin through the activation of the ubiquitously expressed (pro) renin receptor by prorenin and renin that leads to a range of cellular events independent of Ang-II and aldosterone, like the upregulation of pro-fibrotic and pro-inflammatory genes (Hitom et al. 2010). This could explain the fact that renin has been shown to be a significant predictor of mortality in LURIC independent from aldosterone and Ang-II (Tomaschitz et al. 2011).

Nevertheless, the RAAS seems to play a significant role in smoking-induced morbidity and mortality. It is a key player in the homeostatic control of arterial pressure and extracellular volume. The active hormone, Ang-II, is formed by sequential proteolytic cleavage of its precursors. This sequence is initiated by the regulated secretion of renin, the rate-limiting processing enzyme. Active renin cleaves its substrate angiotensinogen to generate Ang-I, which is subsequently converted to Ang-II by ACE. A significant decrease in active renin after smoking has been shown in occasional smokers that could be modulated using alpha-1 and beta-1-blockers (Benck et al. 1999). Further, it has been demonstrated in animal models of pulmonary arterial hypertension that exposure to cigarette smoke leads to increased expression of ACE, and consequently to an increase in Ang-II, and a reduction in ACE2 expression (Yuan et al. 2015). ACE2 hydrolyzes Ang-I to generate the negative regulatory protein Ang-(1–7). Ang-II mediates its effects *via* type 1 Ang-II receptor. This receptor is blocked by commonly used anti-hypertensive drugs that may also have anti-inflammatory effects. A study by Kyvelou et al. (2007) on the anti-inflammatory properties of angiotensin-II receptor blockers has shown a smaller decrease in hsCRP and serum amyloid A induced be these blockers in smokers compared to nonsmokers.

In the present study, we found no significant differences in the concentration of renin, aldosterone, or Ang-II in AS, as compared to NS after adjustment for age, gender, and the use of anti-hypertensive medication. This might be partly explained by the fact that most blood samples were taken in the morning before the patients underwent coronary angiography; so that a longer time had passed since the last smoking event, making the acute effects undetectable. Ang-I was significantly lower only in AS, possibly pointing to an increased conversion rate of inactive Ang-I to active Ang-II. Regarding the association with mortality, Ang-I was not a significant predictor of all-cause mortality in AS, whereas in NS there was an approximately linear inverse association of logarithmically transformed Ang-I with mortality. This finding might support the hypothesis that Ang-I is metabolized differently in smokers as compared to non-smokers. Renin and Ang-II were associated with similar risk increases in both AS and NS.

This study has some limitations. All participants were of European origin and were recruited at a single tertiary care referral center. Therefore, our findings may not be representative for a random population sample or applicable to other ethnicities. Further, we only investigated active smokers and lifetime non-smokers and excluded former smokers from the analyses. Plasma renin activity might have added further insights the complex interaction between smoking and the RAAS. Renin, aldosterone, Ang-I, and Ang-II were only measured once at baseline. The major strengths of the LURIC cohort are, however, the precise clinical and metabolic characterization of the participants and its cross-sectional and prospective design.

In conclusion, the present study is the first to compare plasma levels of renin, angiotensin, and aldosterone between active smokers and never smokers in regard to cardiovascular risk. Unexpectedly, we failed to observe a significant difference between both groups. Nevertheless, increased renin and angiotensin-II were independent predictors of mortality in both groups, while angiotensin-I was associated with reduced risk of

death in never smokers only, suggesting a more complex interaction between smoking and the renin-angiotensin-aldosterone system.

Acknowledgments We extend our appreciation to the participants of the LURIC study; this article would not have been written without their collaboration. We thank the LURIC study team, who were either temporarily or permanently involved in patient recruitment as well as sample and data handling, and the laboratory staff at the Ludwigshafen General Hospital and the Universities of Freiburg and Ulm, Germany. This work was supported by the 7th Framework Program (grant agreement number 201668 and RiskyCAD, grant agreement number 305739) of the European Union.

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 (2016) 26: 83–88
DOI 10.1007/5584_2016_40
© Springer International Publishing Switzerland 2016
Published online: 23 June 2016

Electrodermal Activity in Adolescent Depression

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Abstract

Major depressive disorder (MDD) is characterized by dysphoric mood, which may be accompanied by suicidal ideation. It is supposed that MDD is associated with dysfunction of the autonomic nervous system, but studies in pediatric patients are rare. Therefore, we aimed to study the relationship between MDD and autonomic regulation in adolescence using the electrodermal activity as an index of sympathetic cholinergic control. We examined 25 adolescents suffering from MDD without comorbidities and prior to pharmacotherapy (13 girls, mean age 14.6 ± 0.4 year) and 25 age/gender-matched healthy control subjects. The electrodermal activity was continuously recorded during 5 min of supine rest. The value of this activity in μS was averaged for each minute of the recording. We found that in depressed patients, electrodermal activity was significantly lower each minute of the recording compared to that in the control group. The study demonstrates electrodermal hypoactivity in adolescent patients with MDD, which points to dysfunctional regulation of the sympathetic part of the autonomic nervous system. This finding could represent a potential pathomechanism leading to higher risk of negative health outcomes in pediatric depressed patients. Further research is needed to elucidate the incompletely understood interaction between MDD and autonomic regulatory outputs at young age.

