Bronchial hyperresponsiveness: animal models and clinical aspects

PhD Thesis

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2013

List of papers included in this thesis

I. Petak F, *Czovek D*, Novak Z: ***Spirometry and forced oscillations in the detection of airway hyperreactivity in asthmatic children.*** *Pediatric Pulmonology* 47:(10) pp. 956-965. (2012)

II. *Czovek D*, Novak Z, Somlai C, Asztalos T, Tiszlavicz L, Bozoki Z, Ajtai T, Utry N, Filep A, Bari F, Petak F: ***Respiratory consequences of red sludge dust inhalation in rats.*** *Toxicology Letters* 209:(2) pp. 113-120. (2012)

III. *Czovek D*, Petak F, Donati Y, Belin X, Pache JC, Barazzzone C, Habre W: ***Prevention of the early phase of hyperoxia-induced lung injury by sildenafil or vasoactive intestinal peptide.*** *Submitted to Respiratory Research* (2013)

1 INTRODUCTION

Asthma, the most frequent chronic lung disease in childhood, is a considerable health problem worldwide. Besides biophysical and economic/psychosocial causes, environmental factors can also pose a high risk of the development of asthma or asthma-like symptoms. Exposure to indoor/outdoor allergens, tobacco smoke or air pollution and an urban lifestyle may also play a part in the emergence of the disorder. Such stimuli can provoke an immune response leading to an imbalance in the production of anti-inflammatory/inflammatory cytokines. The chronic presence of inflammation results in a hypersensitive reaction in the airways, which is a characteristic feature of asthma. This bronchial hyperresponsiveness (BHR) is manifested in exaggerated responses to a wide range of seemingly harmless stimuli involving a constriction of the airway smooth muscle, an elevated secretion of the submucosal glands and swelling of the bronchial wall. The mechanisms underlying BHR are not completely understood. In humans, the effects of environmental and genetic factors in the pathogenesis of asthma cannot be distinguished. Inbred animals, however, may serve as ideal models via which to investigate the roles of environmental factors and inflammation without the confounding influence of the genetic background.

1.1 Dust inhalation and BHR

Airway irritation following the inhalation of ambient particulate matter contributes to an increased prevalence and the exacerbation of asthma. The unfortunate red sludge disaster that occurred when the pond dam of a red sludge reservoir plant burst on October 4 2010 gave rise to an extreme situation and health hazard in the area near the Hungarian town of Ajka. Health professionals agreed that the most important health risk was related to the inhalation of the red sludge dust (RSD) formed when the red sludge dried and its particles were swept into the atmosphere by the wind. Since the atmosphere may also be contaminated the RSD if the common dry storage method is used for the deposition of this byproduct, this environmental threat may additionally involve workers or inhabitants in regions neighboring plants where alumina is produced from bauxite by the Bayer process.

1.2 Hyperoxia-induced lung damage

While the adverse pulmonary consequences of O_2 toxicity have been well established, hyperoxia exposure is often required in clinical situations involving severely compromised oxygenation, such as in premature neonates or in patients with acute lung injuries. Since prolonged exposure to an elevated O_2 concentration poses the risk of long-term adverse alterations in the lung architecture and pulmonary function, the prevention of hyperoxia-induced lung injury is of major importance. It is therefore of fundamental importance to develop an animal model with which the short-term effects of hyperoxia on inflammation and BHR can be systematically characterized. With such a model, the effectiveness of preventive

strategies against O_2 toxicity can also be investigated. Many previous studies have highlighted the roles of decrease in nitric oxide (NO) production in the lungs. Thus, restoration of the NO-dependent processes with a PDE_5 inhibitor, such as sildenafil, may provide an effective protection against the development of lung injury following hyperoxia. The lack of the NO-mediated effects may also be compensated by enhancing the vasoactive intestinal peptide (VIP) pathway, which additionally regulates the smooth muscle tone, and potentiates the release of NO from the endothelium and the VIP/NO-containing nerve fibers. All these mechanisms indicate that influencing these pathways may be beneficial in the protection against hyperoxia-induced lung injury.

1.3 Detection of BHR

The assessment of BHR plays a key role in the diagnosis of asthma. In older children or adults, the BHR can be detected by performing spirometry during airway challenges. The airway obstruction leads to a reduced airflow during forced exhalation (e.g. the average forced expired flow between 25% and 75% of the volume expired- FEF_{25-75}) and smaller partial expiratory lung volumes (e.g. the forced expiratory volume in the first second of expiration- FEV_1). The forced vital capacity (FVC) may be normal or slightly decreased because of the hyperinflated lungs in asthma. Although forced expiratory maneuvers furnish valuable parameters to through which detect BHR, such measurement of the lung function preschool children is limited by their inability to provide the close cooperation necessary for spirometry. The forced oscillation technique (FOT) requires no special breathing maneuvers and measures the input impedance of the respiratory system (Zrs) with, its real part directly reflecting the overall airway caliber and the imaginary part related to the elasticity of the respiratory tissues. Thus, in contrast with spirometry, the FOT provides direct information on the mechanical properties of the airways and the respiratory tissues. Despite the potential of the FOT to detect early bronchoconstriction following different challenges, the sensitivity of this method for the detection of BHR has not been systematically compared with that of the gold standard spirometry in asthmatic children.

2 AIMS

The main goals of the studies included in the present thesis are related to the attainment of a greater understanding of the underlying mechanisms of BHR. Specifically, the studies were designed with three particular aims:

- To investigate the consequences of chronic RSD inhalation on the airway and respiratory tissue mechanics in an animal model. We further set out to explore whether chronic RSD exposure leads to BHR, which is the hallmark feature of airway susceptibility.
- To unravel the links between the morphological, biochemical and functional changes in the early phase of hyperoxia exposure and to assess the effectiveness of preventive

treatment by enhancing the NO/cGMP pathway in an animal model of short-term O_2 exposure in immature rats. We hypothesized that sildenafil and VIP have the potential to exert protection in the early phase of hyperoxia-induced lung damage and BHR by inhibiting the initiation of the cascade mechanism that leads to an irreversible lung injury in developing lungs.

