The impacts of anti-inflammatory phosphodiesterase inhibitors on a murine model of chronic pulmonary inflammation

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Abstract

Chronic obstructive pulmonary disease (COPD) often tends to respond poorly to glucocorticoid (GC) therapy. Reduced Histone deacetylase-2 (HDAC-2) activity is an important mechanism behind this GC insensitivity. In this study, we investigated the effects of three phosphodiesterase inhibitors (PDEIs), with anti-inflammatory propensity, on cigarette smoke (CS) induced pulmonary inflammation and HDAC-2 activity. Male C57BL/6 mice were exposed to cigarette smoke (CS) over the course of 30 weeks. Administration of the PDEIs commenced from the 29th week and followed a schedule of once daily treatments, 5 days a week, for 2 weeks. Roflumilast (ROF) was administered intragastrically (5 mg·kg-1), while pentoxifylline (PTX) (10 mg·kg-1) and theophylline (THEO) (10 mg·kg-1) were administered intraperitoneally, either alone or in combination with a GC (triamcinolone acetonide or TRI, 5 mg·kg-1, i.m., single injection). Lung morphometry, as well as the activity of HDAC-2, pro-inflammatory cytokines and reactive oxygen species (ROS) were assessed at the end of the 30 week course. CS exposure was associated with a reduction in HDAC-2 activity and the up-regulation of ROS expression. PTX, ROF and THEO administration led to the partial restoration of HDAC-2 activity, however combining TRI to any of these PDEIs did not synergistically augment this effect. The restoration of HDAC-2 activity was favorably associated with the reduction of ROS expression.Inactivation of HDAC-2 due to long-term CS exposure is closely related to exaggerated oxidative stress, and this reduced HDAC-2 activity could partially be restored through the use of PDEIs. This finding provides a potential novel approach for further clinical research.

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Conflict of interest

The authors declare no conflict of interest.

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Data available statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Abstract

BACKGROUND AND PURPOSE

Chronic obstructive pulmonary disease (COPD) often tends to respond poorly to glucocorticoid (GC) therapy. Reduced Histone deacetylase-2 (HDAC-2) activity is an important mechanism behind this GC insensitivity. In this study, we investigated the effects of three phosphodiesterase inhibitors (PDEIs), with anti-inflammatory propensity, on cigarette smoke (CS) induced pulmonary inflammation and HDAC-2 activity.

EXPERIMENTAL APPROACH

Male C57BL/6 mice were exposed to cigarette smoke (CS) over the course of 30 weeks. Administration of the PDEIs commenced from the 29^{th} week and followed a schedule of once daily treatments, 5 days a week, for 2 weeks. Roflumilast (ROF) was administered intragastrically (5 $mg{\cdot}kg^{-1}$), while pentoxifylline (PTX) (10 $mg{\cdot}kg^{-1}$) and theophylline (THEO) (10 $mg{\cdot}kg^{-1}$) were administered intraperitoneally, either alone or in combination with a GC (triamcinolone acetonide or TRI, 5 $mg{\cdot}kg^{-1}$, i.m., single injection). Lung morphometry, as well as the activity of HDAC-2, pro-inflammatory cytokines and reactive oxygen species (ROS) were assessed at the end of the 30 week course.

KEY RESULTS

CS exposure was associated with a reduction in HDAC-2 activity and the up-regulation of ROS expression. PTX, ROF and THEO administration led to the partial restoration of HDAC-2 activity, however combining TRI to any of these PDEIs did not synergistically augment this effect. The restoration of HDAC-2 activity was favorably associated with the reduction of ROS expression.

CONCLUSIONS AND IMPLICATIONS

Inactivation of HDAC-2 due to long-term CS exposure is closely related to exaggerated oxidative stress, and this reduced HDAC-2 activity could partially be restored through the use of PDEIs. This finding provides a potential novel approach for further clinical research.

Abbreviations

COPD, chronic obstructive pulmonary disease;

HDAC-2, histone deacetylase-2;

CS, cigarette smoke;

Lm, mean linear intercept;

ROS, reactive oxygen species;

BALF, bronchoalveolar lavage fluid;

ROF, roflumilast;

PTX, pentoxifylline;

THEO, theophylline;

TRI, Triamcinolone acetonide.