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Keywords
Autonomic nervous system • Cardiovascular risk • Major depressive disorder • Pediatric patients • Sympathetic activity

1 Introduction

Major depressive disorder (MDD) is a mental disorder characterized by psychological symptoms, e.g., dysphoric or irritable mood, feelings of worthlessness, and thoughts of death, including suicidal ideation (APA 2013). It is supposed that MDD is associated with dysfunction of the autonomic nervous system, which represents a key regulatory system for the maintenance of homeostasis, adaptability, and physiological flexibility of the organism. For instance, impaired sympathetic function is probably one of the mechanisms involved in increased cardiovascular risk associated with MDD (Carney and Freedland 2009). Therefore, detailed study of the relationship between MDD and dysregulation of the autonomic nervous system could bring novel important information about pathomechanisms of depression-related negative health outcomes.

There are only a few noninvasive methods for the assessment of sympathetic function. One of the suitable markers reflecting sympathetic regulation is electrodermal activity (EDA). EDA is represented by skin conductance that depends on the amount of sweat produced by eccrine sweat glands. When the sweat duct is filled with sweat, more conductive area originates on the nonconductive corneu (Cacioppo et al. 2007). While the sweat glands are innervated by sympathetic fibers, their synaptic neurotransmission is mediated almost exclusively by acetylcholine. Thus, EDA provides a direct representation of solely sympathetic cholinergic activity in contrast to the majority of systems regulated by the two main branches of the autonomic nervous system (Fowles 1980).

EDA is accepted as a noninvasive marker of sympathetic arousal in psychophysiological research (Jacobs et al. 1994). It has become a frequently used tool in the research on mental disorders, including MDD. Interestingly, many studies in the last decades have shown that depressed patients have a lower EDA compared with controls (Wolfersdorf et al. 1996; Williams et al. 1985; Ward et al. 1983; Dawson et al. 1977). These results led to the assumption that reduced EDA could be an important biopsychological trait in the etiology of depression. However, previous studies have evaluated EDA in adult depressed patients and studies at adolescent age are very rare (Crowell et al. 2012). Therefore, in the present study we set out to determine the baseline EDA in adolescent patients suffering from MDD, prior to pharmacological treatment.

2 Methods

The study was approved by the Ethics Committee of Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovakia and it comported with Declaration of Helsinki. All subjects and their parents were instructed about the study protocol and they gave written consent to participate at study entry.

2.1 Subjects

We examined 25 patients (F/M – 13/12) with MDD of the mean age of 14.4 ± 0.4 (range 11–17 years) and 25 gender- and age-matched healthy control subjects. The persons suffering from MDD were recruited from the inpatients admitted to a Psychiatric Clinic. The diagnosis of MDD, a single episode without psychotic symptoms (e.g., mood congruent or incongruent delusions, hallucinations) and other psychiatric disorders (e.g., ADHD, conduct disorders,

anxiety disorders), was classified by a thorough clinical investigation based on unstructured diagnostic interview conducted by a staff psychiatrist, specialized in child/adolescent disorders, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-V). The patients were examined before pharmacotherapy during the first week of hospitalization.

The exclusion criteria for both MDD and control group were: history of cardiovascular, respiratory, endocrinological, neurological, metabolic, or infectious diseases. The control subjects have never been treated for any mental disorder.

2.2 Study Protocol

All subjects were examined after breakfast in a quiet room under standard conditions (temperature: 22–23 °C, humidity: 45–55 %), with the minimization of stimuli, in the morning between 8:00 and 12:00 a.m. After 10 min of rest in the sitting position, the subject changed position into supine and remained at rest for further 5 min. Then, EDA was continuously recorded for another 5 min using a biofeedback device ProComp Infinity (Thought Technology Ltd., Canada). According to the general recommendations, sensors were placed on the medial phalanges of the second and the fourth finger of the non-dominant hand (Cacioppo et al. 2007).

2.3 Data Analysis

The recordings were visually checked and rare artifacts were removed manually. For a more accurate assessment of EDA changes, a 5-min recording time in the supine position was divided into five consecutive intervals of 60 s each. The mean value of EDA (in μS) was evaluated for each interval.

Data were expressed as means ± SE. The non-Gaussian/Gaussian distribution was ascertained by the Lilliefors test. The Mann-Whitney *U* test was used for between-groups comparison. A p-value of less than 0.05 defined the statistical significance of differences. Statistical analyses were performed using a commercial statistical package SYSTAT 10 for Windows (SSI, Richmond, CA).

3 Results

Recordings of electrodermal activity show that it was diminished in the supine resting position in adolescent patient with major depressive disorder compared with that in healthy subjects (Fig. 1a, b). A detailed analysis revealed that EDA was significantly lower in depressed patients, as compared with control subjects, during each minute interval of the 5-min recording time ($p < 0.01$; Table 1).