- To establish which FOT parameter is most appropriate for the detection of BHR in asthmatic children, and more specifically to compare the sensitivities of the FOT and spirometry during airway challenges involving the inhalation of various direct stimuli commonly utilized in clinical practice (His and MCh).

3 MATERIALS AND METHODS

The RSD exposure protocol was approved by the Experimental Ethics Committee of the University of Szeged (I-74-17/2010) and the Animal Health and Welfare Office of the local authorities in Hungary. The experimental protocols on Sprague-Dawley rats were approved by the Experimental Ethics Committee of the University of Geneva and the Animal Welfare Committee of the Canton of Geneva (1051/3691/II). The clinical research protocol was approved by the Clinical Ethics Committee of the University of Szeged (Ref. No. WHO2803).

3.1 Lung function measurements

3.1.1 Respiratory mechanics- FOT

The frequency ranges studied in the various protocols were chosen in accordance with the research question and the measurement conditions. Thus, a low-frequency oscillation technique (LFOT) was applied in rats in order to assess the airway and respiratory tissue mechanics (elastance- H and damping- G) separately. Otherwise, the medium frequency range was studied in spontaneously breathing children since it provides detailed information concerning the mechanical status of the airways and the respiratory tissue elasticity without the need for an apneic period.

3.1.1.1 Measurements in rats

Zrs was estimated with the LFOT, as described in detail previously. Briefly, the tracheal cannula was connected to a loudspeaker-in-box system generating a composite signal containing 23 components at low frequencies (0.5-20.75 Hz). The forcing signal was driven through a wave-tube into the trachea during 6-s apneic periods. Two identical pressure transducers were used to measure the lateral pressures at the loudspeaker and at the tracheal end of the wave-tube. A deep inspiration to a pressure of 30 cmH_2O was applied before the first FOT measurement so as to standardize the volume history. The mechanical ventilation was then paused at end-expiration, and 4-6 6-s-long recordings were collected at 1-min intervals between each measurement under baseline conditions and following MCh provocations. Zrs was calculated by applying the transmission line theory. A model including

frequency-independent resistance (Raw), inertance (Iaw) and a constant-phase tissue compartment with tissue parameters for G and elastance H was fitted to the ensemble-averaged Zrs spectra. The lung tissue hysteresivity (η) was calculated as η=G/H.

3.1.1.2 Measurements with the classical setup in human patients

The FOT measurements were performed in accordance with ERS guidelines. To measure Zrs during spontaneous breathing, a commercially available device (i2m, Chess Medica, Oostakker, Belgium) was applied. Briefly, the forcing signal between 4 and 48 Hz was applied to the child via a disposable bacterial filter and a mouthpiece. Zrs was obtained from 8-s-long measurements while the child performed normal breathing during the oscillatory measurements. During the data-recording periods, the children sat upright, wearing a noseclip and breathing quietly through the mouthpiece. They were asked to support their cheeks with their palms during these maneuvres in order to minimize upper airway shunting. The Zrs recordings were accepted if the coherence functions between the pressure and flow signals were generally greater than 0.95, no leak was noted around the mouthpiece, and no technical artefact occurred due to coughing, swallowing or glottis closure.

The changes in the resistive properties of the respiratory system were evaluated by calculating the average resistance between 4 and 24 Hz (R_{4-24}) and by extracting the resistance at 6 Hz (R_6; the lowest frequency where the resistance was reliably measured). The elasticity of the respiratory system was assessed by calculating the sum of all negative values in the imaginary part of Zrs from 4 Hz, which corresponds to the area between the reactance curve and the x axis (AX), and resonant frequency (Fr) was also extracted.

3.1.2 Spirometry

Forced expiratory flow-volume curves were measured by means of a commercial spirometer, including a screen pneumotachograph equipped with a differential pressure transducer. The flow signal was integrated to obtain the changes in lung volume during the forced expiratory maneuvers. FEV_1, FVC and FEF_{25-75} were extracted from the recordings. Three technically acceptable reproducible measurements were made, and the highest values of the spirometric parameters were extracted for the final analyses.

3.1.3 Plethysmography in rats exposed to hyperoxia or room air

End-expiratory lung volume (EELV) was measured at a positive end-expiratory pressure (PEEP) of 2.5 cmH_2O by using a whole-body plethysmograph, as detailed previously. The rats were anesthetized and the trachea was cannulated. Briefly, the rats were placed in a supine position in a sealed Plexiglas chamber. The tracheal cannula was connected to the respirator and also to a pressurized (2.5 cmH_2O) loudspeaker chamber. Before the measurement, the mechanical ventilation was paused, the plethysmograph was opened to the atmosphere and the trachea was opened to the loudspeaker chamber to equilibrate the lungs to a pressure of 2.5 cmH_2O. The airway opening and the plethysmograph box were then closed until the first few breathing efforts generated by the animal against the closed trachea. Six-to-

eight breathing maneuvers were recorded for 10 s by measuring the tracheal (P_{tr}) and box (P_{box}) pressure changes. The recordings of P_{box} were then corrected for the thermal properties of the plethysmograph. Via Boyle's law, EELV was calculated from the relationship between the corresponding changes in P_{tr} and P_{box}.

3.2 Treatment exposures and experimental protocol

3.2.1 RSD inhalation

Certified red sludge samples were obtained from the local office of the National Directorate General for Disaster Management in Devecser 10 days after the disaster. The red sludge was dried and mechanically ground to form a powder containing fine particles. The particle size distribution was determined. Two groups of male Wistar rats were studied (weight range 350-455 g). The animals in the RSD group were exposed to RSD in the exposure chamber for 8 h a day for 2 weeks (n=10). The animals in the control group underwent the same procedure except that they were allowed to breathe room air (n=11). The rats in both groups had access to food and water *ad libitum* throughout the entire exposure period. The experiment was performed on day 15 (Fig. 1.).