Introduction

Chronic obstructive pulmonary disease (COPD) affects approximately 13% of people over the age of 40 (Fang et al., 2018) and was the fifth leading cause of death in China in the year of 2016 (Collaborators, 2017). Unlike bronchial asthma, in which the majority of the patients' symptoms can be well controlled using glucocorticoids (GCs), the response to GC treatment in patients with COPD is not as effective as the response seen in asthmatic patients. This insensitivity to GCs is associated with a reduction in histone deacetylase-2 (HDAC2) activity, and in line with COPD clinical severity (Barnes, Ito & Adcock, 2004; Footitt et al., 2016; Ito et al., 2005). Excessive inflammation, together with enhanced oxidative stress is an important mechanism for this decrease in HDAC-2 activity in patients with COPD (Barnes, 2016; Footitt et al., 2016). Restoration of GC sensitivity is postulated as a novel approach for COPD management (Marwick, Adcock & Chung, 2010; Mitani, Ito, Vuppusetty, Barnes & Mercado, 2016).

Theophylline (THEO), pentoxifylline (PTX) and roflumilast (ROF) are three commonly prescribed phosphodiesterase (PDE) inhibitors for anti-inflammatory treatment. THEO is prescribed as a bronchodilator while PTX is indicated for occlusive extremity artery disease (intermittent claudication)(Chopra, Chopra, Aggarwal & Parashar, 1988). ROF is a relatively new agent that selectively inhibits PDE-4 on inflammatory cells (Kawamatawong, 2017; Sakkas, Mavropoulos & Bogdanos, 2017), and is indicated in adults with severe COPD.

Certain laboratory studies have claimed that GC sensitivity could be re-established by using THEO to up-regulate HDAC-2 expression (Ranjani & Vinotha, 2017; To et al., 2010), however adding THEO to inhaled GCs did not result in improved clinical outcomes (Devereux et al., 2018). We consider whether other anti-inflammatory PDE inhibitors (PDEIs), such as PTX and ROF, had a similar or even greater effect on restoring GC-sensitivity by restoring HDAC-2 activity, thereby providing a novel avenue for clinical investigation.

Methods

Study design

In order to compare the effects of THEO, PTX and ROF when used alone and in combination with a GC, the mice were randomly divided into nine groups ($n = 10$ for each group): (1) Sham CS exposure; (2) CS exposure; (3) CS exposure with ROF administration; (4) CS exposure with PTX administration; (5) CS exposure with THEO administration; (6) CS exposure with triamcinolone (TRI) administration; (7) CS exposure with co-administration of ROF and TRI; (8) CS exposure with co-administration of PTX and TRI; (9) CS exposure with co-administration of THEO and TRI. The course set for CS exposure was 30 weeks and the medication intervention started at the 29^{th} week of the course and continued for 2weeks.

Animals

Six to seven -week old male wild-type C57BL/6 mice (18-22 g body weight) were purchased from Vital River Laboratory and Animals Co., Ltd. (Beijing, China). The animals were housed in a specific pathogen free environment, at a temperature of 22-26 with humidity of 60-70%, where a 12-hour day/night cycle was maintained. The mice had free access to standard laboratory food and fresh water. The laboratory animal management rules of Tongji Medical College of Huazhong University of Science and Technology were strictly abided by.

Cigarette smoke exposure

The animals in different groups were caged separately in a series of transparent chambers (each sized 28 x 21 x 17 cm^3) that were arranged in parallel and connected to a central CS supply, where smoke from the ignited cigarettes (Hongjinglong, Wuhan, China) was automatically pumped evenly into the chambers. Each cigarette contained 10 mg of tar, 0.8 mg of nicotine, and 12 mg of carbon monoxide. During each 60-minutes episode of smoke exposure, eight cigarettes without filters were combusted in turn, generating an air mixture containing 8% CS (the PM 2.5 particles in the air mixture was 45 +- 8.37 mg/m^3). The mice allocated to the sham CS exposure were caged in the same environment but exposed only to fresh air for 60 minutes. These 60 minutes CS exposure or sham -CS exposure episodes were conducted twice a day, 5 days a week, for 30 weeks.

