European Journal of Pharmacology 659 (2011) 79–84

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, Gastrointestinal and Urogenital Pharmacology

Disease-modifying effect of ASP3258, a novel phosphodiesterase type 4 inhibitor, on subchronic cigarette smoke exposure-induced lung injury in guinea pigs

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ARTICLE INFO

Article history:
Received 11 September 2010
Received in revised form 23 January 2011
Accepted 14 February 2011
Available online 5 March 2011

Keywords:
Phosphodiesterase 4
Chronic obstructive pulmonary disease
Cigarette smoke
Guinea pig
ASP3258
Roflumilast

ABSTRACT

ASP3258 is a novel, orally active, selective phosphodiesterase (PDE) 4 inhibitor which has an improved therapeutic window over second generation compounds such as roflumilast and cilomilast. Here, we investigated the effect of ASP3258 on cigarette smoke exposure-induced lung injury in guinea pigs, a well-defined model for chronic obstructive pulmonary disease (COPD). COPD-like lung injury was induced by repeated cigarette smoke exposure (10 cigarettes/day, 5 days/week, for 4 weeks). Orally administered ASP3258 (0.3, 1, and 3 mg/kg) dose-dependently suppressed pulmonary accumulation of mononuclear cells and neutrophils, and the inhibitory effect of ASP3258 (1 mg/kg) was almost the same as that of roflumilast (1 mg/kg). In contrast, a glucocorticoid prednisolone (10 mg/kg, p.o.) did not show any effect. Histological examination revealed that ASP3258 treatment significantly inhibited infiltration of neutrophils and macrophages into either or both alveolar or peribronchiolar areas, as well as hyperplastic and squamous metaplastic changes of epithelium in the bronchi. Decreasing trends in histological scores for accumulation of lymphocytes in the alveoli and alveolar wall thickening were also observed in ASP3258-treated animals. Further, ASP3258 attenuated augmentation of matrix metalloproteinase-9 activity in the bronchoalveolar lavage fluid. These findings suggest that ASP3258 has therapeutic potential for treating COPD not only through inhibition of pulmonary cellular accumulation but also by preventing lung structural alterations initiated by repeated cigarette smoke exposure. To our knowledge, this is the first paper demonstrating that PDE4 inhibitors exert significant inhibitory effects on subchronic cigarette smoke exposure-induced lung injury in guinea pigs.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a common and debilitating chronic inflammatory disease characterized by progressive and largely irreversible airflow limitation and is predicted to become the third leading cause of death worldwide by 2020. Cigarette smoking is a major causal factor for ongoing pulmonary inflammation in the airways and lung parenchyma, and the degree of inflammation is correlated with the severity of airflow limitation. Macrophages, neutrophils, and $CD8^+$ lymphocytes are predominantly involved in the inflammatory response, and various inflammatory mediators derived from these cells, such as cytokines, chemokines, and proteases contribute to COPD development (Pauwels et al., 2001; Barnes et al., 2003; Saetta et al., 2001).

Suppression of the inflammatory responses is thus a rational approach to treating COPD. Although glucocorticoids are the most effective agents in the treatment of inflammatory diseases such as asthma, they have been found to be largely ineffective in attenuating inflammation in COPD patients (Barnes et al., 2003). For this reason, developing novel anti-inflammatory agents with mechanisms of action differing from those of glucocorticoids is an urgent issue in this area. Phosphodiesterase 4 (PDE4) inhibitor, which has potent anti-inflammatory activity, is one possible candidate. PDE4 is the main cAMP-metabolising enzyme in immune and inflammatory cells and airway smooth muscle, and its inhibition suppresses the recruitment and activation of several inflammatory cells, including macrophages, neutrophils, and $CD8^+$ lymphocytes (Souness et al., 2000; Giembycz, 2000). Previous clinical studies have found that second generation PDE4 inhibitors cilomilast and roflumilast significantly improve lung function and reduce the rate of exacerbation in patients with COPD (Rennard et al., 2006; Calverley et al., 2007), and more recently, roflumilast has been approved by the European Medicines Agency for the treatment of COPD (Fabbri et al., 2010). Although cilomilast and roflumilast have shown improved safety margins over the prototype, rolipram, between anti-inflammatory activities and class-specific side effects such as nausea and diarrhea, their therapeutic use is still likely to be limited due to the compounds' adverse effects (Rennard et al., 2006; Calverley et al., 2007).

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doi:10.1016/j.ejphar.2011.02.042

We recently discovered a novel orally active PDE4 inhibitor ASP3258 which has a wider therapeutic window than second generation PDE4 inhibitors cilomilast and roflumilast (Kobayashi et al., in press). Here, we investigated the effects of ASP3258, roflumilast, and prednisolone on repeated cigarette smoke exposure-induced lung injury in guinea pigs, a well-defined experimental model for COPD (Wright and Churg, 2002; Kubo et al., 2005). Further, using this model, we investigated the detailed mechanism involved in the therapeutic effects of PDE4 inhibitors on COPD, which remain largely unknown compared to asthma.

2. Materials and methods

2.1. Animals

Male Hartley strain guinea pigs were purchased from SLC (Shizuoka, Japan). The animals were maintained in ordinary animal cages, with food and water available *ad libitum*. Animals aged 7 weeks and weighing 400–600 g at the start of the experiments were used. All experiments were performed in accordance with the regulations of the corporate Animal Ethical Committee.