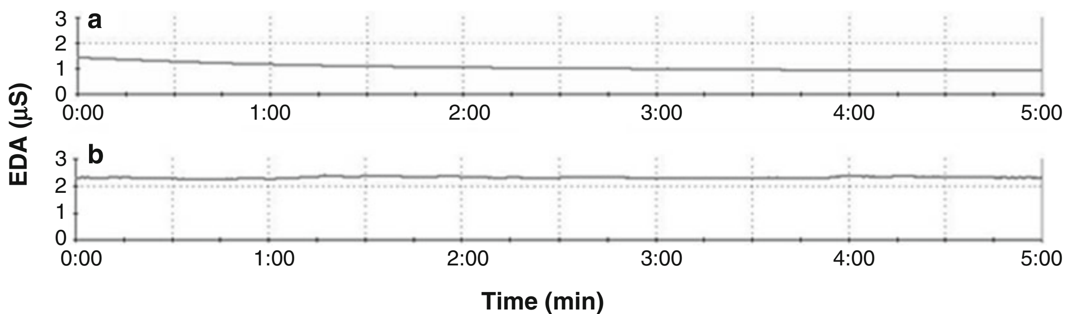

Fig. 1 Recordings of electrodermal activity (EDA) at rest in adolescents with major depressive disorder (**a**) and in healthy subjects (**b**). EDA – electrodermal activity

Table 1 Electrodermal activity (EDA), expressed in μS, in adolescents with major depressive disorder and healthy control subjects

Recording time (min)	Depressed (n = 25)	Healthy (n = 25)	p
1	1.46 ± 0.21	2.74 ± 0.33	p = 0.002
2	1.26 ± 0.19	2.52 ± 0.30	p = 0.001
3	1.22 ± 0.20	2.35 ± 0.29	p = 0.001
4	1.21 ± 0.20	2.20 ± 0.30	p = 0.004
5	1.18 ± 0.20	2.18 ± 0.29	p = 0.002

Data are means ± SE

4 Discussion

The major finding of this study was a significantly reduced EDA, indicating sympathetic hypoarousal, in depressed adolescents. Some previous studies have shown reduced EDA in adult depressed patients (Wolfersdorf et al. 1996; Williams et al. 1985; Ward et al. 1983), but others have reported no difference between depressed and healthy subjects (Toone et al. 1981). Nonetheless, there is a consistent impression that the majority of reports point to the presence of electrodermal hypoactivation in adult depressed patients (Miller 1995). We now extend and strengthen those findings by showing dampened electrodermal activity in depression of the adolescent age as well.

The mechanism of electrodermal hypoactivity remains debatable. It is well-known that EDA is determined by the integration of central and peripheral regulatory mechanisms. In the context of central regulation, complex interaction of cortical and subcortical structures forms three major systems: limbic-hypothalamic circuit, premotor cortex-basal ganglia system, and reticular formation. Thermoregulatory and emotionally driven limbic-hypothalamic system involves the excitatory effect of amygdala, the inhibitory role of hippocampus, and the intense connections with the ventromedial prefrontal cortex (vmPFC) (Boucsein 2012). Importantly, vmPFC is responsible for the EDA regulation during restful states in a manner that increased vmPFC activity is associated with decreased EDA. However, it is worth noting that vmPFC shows an internal functional heterogeneity and reduced EDA is predominantly caused by activation of its posterior region (Zhang et al. 2014). Further, vmPFC seems significantly involved in the pathomechanism of depression. The activity of the posterior part of vmPFC is related to a negative mood and it is enhanced in MDD patients. In contrast, activation of the anterior part of vmPFC is related to a positive mood, positively correlates with EDA, and it is decreased in MDD patients (Zhang et al. 2014; Myers-Schulz and Koenigs 2012). It is then a rational assumption that the present finding of reduced EDA in depressed adolescents could result from the imbalance between the activities of anterior and posterior regions of vmPFC. This assumption is in line with the contemporary neural models of depression which posit that dysfunction of medial prefrontal network and related limbic structures represents a key pathomechanism of emotional, behavioral, and other cognitive aspects of MDD. The rationale of this theory is based on the findings of distinct alterations in the gray matter volume, cellular elements, neurophysiological activity, receptor pharmacology, and gene expression in mood disordered subjects. The structures outlined above also exert a modulatory influence over the autonomic functions *via* connections with the hypothalamus and the brainstem, and thus are capable of altering the activity of peripheral organs, e.g., the cardiovascular system or sweat glands (Price and Drevets 2010). Yet this issue is still discussed and a straightforward effect of one brain area on the complex dynamic emotional and autonomic regulation is certainly too simplistic. The exact mechanisms underlying the function of the

prefrontal-limbic network in the MDD-linked autonomic dysregulation remain to be settled.

Another mechanism of electrodermal activity regulation could include the premotor cortex-basal ganglia system which partakes in setting specific motor actions (Boucsein 2012). The MDD is associated with variable abnormalities of behavioral systems (Kasch et al. 2002), which could be related to EDA (Fowles 1980). However, in the present study, recordings of EDA were performed under a resting condition, so that the influence on EDA of this neural circuit is rather unlikely.