Interventional Medication

The PDEIs dosages were determined from revious studies (Martorana, Beume, Lucattelli, Wollin & Lungarella, 2005; Sun, Li, Gong, Ren, Wan & Deng, 2012; Wang, Chen, Zhang, Hu & Peng, 2012), i.e., 5 $mg*kg^{-1}$ for ROF, 10 $mg*kg^{-1}$ for both PTX and THEO. Determination of the TRI dosage was done by converting the equivalent dose for human beings, i.e., 24 mg TRI for a 60 kg adult, based on body surface area and adapting it accordingly for the mice (Reagan-Shaw, Nihal & Ahmad, 2008). ROF was suspended in sterilized water and given intragastrically once a day, five days a week for 2 weeks, commencing from the 29^{th}week; PTX and THEO were dissolved in sterilized water and given intraperitoneally once a day, five days a week for 2 weeks, commencing from the 29^{th} week; TRI (5 $mg*kg^{-1}$) was administered intramuscularly through a single injection in the 29^{th} week due to its long lasting effect. All the mice were humanely euthanized with an overdose of sodium pentobarbitone (50 $mg*kg^{-1}$) at end of the 30 week course.

Morphometry and Biomarkers assessment

The animals underwent endotracheal intubate soon after euthanasia. For further orphologic and morphometric study, one side of the lungs was prepared through the intratracheal instillation of a 4% paraformaldehyde solution with a hydrostatic pressure of 20 cm that was maintained for at least 20 minutes, before being dissected and sent for additional sample processing procedures, e.g., fixation in 4% paraformaldehyde, paraffin embedding, sagittal slice preparation and staining with hematoxylin-eosin (HE), etc.. Morphometric studies were conducted in accordance with methods previously reported (Knudsen, Weibel, Gundersen, Weinstein & Ochs, 2010), briefly, twenty non-overlapping fields in each slice were randomly selected under an Olympus DP72 camera system (x40 magnification power). The degree of airspace enlargement or emphysema was assessed by mean linear intercept (Lm, μm) using STEPanizer software (Bern, Switzerland) based on the number of intersections of a given line across the alveolar septa. The morphometry was conducted in a blind manner and the scores separately calculated by two experts in pulmonary anatomy, before being averaged and used as the final scores.

The other side of the lungs underwent intratracheal instillation of 0.5 ml of ice-cold phosphate-buffered saline (PBS), three times, while the contralateral main bronchus was ligated. The bronchoalveolar lavage fluid (BALF) was recovered by gentle aspiration and subjected to filtration and centrifugation at 750g for 10 min at 4 ºC. The supernatant was stored at -80 ºC for the subsequent measurements. The lungs of the mice not subjected to BAL or paraformaldehyde fixation were excised and stored at -80 ºC for HDAC-2 assessment. The expression of IL-8 and TNF-α in the BALF were measured using a commercial ELISA kit (Neobioscience, Wuhan, China) according to the manufacturer's instructions.

For assessment of reactive oxygen species (ROS), the frozen sections (6 μm) of the lung tissue were stained with DHE (2 μmol/L) in a humidified and light-protected chamber at 37 ºC for 30 min as described (Faller et al., 2013). Fluorescent images were photographed under a confocal laser scanning microscope (Olympus) and the intensity of fluorescence was measured using Image-Pro Plus 6.0® (Media Cybermetics, Bethesda, MD) software and expressed as arbitrary units of fluorescence.

The nuclear components in the homogenized lung tissue were extracted using a nuclear extraction kit (As-

pen, Wuhan, China). HDAC-2 activity in the nuclear extracts was quantified using an HDAC-2 assay kit (Epigentek Group, Brooklyn, NY), in accordance with the manufacturer's instructions (Sugiura et al., 2012).

Statistical Analysis

Statistical analyses were performed using Graphpad Prism software 5.0 (GraphPad Software Inc., La Jolla, California). Data were first tested for normal distribution. Parametric (such as ANOVA and *post hoc*Dunnett's comparisons) and non-parametric (such as Kruskal-Wallis test) statistics were applied where appropriate. Stepwise regression was applied to the pooled data, exploring the variables which were relevant to the decline of HDAC-2 activity. $P < 0.05$ was regarded as statistically significant.

Materials

ROF, Dihydroethidium (DHE) and Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich, German. PTX was purchased from Enzo Life Sciences, America. THEO was purchased from Medchem Express, America. Triamcinolone acetonide (TRI) was purchased from Xianju Pharmaceutical Co., Ltd. (Zhejiang, China). Phosphate Buffered Saline (PBS) was purchased from HyClone, America.

Results

Body weight

Animals with long-term CS exposure gained body weight slower than the animals in the sham CS exposure group, a significant difference in body weight was notable from the 24^{th} week of the course ($P < 0.05$, Figure 1).