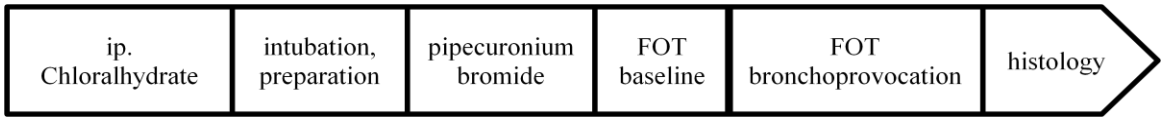

Figure 1. Experimental protocol after RSD or room air exposure on day 15.

3.2.2 Hyperoxia-exposure and treatments; experimental protocol

Weanling male Sprague-Dawley rats (80-125 g) were exposed to either hyperoxia (>95% O2) or normoxia (room air, Group C, n=8) for 72 hours. The hyperoxia-exposed animals were randomized to 3 groups: no additional treatment (Group HC, n=8), the oral administration of sildenafil (20 mg/day; Group HS, n=7) or the i.p. injection of VIP (150 μg/day; Group HV, n=8), in both cases starting simultaneously with the commencement of the exposure to hyperoxia. During the 72-hour oxygen (O_2) or room air exposure the rats were kept in a normobaric Plexiglas chamber. The chamber was opened for a short time (<5 min) to allow delivery of the daily treatment. The $O_2$and CO_2 levels were checked twice a day.

On the day 4, rats were first anesthetized and tracheostomized, mechanically ventilated (8 ml/kg, 110 breath/min, PEEP 2.5 cmH_2O). After completion of the EELV measurements, femoral artery and vein were cannulated for drug delivery and blood gas analysis. Since application of FOT to assess the respiratory mechanics requires apnea, atracurium was administered i.v. before the measurements in order to prevent the spontaneous breathing. The mechanical ventilation was then paused at end-expiration, and 4-6 recordings were collected under baseline conditions. To assess the lung responsiveness MCh was infused i.v. (4-8-16 μg/kg/min) Three Zrs data recordings were collected and ensemble-averaged under a steady-state conditions 6 min after the onset of MCh provocation at each infusion level. After the last

dose, a 15-min period was allowed for the rat to recover and 3 further Zrs recordings were made and ensemble-averaged.

At the end of the experiments, bronchoalveolar lavage fluid (BALF) was collected in order to assess lung inflammation. For histological analyses, the right lung was instilled via the trachea with 4% formol and stained with HE. The left lung was clamped near the bifurcation to avoid its fixation and a piece measuring ~0.5 cm^3 was excised for apoptosis quantification.

3.3 Clinical research study on asthmatic children

Twenty asthmatic children (5 girls and 15 boys, age range 5-18 years) were enrolled into the study. Only patients who did not need salbutamol (Sal) during 8 h before the visit were included in the study. The children visited the pulmonary function laboratory twice in 2 weeks. On the first occasion His provocations were performed, and 2 weeks later the measurements were repeated with MCh challenges. Increasing doses of aerosolized His were administered to the children for 2 min via a face mask, in doubling doses ranging from 0.5 to 16 mg/ml. A set of Zrs recordings and three forced expiratory maneuvers for spirometry were collected after each dose. An interval of at least 5 min was allowed between two consecutive doses. The study was terminated before the highest dose of histamine, or if FEV_1 decreased by more than 20% and/or clinical symptoms of wheeze or a persistent cough were noted. To detect the reversibility of the bronchoconstriction, the challenge protocol was terminated in all children by administering 400 μg of inhaled Sal, followed by the final assessment of Zrs and spirometry. The children revisited the lung function laboratory 2 weeks later. The baseline FOT and spirometry were performed identically as detailed above. Aerosolized saline as the solvent of the agonists was then administered for 2 min, and both the FOT and spirometry were repeated. The bronchoprovocation tests with increasing doses of MCh (0.5 to 8 mg/ml for 2 min) and the broncholysis with Sal was then repeated in the same manner as detailed above.

4 RESULTS

4.1 Basal respiratory mechanical parameters in animal studies

4.1.1 RSD vs. room air inhalation

RSD exposure did not lead to any significant change in Raw ($p=0.65$) or η ($p=0.90$). There was a tendency for G and H to increase in the rats exposed to RSD, but these differences did not reach the level of statistical significance ($p=0.071$ and $p=0.10$, respectively).

4.1.2 Hyperoxia vs. room air exposure

Since a significant difference in the body weight (BW) was found between the groups, the mechanical parameters were corrected by BW. There was no evidence of a statistical significance in the baseline values of Raw·BW following hyperoxia exposure, whereas G·BW

was significantly elevated in Group HC ($p<0.01$ vs. Group C), and H·BW was also higher in Groups HC ($p<0.05$) and HV ($p<0.01$) (Fig. 2.).

Exposure to hyperoxia significantly increased EELV in the rats in Group HC ($p<0.001$ vs. Groups C, HS and HV), but this was blocked by the sildenafil and VIP treatments.

