Original Article

Budesonide ameliorates lung function of the cigarette smoke-exposed rats through reducing matrix metalloproteinase-1 content

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Abstract: Objectives: This study was conducted to investigate an effect of inhaled budesonide on cigarette smoke-exposed lungs with a possible mechanism involved in the event. Methods: Rats were exposed to air (control) and cigarette smoke (smoking) in presence and absence of budesonide. Inflammatory cell count in bronchoalveolar lavage fluid (BALF), lung function testing, mean linear intercept (MLI) in lung tissue, mean alveolar number (MAN) and a ratio of bronchial wall thickness and external diameter (BWT/D) were determined in the grouped rats, respectively. Contents of matrix metalloproteinase (MMP)-1, MMP-2 and tissue inhibitor of metalloproteinase (TIMP)-2 productions in BALF were examined as well. Results: There were significant changes in the above assessments in the smoking rats as compared to those in the control rats (all P < 0.01 and 0.05). Budesonide inhalation significantly decreased the numbers of the BALF cells and partly reversed lung function decline in the challenged rats (P < 0.01 and 0.05). However, this corticosteroid did not influence pathological changes in fine structures of the tobacco smoke-exposed lungs. Treatment with budesonide resulted in an obvious decrease in the MMP-1 but not MMP-2 and TIMP-2 productions (P < 0.05). Conclusion: Inhaled budesonide mitigates the ongoing inflammatory process in the smoked lungs and ameliorates declining lung function through reducing MMP-1 content.

Keywords: Budesonide, cigarette smoke, pulmonary inflammation, lung function and matrix metalloproteinase

Introduction

Chronic obstructive pulmonary disease (COPD) is a prevalent smoking-related disease for which treatment options are limited to either symptom relief and/or the elimination of the known causes such as cigarette smoking [1]. Since one of the risk factors for COPD globally is tobacco smoking which subsequently leads to a series of pulmonary structure changes associated with chronic pulmonary inflammation [2, 3], COPD has ranked as the third-leading cause of death and the number of deaths is projected to increase due to higher smoking rates in many countries [4]. Although there are currently no therapies that effectively cease or reverse disease progression typically characterized by airway remodeling and emphysema, animal model of COPD has been established to provide valuable information for identifying and testing of new therapeutic approaches [5].

Some studies have shown that levels of MMPs are elevated in the BALF from COPD patients [6, 7], and high levels of both MMP and TIMP have been found in sputum from chronic bronchitis [8] and correlated with decrease in lung function [9, 10]. In further investigation, MMPs, particularly in emphysema, probably participate in proteolytic attack on the alveolar wall matrix [11]. It is a widely accepted theory that an important causative factor is extracellular matrix (ECM) remodeling, resulting from aberrant inflammation and disruption of the proteinase-antiproteinase balance [12]. The identity of candidate proteinases has been a subject of much debate since MMPs constitute a large family of enzymes that remodel ECM molecules...
by degrading ECM and the action is regulated by a group of endogenous tissue inhibitors of TIMPs [13].

Budesonide has a potent anti-inflammatory action. When used as an inhaler, the agent goes directly to the inner lining of the inflamed airways to express its effects. Although the role of many corticosteroids in COPD patients is controversial because of questionable benefit and potentially significant drug toxicity [14], regular treatment with budesonide has shown its clinical benefits in reducing the frequency of COPD exacerbation and improving health status in the patients [15].

This study is to investigate an effect (s) of inhaled budesonide on cigarette smoke-exposed lungs with a mechanism by which this steroid may serve to ameliorate the smoking-related lung functional damage.

Materials and methods

Animals and cigarette smoke exposure

Male SD rats 6 (weeks) weighing about 203 g were purchased from the Experimental Centre of Animals at Hebei Medical University. The animals were housed under specific-pathogen-free conditions for the duration of the experiments. All procedures were reviewed and approved by Hospital Research Review Committee.

Animals were randomly divided into three groups of 10 each. The protocol for making the animal model of cigarette smoke-induced COPD was modified with different treatments during a smoking period. The animal model was done with mainstream smoke exposure (20 cigarettes, twice daily) for 4 consecutive months in a cigarette smoke chamber. 4.0 mg budesonide (AstraZeneca, UK) dissolved in 2.0 ml phosphate buffered saline (PBS) was given by aerosol inhalation once a day for a final month. Some of the smoking rats inhaled an equivalent volume of PBS in the last month. Rats in the control group were exposed to air and treated with aerosol of PBS at the same volume in the same time.