Pulmonary Morphology and Morphometry

As expected, emphysema-like structural damage was observed in mice with long-term CS -exposure (Figure 2, panel A), and was associated with a greater Lm compared to the sham CS exposure group (52.70 ± 0.31 μm in CS exposed vs. 32.39 ± 3.55 μm in sham CS exposed, $P< 0.05$). Two weeks of'-treatment with either THEO, PTX, ROF or the addition of TRI with any of these PDEIs, did not mitigate the structural damage (Figure 2, panel A and B, $P > 0.05$).

Pro-inflammatory cytokines in BALF

Long term CS exposure induced the up-regulation of TNF-α (Figure 3, panel A) and IL-8 (Figure 3, panel B) in BALF. Only PTX was able to down-regulate the expression of TNF-α ($P < 0.05$), and the addition of TRI did not improve this inhibitory. PTX, THEO and TRI resulted in the down-regulation of IL-8 expression ($P < 0.05$), and the addition of TRI intensifies this inhibition ($P< 0.05$), not only when used with PTX and THEO but also when used with ROF.

ROS Expression in situ

Expression of ROS in the lung tissue was also augmented by long-term CS exposure (Figure 4, panel A, Table 1). PTX, THEO and TRI but not ROF, inhibited ROS expression. The addition of TRI with PTX and ROF, however, improved this inhibition (Figure 4, Table 1).

HDAC-2 activity in lung tissue

Shown in Figure 5 and Table 1, the lungs of mice with long-term CS exposure had a greater reduction in HDAC-2 activity when compared to the lungs of the mice in the sham CS exposure group. ROF, PTX and THEO were able to partially restore HDAC-2 activity. The addition of TRI to any of the PDEIs, however, did not enhance the restoration of HDAC-2 activity ($P > 0.05$).

Correlations

Multiple stepwise regressions were applied to the pooled data when searching for factors that had a significant impact on HDAC-2 activity, where ROS, IL-8 and TNF-α were set as independent variables. Only ROS

remained in the final equation (adjusted $R^2 = 0.29$, $P < 0.001$), implying that a decline in HDAC-2 activity was related to an increase in ROS activity.

Discussion

The murine model of CS-induced pulmonary emphysema has many similarities to human COPD, including growth delay (Chen, Hansen, Jones, Vlahos, Anderson & Morris, 2008; He, Li, Sun, Zeng, Lu & Xie, 2018), hyper-inflammatory responses and enhanced oxidative stress (Kameyama et al., 2018; Sasaki et al., 2015). By using the C57BL/6 mice strain in this study, we enhanced the susceptibility to CS exposure (Yao et al., 2008). TNF-α and IL-8 were chosen as the representative inflammatory biomarkers because both play key roles in the pathogenesis of CS-induced pulmonary inflammation (de Carvalho et al., 2016; Fujita et al., 2016).

The PDEs are classified into 11 super-families (Kawamatawong, 2017). Involvement of PDE isoforms such as PDE3, PDE4, PDE5 and PDE7 has been demonstrated in the pathogenesis of airway inflammation and hyper-responsiveness (Beute et al., 2018; Dunne et al., 2019; Zuo, Cattani-Cavalieri, Musheshe, Nikolaev & Schmidt, 2019). Similar to previous studies (Marwick et al., 2009; Sun, Li, Gong, Ren, Wan & Deng, 2012), the expression of ROS, IL-8 and TNF-α in mice exposed to long-term CS, was elevated, and was associated with a reduction in HDAC-2 activity and emphysema-like pulmonary damage. As with human COPD, in which no treatment modalities are able to repair pulmonary emphysematous destruction, PTX or ROF therapy in the present study did not mitigate the CS-induced pulmonary emphysema (no reduction in Lm). However, the PDEIs therapy demonstrated some anti-inflammatory merits: IL-8 expression was down-regulated by PTX and THEO, TNF-α was preferentially decreased by PTX and HDAC-2 activity was partially restored when using either of these PDEIs. This restoration was preferentially associated with the downregulation of ROS, rather than a reduction in any single cytokine.