Regarding peripheral regulation, it is generally accepted that human sweat glands have predominantly sympathetic cholinergic innervation from the sudomotor fibers originating in the sympathetic chain. It is assumed that depressed patients may have an abnormal peripheral cholinergic mediation, which could be represented by altered receptor sensitivity (Drevets et al. 2013; Miller 1995). Thus, the peripheral component also should be taken into account. In addition, function of the autonomic nervous system could be affected by pharmacotherapy. The effect of antidepressants has been considered as a possible pathomechanism of reduced EDA in MDD (Schnur 1990). Patients in the present study were examined prior to pharmacological treatment, and thus the influence on EDA of pharmacotherapy seems unlikely either.

In summary, reduced EDA in adolescents with MDD may result from a complex interaction of several pathomechanisms, some of which may still remain unknown. Importantly, altered autonomic regulation expressed by electrodermal hypoactivity is associated with increased risk of negative health outcomes, e.g., cardiovascular complications. Our previous studies of adolescent MDD have revealed impaired autonomic neurocardiac integrity, such as decreased vagal and increased sympathetic cardiac activity, and reduced complexity of the heart rate control (Tonhajzerova et al. 2009, 2010, 2012). This shift in sympathovagal cardiac control, along with electrodermal hypoactivity, could reflect a specific effect on different effector systems of autonomic imbalance in MDD patients.

5 Conclusions

The present study revealed altered sympathetic cholinergic regulation, expressed by electrodermal hypoactivity, in untreated major depression in adolescence. This finding underscores the significance of potential autonomic-mediated risk of early negative health outcomes in depressed patients in a vulnerable adolescent age-period. The exact autonomic regulatory mechanisms underlying the central-peripheral interaction in depressive disorder remain to be further explored by alternative study design.

Acknowledgments This work was supported by National Research Grant VEGA 1/0087/14, Comenius University Grant UK/151/2016, and the project "Biomedical Center Martin" ITMS code: 26220220187, the project is 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 (2016) 26: 89–98
DOI 10.1007/5584_2016_25
© Springer International Publishing Switzerland 2016
Published online: 17 June 2016

Metagenomic Analysis of Cerebrospinal Fluid from Patients with Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of central nervous system of unknown etiology. However, some infectious agents have been suggested to play a significant role in its pathogenesis. Next-generation sequencing (NGS) and metagenomics can be employed to characterize microbiome of MS patients and to identify potential causative pathogens. In this study, 12 patients with idiopathic inflammatory demyelinating disorders (IIDD) of the central nervous system were studied: one patient had clinically isolated syndrome, one patient had recurrent optic neuritis, and ten patients had multiple sclerosis (MS). In addition, there was one patient with other non-inflammatory neurological disease. Cerebrospinal fluid (CSF) was sampled from all patients. RNA was extracted from CSF and subjected to a single-primer isothermal amplification followed by

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NGS and comprehensive data analysis. Altogether 441,608,474 reads were obtained and mapped using blastn. In a CSF sample from the patient with clinically isolated syndrome, 11 varicella-zoster virus reads were found. Other than that similar bacterial, fungal, parasitic, and protozoan reads were identified in all samples, indicating a common presence of contamination in metagenomics. In conclusion, we identified varicella zoster virus sequences in one out of the 12 patients with IIDD, which suggests that this virus could be occasionally related to the MS pathogenesis. A widespread bacterial contamination seems inherent to NGS and complicates the interpretation of results.

Keywords

Cerebrospinal fluid • Idiopathic inflammatory demyelinating disorder • Metagenomics • Multiple sclerosis • Next-generation sequencing

1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system of unknown etiology. Epidemiological studies suggest that the development of MS correlates with genetic predispositions and environmental risk factors such as vitamin D insufficiency, cigarette smoking, high estrogen levels, and changes in dietary fats, as well as infections (Pender and Burrows 2014; O'Gorman et al. 2012; Zawada 2012; Kakalacheva et al. 2011; Brahic 2010). The possible role of infections in MS pathogenesis is supported by the uneven worldwide distribution of the disease, its inflammatory character, and human migration studies indicating an increase in disease risk when moving from low to high MS prevalence areas (Ascherio and Munger 2007; Marrie 2004).

In the last decades, over 20 infectious agents (viruses, bacteria, and fungi) have been proposed as a potential cause of MS (O'Gorman et al. 2012; Zawada 2012; Kakalacheva et al. 2011). The relationship between various infections and MS development has been supported by the detection of specific antibodies in the serum and cerebrospinal fluid (CSF), and by the presence of pathogens' nucleic acids and proteins in CSF. The mechanisms explaining how infections might trigger autoreactive immune response include molecular mimicry, viral support of autoreactive cell survival, epitope spreading, and bystander activation (Zawada 2012; Kakalacheva et al. 2011; Brahic 2010).