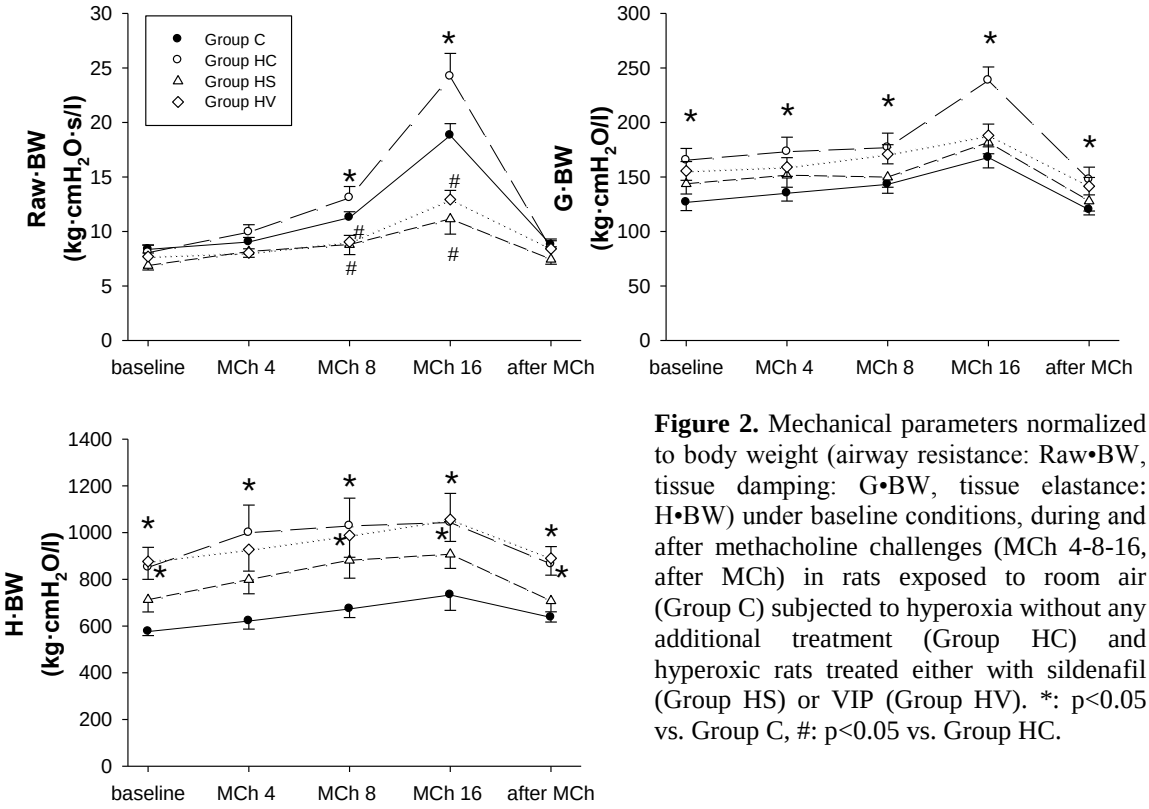

Figure 2. Mechanical parameters normalized to body weight (airway resistance: Raw•BW, tissue damping: G•BW, tissue elastance: H•BW) under baseline conditions, during and after methacholine challenges (MCh 4-8-16, after MCh) in rats exposed to room air (Group C) subjected to hyperoxia without any additional treatment (Group HC) and hyperoxic rats treated either with sildenafil (Group HS) or VIP (Group HV). *: $p<0.05$ vs. Group C, #: $p<0.05$ vs. Group HC.

4.2 Changes in lung mechanics following bronchial challenges

4.2.1 MCh challenges after RSD or room air exposures

MCh caused marked dose-dependent increases in Raw, G and η ($p<0.001$), while the increases in H were less pronounced, but still statistically highly significant ($p<0.001$).

The significant interactions revealed between MCh and RSD by two-way ANOVA ($p=0.005$) indicated that RSD inhalation affected the magnitude of the MCh-induced increases in Raw, i.e. BHR developed in the RSD group. The presence of BHR following chronic RSD inhalation was also reflected in the significantly lower ED_{100} values in the RSD group than in the controls (5.3±0.2 vs. 7.5±0.9 μg/kg, respectively, $p=0.041$). The tissue parameters were not affected significantly by RSD inhalation (Fig. 3.; $p=0.69$, $p=0.15$ and $p=0.37$ for G, H and η, respectively).

4.2.2 MCh provocations after exposure to hyperoxia or room air

The development of BHR was apparent from the significantly greater responses to MCh in the rats in Group HC ($p<0.05$ and $p<0.001$, at the second and third doses of MCh vs. Group C, respectively), whereas a significant reduction in the lung responsiveness to MCh was obvious in the hyperoxic rats treated with sildenafil (Group HS, $p<0.001$ at the third dose of MCh vs. Group C) or VIP (Group HV, $p<0.001$ at the third dose of MCh vs. Group C). The enhanced lung responsiveness in Group HC was also manifested in the significantly greater increases in G·BW in response to MCh ($p<0.005$), whereas there was no evidence of an enhanced response in this parameter following either sildenafil or VIP treatment. No statistically significant treatment-dependent differences were observed in the slight MCh-induced elevations in H·BW. All mechanical parameters exhibited a complete recovery by 15 min after the last MCh dose (Fig. 2.).