Preparation of BALF and cell count

Rats were anesthetized and lungs were lavaged by instillation and withdrawal of 3.0 ml PBS through a tracheal cannula, and an equal volume of BALF was collected from individual rats. The BALF supernatants were obtained with centrifuge (1500 rpm × 15 min) at 4°C. The protein concentration of the cleared supernatant was determined by BCA assay (Pierce Biotechnology, Rockford, IL). Cleared supernatants were stored at -70°C for the enzyme-linked immunosorbent assays (ELISA). The cell pellet collected from each sample was applied to a glass slide using a cytocentrifuge (1000 rpm × 10 min) and then the slide was stained with Hema 3 Stain Set (Fisher Scientific) for the differential count of cells. The relative proportion of different cells was determined morphologically by counting 300 cells/slide and then was factored to the number (× 10^5/ml) of total BALF cells collected in each group.

Morphometry

Specimens of right lung were taken from the harvested lung. Each one was cut in three pieces juxtaposed to each other, and of equal size. The middle part was inflated in PBS containing 4% (v/v) formalin under vacuum (13 kPa) for 20 min using a routine water stream-driven device (water aspirator). The specimens from groups were then dehydrated and embedded in paraffin. Sections (4 μm) were cut, and care was taken to prevent overstretching. The sections were stained with hematoxylin-eosin (H&E) for calculation of fine structures of lung tissue.

The degree of parenchymal destruction was determined by a microscopic point count technique, which was performed using a transparent sheet with 50 counting points. The sheet was laid on an A 5-size print, on which the microscopic images from the stained sections were projected using PC-Image software. Images free of large bronchi, vessel, collapsed tissue, or extensive fibrosis were selected. From each lung specimen, generally 8 representative non-overlapping fields were selected.

MLI, MAN and a BWT ratio were calculated in morphological analysis. MLI was determined for each region studied on an overlay consisting of horizontal and vertical lines. All intercepts with alveolar septal number (ASN) were counted at the intersection point of the two lines in the central field of the view under microscope. The total length (L) of all the lines together divided by the number of intercepts gives the mean linear intercept for the region studied. A
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Figure 1. BALF cell count. Rats were exposed to air (control) and cigarette smoke (smoking) in presence and absence of inhaled budesonide alone. The numbers of leukocytes (A), neutrophils (B), macrophages (C) and lymphocytes in the BALF samples from the rats were examined, respectively. Data were expressed as Mean ± SD (n = 10).

formulas is shown as MLI = L/ASN μm), which is used to estimate an average diameter of a single alveolus in size. MAN was determined according to alveolar number (AN) in each field of view and a square area (SA) of the field. A formula is shown as MAN = AN/SA (number/μm2), which is an indicator for density of alveoli. Bronchial wall dimension was measured by a BWT/D ratio.

Lung function testing

Lung function was examined after rats were anaesthetized by an intraperitoneal injection of 10% chloral hydrate (3 ml/kg) and maintained with an appropriate plane of the anesthesia. The trachea was opened with an inverted T-shaped incision in the position between the 2nd and the 3rd cartilage ring, rapidly intubated, and placed the animal into an apparatus for measuring the volume of air inspired and expired by the lungs. (Beijing Rambo Technology Co. Ltd. Beijing, China). The one of the exports of the T-typed cannula in the trachea was connected to a pressure transducer applied to a pulmonary mechanics analyzer and another one was used for administration of air to expand the lungs of the experimental rats.

A ratio of forced expiratory volume at 0.3 s and forced vital capacity (FEV0.3/FVC), dynamic lung compliance (Cdyn), and inspiratory (RI) and expiratory (Re) resistance were assessed by injection of 6.0 ml air into the T-typed cannula in the trachea of the anaesthetized rats and parameters of lung function collected from the rats were automatically recorded by the analyzer.

Determination of MMP-1, MMP-2 and TIMP-2 productions

Values of optical densities (OD) for the specific protein contents in BALF were examined using immunohistochemistry and computer-based
image analysis system after a total protein concentration was measured by BCA Assay. The OD value of the protein level was calculated using the formula: $\text{OD value} = \text{Background OD} - \text{measured value}$ (an average value from 5 tests). The contents of MMP-1, MMP-2 and TIMP-2 productions in the samples from groups were determined using antibodies against the murine MMP-1, MMP-2 and TIMP-2 from Bio-Rad (Hercules, CAUS) according to the manufacturer's directions. Briefly, 100 µl of the substrate solution was added to 100 µl of sample in microtiter plates and incubated for 30 min at room temperature. The reaction was stopped by adding 50 µl of 4 M sulfuric acid, and the OD values were read in a microtiter autoreader at 450 nm.

Statistic analysis

Data were expressed Mean ± Standard Deviation (SD) on the results. Statistical analysis was performed using Statistical Package for the Social Science (SPSS, version 16.0). Comparisons from groups were performed by one-way analysis of variance (ANOVA). Student’s paired t-test was used to compare measurements of individual groups. $P$ values less than 0.05 were considered significant.