Several pathogenic candidates have been proposed: Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), human cytomegalovirus (CMV), herpes simplex viruses (HSV) type 1 and 2, human endogenous retrovirus (HERV), measles virus (MeV), or even nonpathogenic torque teno virus (TTV) (Pender and Burrows 2014; Borkosky et al. 2012; Zawada 2012; Zivadinov et al. 2006; Swanborg et al. 2003; Sanders et al. 1996; Norrby et al. 1974). Among bacteria, *Pseudomonas aeruginosa* has been postulated to be involved in MS pathogenesis, as it was shown by Hughes et al. (2001) that specific antibodies are higher in MS patients than in controls. Another study reported that *Chlamydia pneumoniae* IgG antibodies are significantly higher in CSF of MS patients than in control patients and *Chlamydia pneumoniae* DNA has been detected in the CSF and brain of MS patients (Swanborg et al. 2003; Krametter et al. 2001). Other potential pathogenetic candidates underlying MS are fungi whose toxins can be at play in the destruction of astrocytes and oligodendrocytes, leading to myelin degradation (Zawada 2012; Benito-Leon et al. 2010). Despite numerous studies above outlined, there is still no definitive evidence that any particular pathogen is the cause of MS (Brahic 2010).

Most studies on the potential infectious agents in MS concentrate on selected pathogens. There are only few reports that address the microbial flora in MS patients and those that do address it, deal with the intestinal microbiota, especially after the discovery of its role in the dysregulation of innate and adaptive immune response, central nervous system demyelination, and the development of inflammatory bowel disease (Hansen 2015; Joscelyn and Kasper 2014; Round and Mazmanian 2009). The gut microbiome in MS patients has been characterized by a microarray analysis of bacterial 16S ribosomal RNA and changes in the abundance of some taxonomic units, including a lower level of *Faecalibacterium*, have been observed (Cantarel et al. 2015). Miyake et al. (2015) have reported dysbiosis in the structure of gut microbiota in MS patients, compared to healthy controls, consisting of differences in abundances of 21 different species in fecal samples assessed by pyrosequencing. There is still lack of information about bacterial, viral, fungal, and parasitic sequence composition in CSF of MS patients. A new light on potential infectious etiology of MS may be provided by next-generation sequencing (NGS) based metagenomics which enables a simultaneous analysis of numerous microorganisms (Miller et al. 2013; Padmanabhan et al. 2013; Sleator et al. 2008).

In the present study we conducted metagenomic sequencing of cerebrospinal fluids of patients with idiopathic inflammatory demyelinating disorders (IIDD) of the central nervous system, using a single-primer isothermal amplification followed by NGS and comprehensive data analysis (Perlejewski et al. 2015).

2 Methods

2.1 Patients

The study protocol was approved by the Internal Review Board for Medical Research of Warsaw Medical University in Warsaw, Poland. All patients gave written consent for study procedures. The study included 13 patients. There were 12 patients with idiopathic inflammatory demyelinating disorder (IIDD) of the central nervous system; 7 women and 5 men, aged from 22 to 52 years. Ten patients had MS diagnosed on the basis of Polman et al.'s (2011) criteria, one patient had clinically isolated syndrome, and another one had recurrent optic neuritis. In addition, there was one patient with other non-inflammatory neurological disease. CSF was collected through lumbar puncture in all patients during hospitalization at the Department of Neurology of Warsaw Medical University.

2.2 RNA Isolation, Sequencing and Data Analysis

Total RNA was extracted from 500 μl of CSF using the single-step RNA isolation method of Chomczynski (1993) with TRIZOL LS Reagent (Life Technologies; Carlsbad, CA). All samples were elaborated with a single-primer isothermal amplification technique marketed by NuGEN (Ovation RNA-Seq V2; NuGEN, San Carlos, CA). The amplified products were purified using Agencourt AMPure XP beads (Beckman Coulter; Pasadena, CA) and measured with Qubit 2.0 Fluorometer (Life Technology; Carlsbad, CA). Libraries for NGS were prepared using Nextera XT Kit (Illumina; San Diego, CA) following the manufacturer's protocol. In the first step, cDNA was fragmented using transposon-based method and at the same time sequences were marked with indexes by PCR. Subsequently, PCR products were purified with 1.8 volumes of AMPure XP beads (Beckman Coulter; Pasadena, CA). The quality and the length of the sequence library for each sample were measured with a Bioanalyzer (Agilent Technologies; Santa Clara, CA) and either DNA 1000 or DNA HS kit. Finally, samples were pooled equimolarly and sequenced on Illumina HiSeq 1500 (100 nt, paired-end reads).

Raw reads were trimmed by the following procedures: 1/adaptor removal using cutadapt-1.2.1 (Martin 2011); 2/artifact sequence removal using fastx artifact filter; 3/trimming bases with the quality below Q20 (phred quality score) from

3′ end of each read and removing reads shorter than 50 bp using fastq quality trimmer (FASTX-Toolkit 2016). Then, trimmed sequences were mapped onto the human reference sequence (hg19) with the Stampy software (Lunter and Goodson 2011). The unmapped sequences were compared using blastn program against unfiltered NCBI-nt database with e-value cutoff of 1e-5. The taxonomic information of each sequence was assigned and the abundance of identified microorganisms was presented by text mining of blastn output files using BioRuby scripts (Goto et al. 2010).

3 Results

We obtained 441,608,474 reads after sequencing and quality trimming. The highest number of reads was obtained in a CSF sample taken from the patient with other non-inflammatory neurological disease (42,625,952). The number of reads in the IIDD patients ranged from 26,809,197 (Pt. 8) to 40,972,314 (Pt. 9) (Table 1).