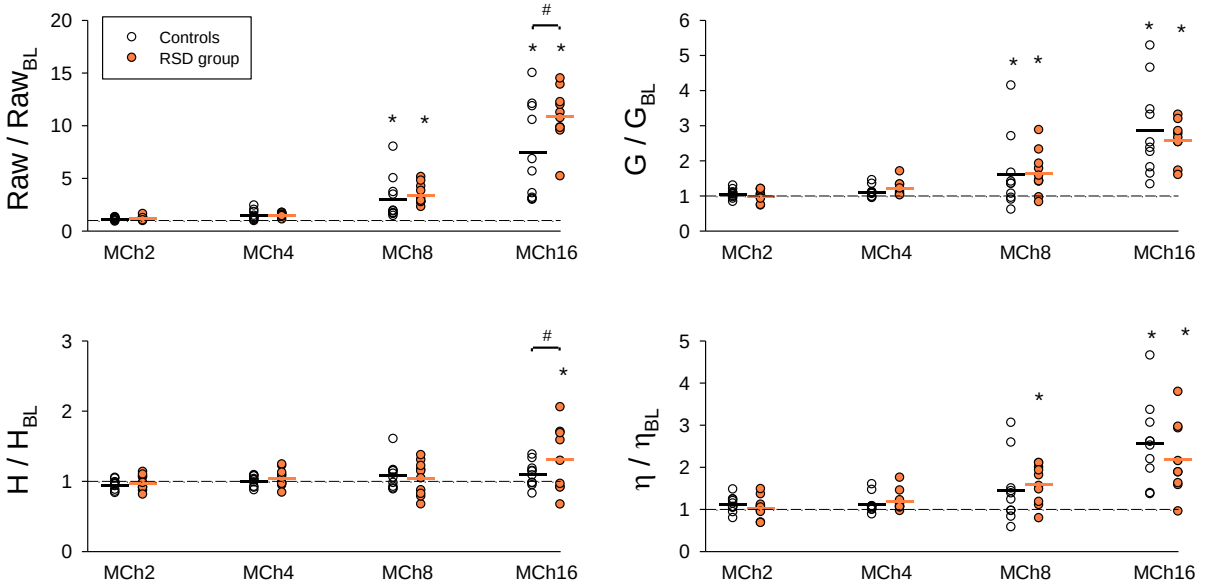

Figure 3. Airway and respiratory tissue parameters measured after MCh challenges relative to the baseline (BL) values in the control rats (closed circles) and in rats exposed to RSD (open circles). Horizontal lines denote the group mean values. *: $p<0.05$ vs. the baseline; #: $p<0.05$ between the groups.

4.3 Inflammation and histologycal changes

4.3.1 Inflammatory response after RSD inhalation

Inhalation of RSD led to mild-to-moderate perivascular infiltration ($p=0.003$; in decreasing sequence of particle amount in the lymphocytes, plasma cells, eosinophils, mastocytes and neutrophils) and the perialveolar infiltration ($p<0.001$; in decreasing sequence in macrophages, lymphocytes and plasma cells) of inflammatory cells with no difference in BALT hyperplasia ($p=0.43$). In the alveolar areas RSD particles were recovered as fine, granular and pigmented cytoplasmatic inclusions in the alveolar macrophages. Chronic exposure to RSD induced the development of mild hyperemia ($p=0.03$).

4.3.2 Hyperoxia-induced inflammation

Hyperoxia led to an increased number of inflammatory cells in the BALF collected from the animals in Group HC ($p<0.001$ vs. Group C and $p<0.01$ vs. Groups HS and HV) with an increased percentage of PMN cells ($p<0.001$ vs. Groups C, HS and HV) and an elevated protein content of the supernatant ($p<0.001$).

The lung sections of untreated animals subjected to hyperoxia revealed an aberrant alveolarization with decreased alveolar septation and consequently enlarged alveolar spaces in comparison with normoxia-exposed lungs. These changes are expressed by the significantly increased mean linear intercept (MLI) value ($p<0.001$ vs. Group C). When sildenafil or VIP treatment was applied concomitantly with hyperoxia, the normal alveolarization was preserved, as indicated by the MLI values, which exhibited no statistically significant difference from those for the normoxia group.

4.4 Results of the clinical study

4.4.1 Basal lung functional and mechanical parameters in asthmatic children

The children had normal basal lung function parameters with a mean percentage predicted value for FEV_1 of 104% (range 93-136%) and a mean respiratory resistance between 4 and 24 Hz (R_{4-24}) of 91% (range 74-105%).

4.4.2 Detection of BHR

Changes observed in the spirometric and FOT parameters following His challenges are depicted in Fig. 4. Increasing doses of aerosolized His caused statistically significant decreases in the lung volume/flow indices determined by spirometry ($p<0.001$ for FEV_1 and FEF_{25-75}; $p=0.011$ for FVC). The parameters extracted from the real and imaginary parts of the Zrs spectra exhibited dose-dependent elevations after His provocation ($p<0.001$ for R_{4-24}, R_6 and Fr, $p=0.005$ for AX). The data analyses revealed that the changes in the parameters determined with the FOT were statistically detectable at lower doses of His (after 0.5 mg/ml in R_{4-24}, R_6 and Fr; and after 2 mg/ml in AX) than those obtained by spirometry (after 1 mg/ml in FEV_1, FVC and FEF_{25-75}).

Inhalation of saline following the baseline recordings did not cause significant changes in any of the lung function parameters ($p=0.47$, 0.34, 0.75, 0.98, 0.84, 0.94 and 0.55 for FEV_1, FEF_{25-75}, FVC, R_{4-24}, R6, AX and Fr, respectively). Inhalation of increasing doses of MCh resulted in dose-dependent decreases in FEV_1, FEF_{25-75} ($p<0.001$ for both) and FVC ($p=0.002$). All parameters obtained with the FOT displayed dose-dependent significant increases following MCh inhalation ($p<0.001$ for all). The changes in the spirometric indices became statistically significant after a MCh dose of 1.0 mg/ml. Inhalation of even the smallest concentration of MCh (0.5 mg/ml) was sufficient to cause statistically detectable increases in the FOT parameters R_{4-24}, R_6 and Fr, while significant increases in AX were observed after a MCh dose of 1 mg/ml.

Comparison of the parameters obtained by spirometry and FOT

4.4.2.1 <u>Relationships between the parameters detected</u>

Following His challenges, strong and statistically significant correlations were observed between FEV_1 and R_{4-24} (R^2=0.6, p<0.0001) and between FEF_{25-75} and R_6 (R^2=0.47, p<0.0001).