Results

Inhaled budesonide decreases the number of leukocytes and differential cells

Cellular compositions in BALF from smoking rats were examined in presence and absence of the corticosteroid and the results are shown Figure 1A-D. A high level of inflammatory cell infiltrates was clearly observed in the samples from smoking rats. In contrast, there were significant increases seen in counts of leukocytes (A), neutrophils (B), macrophages (C) and lymphocytes (D) between the smoking and the control rats ($P < 0.01$ or 0.05). Treatment with budesonide inhalation alone significantly decreased the numbers of these cells but not lymphocytes in the smoking rats (all $P < 0.01$). In further analysis, the count of lymphocytes had a slight decrease without a statistical difference.

Inhaled budesonide does not improve pathological changes of lungs

Changes in fine structures of the cigarette smoke-challenged lungs were examined using MLI, MAN and BWT/D measurement technique and the results are shown in Figure 2A-C. The average values of MLI ($\mu$m), MAN (number/$\mu$m$^2$) and a BTW/D ratio were shown as $26.1 \pm 4.2$, $20.2 \pm 2.4$ and $0.14 \pm 0.03$ in the control group; $36.6 \pm 3.2$, $12.4 \pm 2.3$ and $0.023 \pm 0.06$ in smoking group; $33.5 \pm 2.6$, $14.5 \pm 2.5$ and $0.20 \pm 0.05$ in budesonide group, respectively. In contrast, enlargement of air spaces with the decreases in alveolar density and the increased BTW/D ratio was observed in the smoked lungs as compared to those of the control and the budesonide-treated rats ($P < 0.01$ and 0.05). Treatment with budesonide may slightly reverse the values of MLI, MAN and BDW/D, but there were no significant differences seen in these measurements between the smoking rats treated with and without budesonide.

Inhaled budesonide ameliorates lung function

Lung function testing was carried out at the end of the challenge procedure and the results are
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shown in Figure 3A, 3B. There were significantly decreases found in measurements of FEV_{0.3}/FVC and Cdyn in the smoking rats as compared to the rats in other two groups (P < 0.01 or 0.05). The average values for FVC/FVC_{0.3} (%) and Cdyn (cmH\textsubscript{2}O) were shown as 52.0 ± 8.1 and 0.0356 ± 0.0021 in the control group, 36.0 ± 7.0 and 0.0306 ± 0.0026 in smoking group and 43.0 ± 4.0 and 0.0328 ± 0.0014 in budesonide group, respectively. In contrast, the values of FVC/FVC_{0.3} and Cdyn in smoking rats were obviously lower than those of the control animals (P < 0.01 or 0.05). Treatment with budesonide may partly reverse the lowering FVC_{0.3}/FVC and Cdyn by 16.3% and 6.7% vs. the smoking group. There were statistical differences seen in both measurements between the smoking rats treated with and without budesonide (P < 0.05).

Airway resistance was synchronously determined and the results are shown in Figure 2C, 2D. The average values (%) for Re and Ri were shown as 1.97 ± 0.21 and 1.56 ± 0.22 in the control, 2.29 ± 0.11 and 1.78 ± 0.13 in smoking and 2.13 ± 0.11 and 1.69 ± 0.10 in budesonide group, respectively. In contrast, Re and Ri in the smoking group obviously increased over the control group (P < 0.01 and 0.05). Treatment with budesonide resulted in a significant decrease in Re by 7.5% but not Ri in the challenged rats (P < 0.05).

Inhaled budesonide decreases MMP-1 level

Contents of MMP-1, MMP-2 and TIMP-2 protein productions in the BALF samples from the experimental rats were examined and the results are shown in Figure 4A-C. Levels (ng/ml) of MMP-1, MMP-2 and TIMP-2 productions

Figure 3. Change in lung function. Changes in FEV_{0.3}/FVC (A), Cdyn (B), Re (C) and Ri (D) were observed in the experimental rats exposed to air (control) and cigarette smoke (smoking) in presence and absence of inhaled budesonide. Data were expressed as mean ± SD (n = 10).
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Figure 4. Change in MMP-1, MMP-2 and TIMP-2 productions. Contents (ng/ml) of MMP-1 (A), MMP-2 (B) and TIMP-2 (C) productions in BALF were determined in rats exposed to air (control) and cigarette smoke (smoking) in presence and absence of budesonide. Data were expressed as mean ± SD (n = 10).

displayed as 3.2 ± 0.8, 3.0 ± 0.9 and 0.32 ± 0.13 in control; 4.1 ± 0.5, 4.5 ± 0.9 and 0.49 ± 0.12 in smoking; 3.4 ± 0.7, 3.7 ± 0.8 and 0.51 ± 0.13 in the budesonide-treated rats, respectively. The levels of all three protein productions in the samples from the smoking rats were significantly increased as compared to those of the control rats (all \( P < 0.01 \)). However, treatment with budesonide resulted in an obvious decrease in MMP-1 rather than MMP-2 and TIMP-2 productions in the BALF samples from smoking rats. There was a statistical difference observed between the rats treated with and without the steroid (\( P < 0.05 \)).