Human sequences were the most abundant in all CSF samples from the patients with IIDD (84.35655–97.47609 % of all reads) and in a sample from the patient with other non-inflammatory neurological disease (91.44283 %) (Table 1). In the former samples viral sequences represented 0.00085–0.97591 % of all reads, while in the latter sample they constituted 0.01338 %. Viral sequences detected in the CSF samples obtained from all IIDD patients and from the patient with other non-inflammatory neurological disease matched to bacteriophages. Further, in a sample from the patient with clinically isolated syndrome, 11 reads of varicella-zoster virus (VZV) were found. Bacteria were represented by 0.83873–12.49834 % of reads in samples from IIDD patients and 3.49656 % in a sample from the patient with other non-inflammatory disease. Most abundant bacterial reads mapped to the genomes from *Pseudomonas, Escherichia, Bacillus, Streptococcus, Acinetobacter, Corynebacterium*, and *Moraxella* genera. Fungal reads (0.22931–2.80156 % in samples from the patients with IIDD and 0.40126 % in a sample from the patient with other non-inflammatory disease) represented species from a variety of genera, such as *Malassezia, Ascomycota, Funneliformis, Glomus, Cladosporium, Candida*, and *Alternaria*. Parasites and protozoa constituted 0.04785–0.84515 % of all reads in samples from the patients with IIDD and 0.20770 % of reads in a sample from the patient with other non-inflammatory disease. Representatives of the *Albugo* genus were detected in all investigated samples. Among other parasitic/protozoan reads most mapped to the genomes represented by *Besnoitia, Babesia*, and *Plasmodium* genera. Five most abundant bacterial, fungal, and parasitic/protozoal sequences are shown in Table 2.

4 Discussion

In the present study we demonstrate the results of a metagenomic search for potential infectious agents in CSF of patients with idiopathic inflammatory demyelinating disorder, employing next-generation sequencing. In one of IIDD patients, diagnosed with clinically isolated syndrome, we detected 11 reads which mapped to VZV genome. Finding a DNA virus while analyzing RNA is not unexpected with the methodological approach used. In a previous study we have demonstrated that the Chomczynski RNA extraction, followed by a single-primer isothermal amplification, NGS, and metagenomic data analysis, enables to detect both DNA and RNA sequences (Perlejewski et al. 2015). Interestingly, a relationship between VZV infection and demyelinating disorders has been previously suggested by the demonstration of more frequent presence of VZV-DNA and viral proteins in CSF of MS patients as compared to patients with other neurological diseases or healthy controls (Sotelo et al. 2008; Mancuso et al. 2007). Further, VZV-DNA is more prevalent in CSF and peripheral blood mononuclear cells during MS relapse than in remission (Sotelo et al. 2014; Ordonez et al. 2004).

Table 1 Results of next-generation sequencing (NGS) of cerebrospinal fluid samples from 12 patients with central nervous system idiopathic inflammatory demyelinating disorder (IIDD) and one patient with other non-inflammatory neurological disease. Reads were compared to the NCBI-nt database

Sample ID	Pt. 1	Pt. 2	Pt. 3	Pt. 4	Pt. 5	Pt. 6	Pt. 7	Pt. 8	Pt. 9	Pt. 10	Pt. 11	Pt. 12	Pt. 13
Diagnosis	MS	MS	MS	CIS	RON	MS	MS	MS	MS	MS	MS	MS	OND
Reads after trimming	29,850,818	36,196,465	36,927,824	31,002,606	34,064,072	33,734,539	33,335,714	26,809,197	40,972,314	31,237,666	31,291,530	33,559,777	42,625,952
Human	28,594,910 (95.79272 %)	30,533,365 (84.35455 %)	35,995,798 (97.47609 %)	30,086,680 (97.04565 %)	32,616,258 (95.74973 %)	31,580,310 (93.61417 %)	29,702,485 (89.10109 %)	22,653,060 (84.49735 %)	35,172,506 (85.84457 %)	28,101,524 (89.96038 %)	28,802,337 (92.04515 %)	31,317,498 (93.31855 %)	38,978,376 (91.44283 %)
Viral	1850 (0.00620 %)	1312 (0.00362 %)	1590 (0.00431 %)	263 (0.00085 %)	140,890 (0.41360 %)	2844 (0.00843 %)	2097 (0.00629 %)	10,340 (0.03857 %)	5009 (0.01223 %)	304,852 (0.97591 %)	2453 (0.00784 %)	2671 (0.00796 %)	5702 (0.01338 %)
Bacterial	309,417 (1.03654 %)	4,523,958 (12.49834 %)	309,726 (0.83873 %)	653,442 (2.10770 %)	668,362 (1.96207 %)	569,177 (1.68722 %)	1,771,447 (5.31396 %)	2,364,298 (8.81898 %)	2,730,620 (6.66455 %)	1,954,752 (6.25768 %)	819,745 (2.61970 %)	645,875 (1.92455 %)	1,490,443 (3.49656 %)
Fungal	348,489 (1.16744 %)	301,127 (0.83192 %)	336,543 (0.91135 %)	73,745 (0.23787 %)	78,112 (0.22931 %)	665,311 (1.97220 %)	347,068 (1.04113 %)	237,889 (0.88734 %)	1,022,283 (2.49506 %)	76,744 (0.24568 %)	826,274 (2.64057 %)	940,198 (2.80156 %)	171,043 (0.40126 %)
Archaeal	8 (0.00003 %)	0	6 (0.00002 %)	0	0	473 (0.00140 %)	321 (0.00096 %)	99 (0.00037 %)	102 (0.00025 %)	0	22 (0.00007 %)	18 (0.00005 %)	2057 (0.00483 %)
Parasitic Protozoan	222,058 (0.74389 %)	54,546 (0.15069 %)	108,945 (0.29502 %)	22,349 (0.07209 %)	16,298 (0.04785 %)	278,625 (0.82593 %)	93,886 (0.28164 %)	96,054 (0.35829 %)	139,132 (0.33958 %)	1609 (0.05317 %)	264,459 (0.84515 %)	225,398 (0.67163 %)	88,534 (0.20770 %)
Other*	60,570 (0.20291 %)	213,992 (0.59120 %)	39,762 (0.10767 %)	56,480 (0.18218 %)	468,183 (1.37442 %)	163,876 (0.48578 %)	701,974 (2.10577 %)	863,334 (3.22029 %)	1,012,936 (2.47225 %)	703,031 (2.25059 %)	101,959 (0.32584 %)	84,627 (0.25217 %)	867,090 (2.03418 %)
No match	313,516 (1.05028 %)	568,165 (1.56967 %)	135,454 (0.36681 %)	109,647 (0.35367 %)	75,969 (0.22302 %)	473,923 (1.40486 %)	716,436 (2.14915)	584,123 (2.17882)	889,726 (2.17153 %)	80,154 (0.25659 %)	474,281 (1.51568 %)	343,492 (1.02352 %)	1,022,707 (2.39926 %)