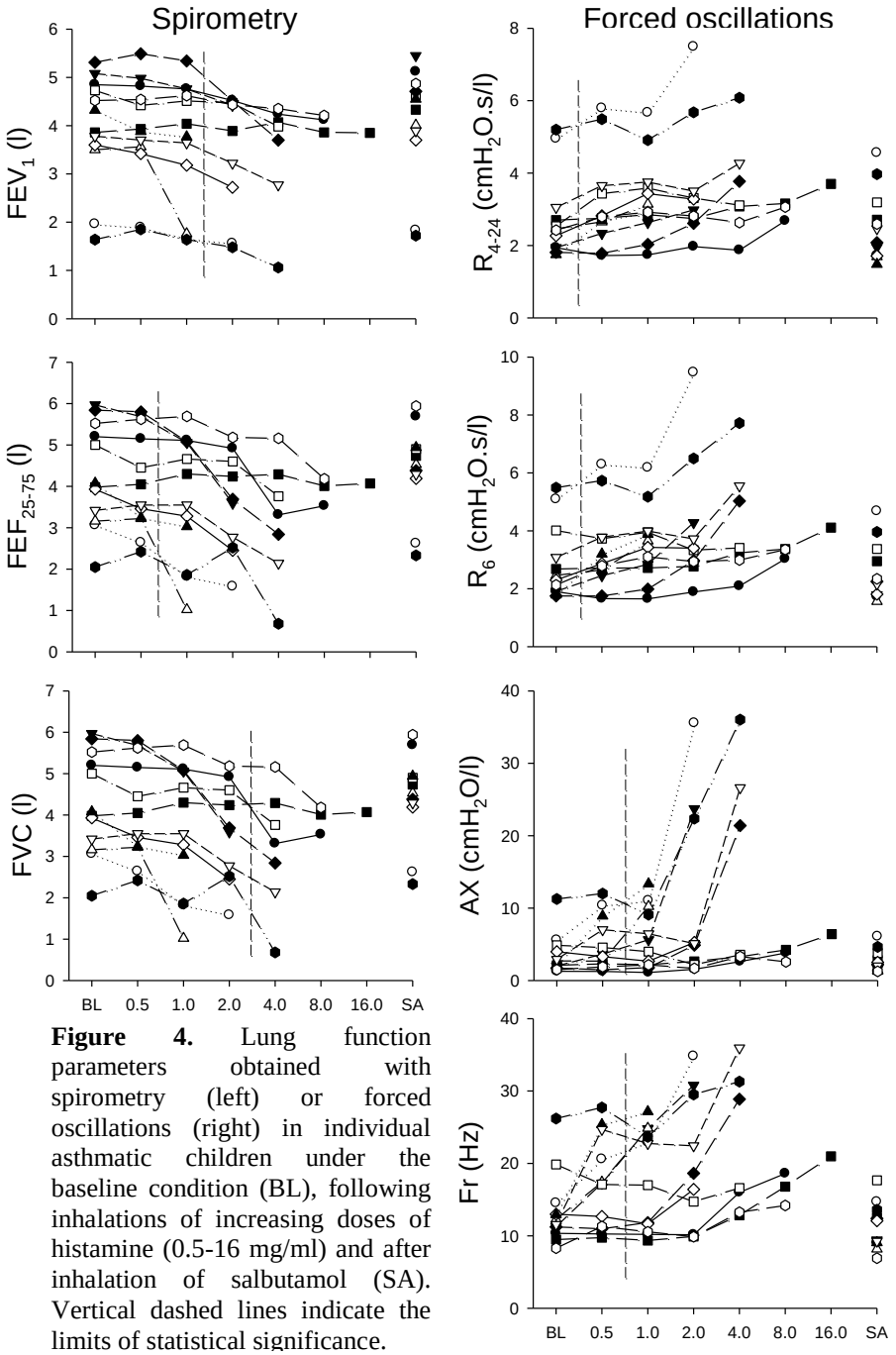

Figure 4. Lung function parameters obtained with spirometry (left) or forced oscillations (right) in individual asthmatic children under the baseline condition (BL), following inhalations of increasing doses of histamine (0.5-16 mg/ml) and after inhalation of salbutamol (SA). Vertical dashed lines indicate the limits of statistical significance.

The correlations between these parameters following MCh challenges were somewhat weaker, but their associations were still close and highly significant (R^2=0.48, p=0.0001 between FEV_1 and R_{4-24} and R^2=0.5, p<0.0001 between FEF_{25-75} and R_6).

4.4.2.2 Variabilities

The smallest variabilities were found for FEV_1 (4.3%) and FVC (4.4%), those for FEF_{25-75}(7.1%), R_{4-24} (10.3%) and R_6 (11.3%) were higher, and the highest were observed for AX (25.6%) and Fr (16.7) in the children who participated in both the FOT and spirometry.
Since the variability in the FOT parameters was greater than that in the spirometric indices, we examined the magnitude of the changes in the resistance parameters where the His and MCh provocation tests became positive according to the ATS/ERS guidelines. When a positive response was detected during the bronchial challenge tests, the changes in R_{4-24} were about twice as high as those in FEV_1 for both of the constrictor agonists studied, while the elevations in R_6 were even more pronounced.

4.4.2.3 SD indices

The sensitivity of the FOT parameter R_{4-24} was greatest following both His and MCh challenges. R_6 and FEF_{25-75} exhibited similarly high sensitivities in the detection of His- or MCh-induced airway narrowing. The parameters AX, Fr, FEV_1 or FEF_{25-75} also demonstrated bronchoconstriction after His and MCh challenges, but only after the second dose of the constrictor agonists.

5 DISCUSSION

Airway susceptibilities involving BHR are posing an increasing worldwide challenge for health professionals. The studies included in the present thesis focus on the environmental factors involved in the pathogenesis of BHR in animal models. Airway irritations leading to chronic inflammation may result from the inhalation of airborne particles and/or an altered O_2 content of the inspired gas mixture. Treatment of BHR related to asthma has been well established by inhibiting the inflammation and by affecting the receptors responsible for the regulation of the airway tone. However, treatment strategies for hyperoxia-induced lung damage that may also induce BHR have not been completely characterized. Thus, interest has emerged in protection against the adverse pulmonary effects of O_2 toxicity. Since the first symptoms of asthma are revealed before school age, a clinical protocol was designed to detect BHR in asthmatic children by using a novel measurement technique that can be applied in pre-schoolers.