Discussion

In this animal model of COPD, the number of leukocytes in BALF significantly increased after the rats exposed to cigarette smoke twice a day for 4 consecutive months, demonstrating an inflammatory response associated with development of the lung model. In further analysis, the count of differential cells displayed a large inflammatory infiltrate, comprised mainly of neutrophils and macrophages with a slight increase of lymphocytes, into the BALF, clearly indicating that these cells were required for the cigarette smoke-driven airway inflammation in the long-term remodeling effect. Treatment with inhaled budesonide was found to have pulmonary effects evidenced by the reduced numbers of neutrophils and macrophages but not lymphocytes, suggesting that the agent mitigated the ongoing inflammatory process in the smoked lungs. Budesonide has a wide range of inhibitory activities against many cell types including neutrophils and macrophages [16]. Moreover, budesonide uptake is rapid because of its high affinity for the glucocorticoid receptor and reversible esterification of budesonide on inhalation may prolong the duration of action in airways and lung tissue [17, 18]. Our findings were given explicit support in treating COPD, even though long-term treatment with the steroid must be associated with a high risk of adverse systemic effects [19].

Main features of micro-anatomy of cigarette smoke-exposed lungs were examined using morphometric techniques that have been involved in the direct and unbiased estimation for the quantitative analysis of lung and bronchial injuries [20-22]. Our results showed that MLI and the BWT/D ratio obviously increased with the reduced MAN in the challenged lungs as compared to those in the control rats, indicating the emphysema-like lesions such as enlargement of distal air space in size and lowering alveolar density as well as tracheal wall thickening established in the animal model of COPD. Since treatment with inhaled steroid did not change fine structures in the diseased lungs, it led us to conclude that the steroid effect was weak in mitigating structural lesions of the lung tissues, and therefore it did not rescue lung architecture in this model.

In terms of lung function examination, our results revealed significant decreases in \( \text{FEV}_{0.3} / \text{FVC} \) and \( \text{Cdyn} \) concomitantly with an obvious increase in Re as compared to the control animals, suggesting excessive lung function decline with certain degrees of severity progressively reached in the rats over the challenge time. In this study, the measurement approach applied for collection of lung function parameters was performed by 6.0 ml air inject-
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ed into the lungs to expand chest of the animals. This offered an advantage over the agonist stimulation because the airflow response is more related to an in vivo condition of airway obstruction. In further observation, treatment with inhaled budesonide slightly but significantly increased in FEV$_{0.3}$/FVC and Cdyn, and decreased in Re, indicating that the agent ameliorated the smoking-induced lung function decline. The findings were consistent with the reports that COPD is characterized by expiratory airflow limitation that is partially reversible, and an inhaled glucocorticoid has a high affinity for the lung increasing its usefulness [23, 24].

To gain such an understanding regarding a precise role of the inhaled steroid in the cigarette smoke-exposed lungs, levels of MMP-1, MMP-2 and TIMP-2 proteins in BALF were measured. Our results showed abundance of MMP-1, MMP-2 and TIMP-2 productions in the samples from the smoking rats, clarifying the inflammatory proteinase, MMP-1, MMP-2 and its cognate inhibitor, TIMP-2 change in the lungs during the challenge process. Since MMPs may be produced by alveolar macrophages [25] and they can collectively degrade all of the protein components of ECM [26], therefore the increased MMPs burden in the lungs may lead to MMP-1 or MMP-2/TIMP-2 imbalance because of a slight increase of TIMP-2 content detected in BALF. Conversely, the change in balance referred to severity of lung injuries as morphometric evidence of emphysema developed with lung-specific expression of MMP-1 [27]. Treatment with budesonide inhalation resulted in an obvious change in MMP-1 but not MMP-2 and TIMP-2 productions, suggesting that the glucocorticoid steroid acted predominantly on MMP-1 and significantly reduced the protein content. MMP-1 is active against collagens and overexpression of the proteinase in mice causes emphysema [28, 29]. This provided evidence that the genesis of emphysema may be related to more than just elastin degradation by elastase, and brought attention to the importance of the lung matrix as a whole in COPD. Since an increase in MMP-1 production affects airway resistance and lung compliance [30, 11], it is reasonable to consider that reduction of the MMP-1 content protects lung function from the smoke-induced injury in the model.

In conclusion, treatment with inhaled budesonide significantly attenuates the lung inflammatory response to the inhalation of cigarette smoke and improves declining lung function through reduction of MMP-1 production in the smoked lungs.

Disclosure of conflict of interest

None.

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