MS multiple sclerosis, *CIS* clinically isolated syndrome, *RON* recurrent optic neuritis, *OND* other non-inflammatory neurological disease
*Sequences related to plants, plant viruses, and synthetic DNA constructs

Table 2 The most frequently identified species/genera in cerebrospinal fluid from 12 patients with Central Nervous System Idiopathic Inflammatory Demyelinating Disorder (IIDD) and one patient with other non-inflammatory neurological disease (OND)

Sample ID	Diagnosis	Viruses*	Bacteria**	Fungi**	Parasites/Protozoa**
Pt. 1	MS		Escherichia (48,060)	Cladosporium (41,876)	Besnoitia (77,858)
			Streptococcus (13,647)	Funneliformis (32,047)	Alexandrium (15,146)
			Staphylococcus (13,309)	Glomus (22,485)	Prorocentrum (9548)
			Salmonella (12,721)	Galactomyces (18,469)	Amphidinium (5908)
			Pseudomonas (10,004)	Rhodotorula (7815)	Plasmodium (5367)
Pt. 2	MS		Acinetobacter (563,147)	Malassezia (90,158)	Albugo (10,690)
			Corynebacterium (368,758)	Ascomycota (28,713)	Strombidinopsis (4458)
			Staphylococcus (269,152)	Pleosporales (13,919)	Pseudoplatyophrya (3229)
			Streptococcus (253,844)	Sclerotium (9855)	Stephanopyxis (2563)
			Actinomycetales (251,931)	Saccharomycetales (7863)	Protostelium (1901)
Pt. 3	MS		Escherichia (59,367)	Cladosporium (38,058)	Besnoitia (37,811)
			Bacillus (36,154)	Galactomyces (28,425)	Alexandrium (5553)
			Streptococcus (18,925)	Funneliformis (15,555)	Albugo (3526)
			Staphylococcus (18,506)	Glomus (11,732)	Stemonitis (3105)
			Micrococcus (14,503)	Candida (9920)	Plasmodium (2842)
Pt. 4	CIS	Varicella-zoster virus (11)	Helicobacter (68,198)	Ascomycota (11,755)	Strombidinopsis (1663)
			Acinetobacter (64,688)	Malassezia (7721)	Albugo (1516)
			Corynebacterium (54,628)	Alternaria (2710)	Euglena (1240)
			Staphylococcus (49,276)	Leptosphaeria (2633)	Nannochloropsis (1232)
			Actinomycetales (30,954)	Zymoseptoria (1858)	Bacillariophyta (1156)
Pt. 5	RON		Corynebacterium (59,747)	Triposporium (4346)	Plasmodium (2760)
			Acinetobacter (49,120)	Mollisina (3997)	Nannochloropsis (2475)
			Bradyrhizobium (39,549)	Podosphaera (3828)	Albugo (2299)
			Micrococcus (39,539)	Melampsora (2973)	Eunotia (1780)
			Klebsiella (29,393)	Pseudogymnoascus (2795)	Babesia (605)

(continued)

Table 2 (continued)