5.1 Animal models

5.1.1 Lung mechanics in animal models

5.1.1.1 Effects of RSD on basal lung function and lung hyperresponsiveness

Despite the high RSD concentration and the evidence that it accessed the lower respiratory tract, no clinically significant adverse changes in the baseline airway or respiratory tissue mechanics were detected. The tendency to higher G and H in the animals in the RSD group may be a consequence of the mild inflammation demonstrated by the lung histology. Since merely mild airway inflammation has no major effects on the basal lung function, the lack of difference between the two groups is consistent with the previous results.

In agreement with previous findings that inhaled particulate matter induced asthma-like symptoms, the chronic airway inflammation that occurred following RSD inhalation led to the development of BHR, which was manifested in significantly elevated responses to MCh and subsequent decreases in ED_{100}. As concerns the mechanism of the emergence of BHR, the involvement of macrophages can be anticipated from the histological findings, since the role of alveolar macrophages in the development of an inflammation response in the small airways was evidenced previously. Chronic dust inhalation drives the influx of macrophages and other inflammatory cells, and these factors may lead to the progress of chronic lung diseases such as asthma, emphysema, bronchiolitis or fibrosis.

5.1.1.2 Respiratory function and lung responsiveness after hyperoxia exposure

The basal Raw was not affected by hyperoxia exposure; this is also in line with the notion that Raw is determined primarily by the geometry of the central conducting airways, which remained unaffected. The 72-h exposure to hyperoxia resulted in increases in the basal values of both viscoelastic tissue parameters (Fig. 2; G·BW and H·BW). Since the hyperoxia-induced increases in G·BW and H·BW were proportional, the enhancement of ventilation heterogeneities was not likely to have played a role in these findings. It seems more probable that perivascular and interstitial edema formation is responsible for these proportional elevations, this being substantiated by the histological findings and the elevated protein level in the BALF. Nevertheless, other processes may also have been involved in the increase in H·BW, since an elevation in elastance was also observed in the VIP-treated rats, despite the lack of manifest edema. Such processes may be related to hyperventilation throughout the exposure, striving to compensate the hyperoxia and causing hypertrophy of the intercostal muscles, leading to stiffening of the entire respiratory system.

It is noteworthy that this functional abnormality is already present despite the lack of remodeling of the bronchial wall at this early stage of O_2 toxicity. Both sildenafil and VIP compensated and even overcompensated the enhanced lung responsiveness. The excessive NO level may explain the diminished BHR by exerting the relaxation potential of the bronchial smooth muscle, which then leads to a decreased response relative to that in naïve animals. Since exposure to hyperoxia causes adverse alterations in the lungs at functional,

structural and molecular levels, we characterized whether the prevention of functional abnormalities by stimulating the NO pathway is due to maintenance of the normal lung structure and biochemical profile.

5.1.2 Mechanisms underlying BHR following hyperoxia exposure

5.1.2.1 Inflammation

Since the inflammation is of great importance in the development of hyperoxia-induced lung damage, and as the inflammatory cells appear in the first few hours of hyperoxia exposure, early inhibition of the inflammatory response might prevent the lung injury. Treatment with sildenafil or VIP offers effective protection against the deleterious inflammatory events in the lungs by inhibiting PMN cell infiltration into the alveolar compartment and reducing the consecutive tissue destruction and pulmonary edema caused by hyperoxia. Overall, lung inflammation with low levels of bronchodilatory NO production, and alveolar destruction with a subsequent decrease in mechanical support to the small airways, are responsible for the enhancement in lung responsiveness.

5.1.2.2 Lung morphology

In our hyperoxia model, the 72-h exposure to O_2 was sufficiently long for an abnormal alveolar structure to be observed in the developing lungs. Compensation of the decreased NO activity, either through stimulation of the cGMP-dependent effects by using sildenafil to activate VEGF, or through enhancement of the NO production by VIP treatment was able to preserve the normal lung development despite maintenance of the hyperoxic environment. MLI measurement confirmed that enlarged alveolar spaces were observed only in the non-treated O_2-exposed animals; in the other groups, the MLI data were normal. This finding points to a preserved physiological alveolar structure.

5.1.2.3 Changes in lung volume

The changes in lung architecture were also reflected in the hyperoxia-induced EELV elevation in Group HC. In this emphysematous condition, air is trapped in the enlarged alveolar spaces at end-expiration and the gas content of the chest is increased. Moreover, the small airways are likely to display a tendency to collapse during expiration due to their structural impairment, and their function may be further compromised by the decreased elastic support of the damaged lung parenchyma, which would be less able to keep the small airways open. Since the sildenafil and VIP treatments prevented the adverse alterations in the alveolar structure, the EELV must have remained normal in these animals.

5.2 Detection of BHR in children

Since spirometry is a routine procedure in lung function laboratories for the detection of BHR in the diagnosis of asthma, demonstration of the similar ability of the FOT is of major importance, since this technique can be carried out reliably in a much younger age group (as

low as 2 years of age). Nevertheless, children of school age were enrolled in the present study, in order to allow a comparison with spirometry.

5.2.1 Relationships between the parameters obtained with the FOT and spirometry

Strong and highly significant correlations were observed between the corresponding FOT and spirometric parameters, which suggests that airway narrowing is faithfully reflected by both techniques. Nevertheless, the correlation analysis does not take into account the variability of the measured parameters, which is of great importance in a comparison of the ability of a lung function test to measure bronchoconstriction with subsequent promise to detect BHR. Wexperienced greater short- and long-term variabilities for the FOT parameters than those measured with spirometry. This implies that greater magnitudes of changes can be regarded as positive responses in the FOT parameters, which corresponds to previous observations and recommendations for higher acceptance limits of clinically important changes.