PTX is the strongest anti-inflammatory agent among the three PDEIs if the inhibitory effects on IL-8, TNF-α and ROS are considered. PTX has never been recommended for COPD treatment, as early clinical trials with PTX monotherapy showed no significant clinical benefits when considering oxygenation and amelioration of pulmonary function in patients with COPD (Fallahi, Ghayumi & Moarref, 2013; Sasse, Causing, Stansbury & Light, 1995). Unfortunately, thus far, there is no agent that can reverse the damage done when impaired lung function is evident, or decrease the decline in the pulmonary function of patients with COPD. Variables such as the reduction rate of exacerbation and improving the quality of life of patients, have become the major study endpoints in most COPD clinical studies.

Recent research revealed that the benefit of ROF treatment in COPD is related to the inhibition of eosinophils rather than neutrophils (Rabe et al., 2018), despite the pathogenesis of COPD being more closely related to CD8 skewness and neutrophil infiltration (Eapen, Myers, Walters & Sohal, 2017). This could suggest that the subjects enrolled in studies concern with the clinical efficacy of ROF, might include some patients with overlapping asthma. Clinical trials based on patients with COPD using a combination of low-dose oral THEO and inhaled corticosteroids did not show significant benefits (Cosio et al., 2016; Devereux et al., 2018). PTX possesses preferential inhibitory effects on CD8 and neutrophils (Costantini et al., 2010; Konrad, Neudeck, Vollmer, Ngamsri, Thiel & Reutershan, 2013), and when combined with a GC, as shown in our current study, its effect on ROS inhibition is stronger than that of THEO and ROF. Therefore, the efficacy of PTX in COPD treatment should be re-evaluated using adequate clinical variables, such as the rate of lung function decline and the frequency of acute exacerbations.

When considered together, we concluded that the reduction of HDAC-2 activity is associated with the up-regulation of ROS. THEO, as well as PTX and ROF, can restore HDAC-2 activity by alleviating oxidative stress.

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Figure caption:

Fig. 1. The mice with long-term CS exposure (presented as black dots) had gained body weight slower than the animals in the sham CS exposure group (presented as circles), a significant difference in body weight was noted from the 24th week ($P < 0.05$). Data presented as mean body weight ± SD ($n = 10$ in each group).

Fig. 2. Long-term cigarette smoke (CS) exposure induced emphysema-like pulmonary morphometry (panel A, 10×magnification), illustrated by a greater mean liner intercept (Lm) when compared to the sham CS exposure group (panel B, $P < 0.05$,). Two week course of treatment with roflumilast, pentoxifylline, theophylline or a combination of a glucocorticoid (Triamcinolone) with any of these phosphodiesterase inhibitors, did not mitigate pulmonary emphysema (panel B, $P > 0.05$).

Fig. 3. The expression of TNF-α (panel A) and IL-8 (panel B) in bronchoalveolar lavage fluid (BALF) samples was up-regulated by long term cigarette smoke (CS) exposure. Only PTX down-regulated the expression of TNF-α, while the addition of TRI did not increase this inhibition. PTX, THEO and TRI but not ROF down-regulated the expression of IL-8. The addition of TRI intensifies the inhibition, not only for PTX and THEO but also for ROF.

Results presented as mean ± SD ($n = 5$ mice for group).*P < 0.05, compared to the Sham-CS group;$^{+}$P < 0.05, compared to the CS group.

Fig. 4. Expression of ROS in the lung tissues was augmented by long-term CS-exposure (panel A, $P < 0.05$). PTX, THEO and TRI but not ROF inhibited ROS expression. The addition of TRI to ROF, however, increased the inhibition (panel B). the addition of TRI to PTX resulted in the synergistic inhibition on ROS expression (panel B, P < 0.05). Data presented as mean +- SD ($n = 5$ mice per group).$^{*}P < 0.05$, compared to the Sham-CS group; $^{+}P < 0.05$, compared to the CS group; $^{\mathrm{P}}P < 0.05$, compared to the CS+TRI group.

Fig. 5. The lungs having had long-term CS exposure had a greater reduction in HDAC-2 activity when compared to the lungs in the sham CS exposure group. ROF, PTX or THEO (but not TRI) partially restored HDAC-2 activity. The addition of TRI to each of the PDEIs, however, did not enhance HDAC-2 activity (P > 0.05). Data presented as mean +- SD ($n = 5$ mice for each group). $^{*}P< 0.05$, compared to the Sham-CS; $^{+}P< 0.05$, compared to the CS group; $^{\mathrm{P}}P< 0.05$, compared to the CS+TRI group.

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