Sample ID	Diagnosis	Viruses*	Bacteria**	Fungi**	Parasites/Protozoa**
Pt. 6	MS		Escherichia (49,987)	Galactomyces (68,600)	Besnoitia (88,141)
			Propionibacterium (40,327)	Funneliformis (43,701)	Stemonitis (10,829)
			Microlunatus (29,628)	Glomus (36,135)	Alexandrium (9832)
			Bacillus (26,898)	Cladosporium (27,492)	Albugo (9047)
			Streptococcus (16,102)	Candida (25,745)	Plasmodium (6520)
Pt. 7	MS		Staphylococcus (471,858)	Knufia (28,791)	Besnoitia (67,537)
			Rothia (226,150)	Funneliformis (25,483)	Stemonitis (1592)
			Pseudomonas (128,641)	Glomus (19,359)	Plasmodium (1045)
			Escherichia (49,170)	Exophiala (10,727)	Polysphondylium (646)
			Lactobacillus (40,061)	Penicillium (7499)	Vermamoeba (628)
Pt. 8	MS		Pseudomonas (922,707)	Funneliformis (20,089)	Besnoitia (62,800)
			Staphylococcus (93,740)	Glomus (14,996)	Albugo (6334)
			Escherichia (84,144)	Malassezia (13,442)	Babesia (2936)
			Stenotrophomonas (61,888)	Debaryomyces (8940)	Stemonitis (1511)
			Corynebacterium (52,014)	Candida (8839)	Plasmodium (1449)
Pt. 9	MS		Pseudomonas (885,872)	Malassezia (199,620)	Besnoitia (72,608)
			Escherichia (160,595)	Brachyalara (34,938)	Amphifilidae (11,494)
			Streptococcus (89,594)	Sclerotium (32,549)	Albugo (7412)
			Rothia (67,661)	Funneliformis (28,706)	Babesia (4581)
			Bacillus (52,881)	Gigaspora (2,2347)	Plasmodium (4580)
Pt. 10	MS		Micrococcus (496,707)	Peniophora (9950)	Plasmodium (3368)
			Lactococcus (119,025)	Malassezia (5551)	Soliformovum (1307)
			Pseudomonas (114,800)	Rhodotorula (3468)	Pylaiella (1154)
			Bradyrhizobium (101,190)	Falciformispora (2664)	Amphifilidae (880)
			Staphylococcus (81,941)	Candida (1637)	Albugo (851)

(continued)

Table 2 (continued)

Sample ID	Diagnosis	Viruses*	Bacteria**	Fungi**	Parasites/Protozoa**
Pt. 11	MS		Kocuria (91,204)	Galactomyces (81,037)	Besnoitia (100,425)
			Streptococcus (43,640)	Funneliformis (52,862)	Alexandrium (10,612)
			Propionibacterium (42,704)	Cladosporium (50,749)	Stemonitis (9079)
			Escherichia (33,717)	Glomus (42,829)	Prorocentrum (6681)
			Micrococcus (31,223)	Candida (28,972)	Albugo (6641)
Pt. 12	MS		Moraxella (107,806)	Galactomyces (102,358)	Besnoitia (104,194)
			Pseudomonas (45,308)	Cladosporium (45,585)	Stemonitis (9481)
			Escherichia (38,946)	Funneliformis (44,676)	Albugo (8374)
			Streptococcus (38,923)	Glomus (36,052)	Polysphondylium (8182)
			Corynebacterium (28,659)	Candida (34,998)	Plasmodium (5422)
Pt. 13	OND		Pseudomonas (259,271)	Funneliformis (20,226)	Besnoitia (59,244)
			Escherichia (161,936)	Glomus (12,842)	Albugo (3081)
			Bacillus (90,784)	Cladosporium (10,331)	Plasmodium (2994)
			Streptococcus (38,627)	Candida (3224)	Babesia (1869)
			Lactobacillus (37,561)	Alternaria (3171)	Bodonidae (1129)

The numbers of sequences representing each species/genera are shown in brackets
MS multiple sclerosis, *CIS* clinically isolated syndrome, *RON* recurrent optic neuritis, *OND* other non-inflammatory neurological disease
*viruses other than bacteriophages; **5 most numerous genera

Although CSF is considered basically sterile, we detected reads that mapped to all the analyzed categories, i.e., viral, bacterial fungal, and parasitic/protozoal. These results are consistent with the observations from other studies which demonstrate a common presence of DNA contamination in metagenomes, which most likely originates from commercial extraction kits and PCR reagents or has an environmental source. The microbial composition depends on the kind of reagents used and its changes occur even on switching from one batch of the same reagent to another one (Weiss et al. 2014). The role of environmental contaminates in metagenomes is also emphasized by the demonstration of different microbial composition in the same sample when analyzed in different facilities (Salter et al. 2014). The low-biomass microbial populations, such as in CSF, seem to be particularly susceptible to contamination in metagenomic studies (Laurence et al. 2014; Salter et al. 2014).

In conclusion, while analyzing CSF samples from 12 patients with idiopathic inflammatory demyelinating disorder we found DNA of varicella zoster virus in one sample. Numerous bacterial, fungal, parasitic, and protozoal sequences were detected in all analyzed samples, which suggests that a widespread contamination, complicating the interpretation of results, is inherent to metagenomic studies.

Acknowledgment This study was supported by grants from the Foundation for Polish Science – 67/UD/SKILLS/2014 and the Polish National Science Center – N/N401/646940.

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 (2016) 26: 99–100
DOI 10.1007/5584_2016
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