5.2.2 SD indicies

In order to take the greater magnitudes of the changes in the FOT parameters into account, the alterations in the lung function indices following the His and MCh challenges were also expressed in SD indicies. This analysis revealed comparable SD indicies for the lung function parameters determined with the FOT and spirometry in the detection of airway narrowing following both the His and the MCh challenges. However, there was a tendency to a greater sensitivity of the FOT parameters relating to the low-frequency part of the Zrs spectra, which was particularly evident for AX. Since AX is extracted from the low-frequency reactance, it primarily reflects changes in the elasticity of the total respiratory system. It therefore seems likely that, besides inducing a constriction of the conducting airways manifested in elevated forced expiratory lung volumes and resistance parameters, the inhalation of the bronchoconstrictor agonists also led to a stiffening of the respiratory tissues. The most probable mechanism responsible for such a change in the respiratory elasticity is the loss of lung volume via the development of air trapping, due to the uneven deposition of the bronchoprovocation agonists, which was earlier clearly demonstrated following challenges with aerosolized His.

6 SUMMARY AND CONCLUSIONS

We investigated the influence of environmental factors on the development of BHR in inbred animals, which excluded the effects of various genetic factors. We demonstrated the development of pulmonary inflammation and subsequent BHR following airway irritations in animal model studies. We also demonstrated the feasibility of detection of BHR in children of school age by using a technique that requires minimal patient cooperation. In all studies included in the present thesis, the basal airway mechanics was unaffected be the altered environment, while the elevated constrictor response of the airway smooth muscle following the application of bronchial challenges was proved by measuring airway properties with the

FOT. Specifically, the findings of the present thesis contributed to the following particular research topics:

- The effects of RSD on the pulmonary system were assessed in an animal model by performing basal lung function measurements, with the assessment of changes in lung responsiveness and also histopathological analyses. When rats were chronically exposed to a high concentration of RSD, the fine powder particles reached the lower respiratory tract, but the alterations in the basal airway and respiratory tissue mechanics were not significant. The mild inflammation that developed around the pulmonary vessels and in the alveolar wall was associated with a mild BHR. Since these mild respiratory symptoms emerged following exposure to extremely high concentrations, our results may suggest that the short-term health hazard as regards the pulmonary system in healthy human adults exposed to RSD inhalation is not greater than that due to urban dust at a comparable concentration.
- In our short-term hyperoxia exposure model, duration of 72 h was sufficient to induce adverse molecular, morphological and functional changes in the lungs. Sildenafil and VIP treatments were able to maintain the normal alveolar structure, inhibit the development of edema and inflammation, and prevent the consequential lung function deterioration by enhancing the activity of NO-mediated pathways involved in the lung damage following hyperoxia. Our findings therefore point to the potential benefit of sildenafil or VIP treatment in the critical acute phase of hyperoxic lung damage, and such treatment should be considered as one of the possible strategies designed to prevent the irreversible functional and structural changes in the developing lungs.

Since the detection of BHR poses an increasing clinical challenge in patients with limited abilities to cooperate, a clinical study was designed to determine the efficiency of the FOT in the detection of BHR following bronchoprovocation tests in asthmatic children. Our clinical findings suggest that

- the resistance and reactance parameters determined with the FOT revealed airway constriction at smaller doses of the agonists than those needed in standard spirometry;
- with regard to the greater variability in the FOT parameters when the FOT is performed concomitantly with spirometry, the two lung function measurement methods have similar sensitivities in the assessment of BHR;
- if the FOT is performed alone, the short-term variability of the resistance parameters decreases markedly and approaches that observed for the spirometric indices.

In summary, the studies included in the present thesis contribute to a better understanding of the BHR that develops in an altered environment by pointing to the complex involvement of inflammatory and cellular mechanisms leading to structural and functional abnormalities. Our clinical study reveals that the use of the FOT may impose less stress on the tested children and may lead to a decrease in the age at which BHR can be detected, so that asthma can subsequently be diagnosed in the preschool age range.

7 ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors.

I thank *Zoltán Novák* who introduced me to clinical research and provided expertise, support and guidance throughout my studies and my experimental work. Thank you for the incessant encouragement, especially before my first presentation, which has had a profound effect on my personal and academic career.

I would also like to express my gratitude to *Ferenc Peták* for his constant support and mentoring, especially during my experimental work. His excellent guidance has been essential in the development of my research carrier. Without your belief and advice I would never have begun my PhD studies.

I am also very grateful to *Walid Habre* for being my host supervisor during my year in Geneva. Thank you for the opportunity to work within your laboratory and for the trust, independence, invaluable advice, training, and support you have provided me.

I wish to express my thanks to *Ferenc Bari,* head of my home institite (Department of Medical Physics and Informatics) for his support, trust, understanding (and for the pressure he you put on me with your recurrent questions).

Thanks to *Mária Balla* for her indefatigable help with my experimental work, and for demonstrating exemplary patience during the very long weekends that we have spent together in the pulmonary function laboratory.

A special thanks goes to *Xavier Belin* for his unique technical support in the animal research projects we have undertaken together in Geneva, and for the never ending days we spent underground without seeing the sun.

I further express my deep gratitude to my friends from Geneva; *Fabiola, Iliona, Camille and Yves*. They were always with me in the challenging moments during and following my stay. Thank you very much for making my year in Geneva unforgettable and enjoyable.

I would like to express my thanks to my friends and collegaues for the nice moments and the pleasant time spent at work in your company. Thank to *Marietta, Magdi, Anna, Darren, Kati, Juli, Zsuzsi* and also to *Bence* for your motivation and humour.

I also wish to thank my *parents*, *sister* and *brother* who have always provided exceptional motivation over the years. Thank you for your love, trust and opportunities you have given me in life.

I wish to express my grateful thanks to *Zoltán Hantos* not only for his professional help, advice and support but also for his invaluable friendship. Zoli, thank you very much for looking after me, and for your help and support in all circumtances. As my gratitude is far too personal I prefer to express it to you in the company with a couple of beers.

Last but by far not least, I would like to thank *Csaba* for his patience, support, and love; even in the most difficult moments. Without you, these years would have been more complicated and less enjoyable. Your particular sense of humor sweetened my days and always provided me with an optimistic attitude. Köszönöm szépen!