2.2. Drugs

ASP3258 (3-[4-(3-chlorophenyl)-1-ethyl-7-methyl-2-oxo-1,2-dihydro-1,8-naphthyridin-3-yl]propanoic acid) and roflumilast were synthesized at Astellas Pharma Inc. (Tsukuba, Japan). Prednisolone was purchased from Nacalai Tesque (Kyoto, Japan). These compounds were suspended in 0.5% (w/v) methylcellulose (Shin-Etsu Chemical Co., Tokyo, Japan) solution and orally administered at 3 ml/kg. The normal and control groups were treated with vehicle (0.5% methylcellulose).

2.3. Cigarette smoke exposure-induced lung injury model

Cigarette smoke exposure was performed using commercially available non-filter cigarettes (Peace brand cigarettes; Japan Tobacco Inc., Tokyo, Japan) and a cigarette smoke generator SG-200 and inhalation apparatus made up of 20 individual chambers (Sibata Scientific Technology Ltd., Tokyo, Japan) as described previously (Kubo et al., 2005). Briefly, each animal was placed into an individual chamber and exposed to diluted cigarette smoke in a conscious and restrained state. Animals were repeatedly exposed to the smoke of 10 cigarettes/day, 5 consecutive days/week, for 4 weeks. Each cigarette was puffed 15 times for 3 min at a rate of 5 puffs/min. One puff meant drawing 35 ml of cigarette smoke into a 50 ml syringe, and then blowing this cigarette smoke, which was diluted to 4% with air, into the apparatus. Fresh room air inhalation was performed for 1 min every 3 min of cigarette smoke exposure. According to the manufacturer's specifications, each cigarette contained 2.4 mg of nicotine and 24 mg of tar. All drugs and vehicle were orally administered 1 h before the start of each cigarette smoke exposure session. Age-matched non-smoke-exposed and vehicle-administered animals were used as normal group animals.

2.4. Bronchoalveolar lavage

Guinea pigs were sacrificed under urethane anesthesia (1.2 g/kg, i.p.), after which their tracheas were cannulated. The lungs were lavaged with 5 ml of ice-cold saline containing 1 U/ml heparin five times via the cannula. Bronchoalveolar lavage (BAL) fluid was centrifuged at 400×*g* for 10 min at 4 °C. The resultant cell pellet was resuspended in 2 ml of ice-cold heparinised saline to measure total cell count, and the supernatant was stored at −80 °C until use. BAL was performed 4–6 h after the final cigarette smoke exposure. The total number of leukocytes in the BAL fluid was counted using an automated cell counter (Celltac-α; Nihon Kohden, Tokyo, Japan), and differential cell count was performed using a cytospin preparation stained with Diff-Quik (Sysmex International Reagent Co., Ltd., Kobe, Japan). A minimum of 300 cells were identified and differentiated as mononuclear cells, neutrophils, or eosinophils using the standard morphological criteria.

2.5. Histological evaluation

To avoid possible traumatic damage due to BAL, histological assessment of the lung tissue was performed in separate animals. Animals to be evaluated were sacrificed 4–6 h after the final cigarette smoke exposure, after which the lungs were removed and fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 2-μm sections, and stained with hematoxylin and eosin. The bronchus and parenchyma sections were analyzed in a blind fashion, and the degrees of observed pathological changes including hyperplasia/squamous metaplasia of epithelium; infiltration of neutrophils, macrophages, and lymphocytes into the bronchiole or the alveolus; and thickening of the alveolar wall were scored as follows: 0, none; 1, slight; 2, mild; 3, moderate; and 4, severe.

2.6. Gelatin zymography

BAL fluid supernatant samples were concentrated 5-fold using Centricon YM-3 filters (Millipore, Billerica, MA, USA). The concentrated samples and positive controls (purified human metalloproteinase (MMP)-2 and -9; Chemicon, Temecula, CA, USA) were loaded onto 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 1 mg/ml gelatin under nonreducing conditions. After electrophoresis, the gel was washed for 2 h in 50 mM Tris–HCl (pH 7.5) containing 0.1 M NaCl and 2.5% Triton X-100 to remove the SDS. The gel was then rinsed in water and incubated overnight at 37 °C in development buffer (50 mM Tris–HCl [pH 7.5] containing 20 mM $CaCl_2$). After development, the gel was stained with staining solution (0.25% Coomassie blue R250 in 45% methanol/10% acetic acid/45% H_2O) and subsequently washed with destaining solution (30% methanol/10% acetic acid/60% H_2O). Gelatinolytic activities were detected as clear bands of gelatin lysis against a blue background stain. Band density was quantified using ImageQuant software (GE Healthcare UK Ltd., Buckinghamshire, UK).

2.7. Statistical analysis

All statistical analyses were conducted using the SAS system (SAS Institute Inc., Cary, NC, USA). Data were expressed as means ± S.E.M. The statistical significance of differences between groups was determined using Student's t-test, Dunnett's multiple range test, or Wilcoxon rank sum test. Values of $P<0.05$ were considered significant.

3. Results

3.1. Effects of ASP3258, roflumilast, and prednisolone on pulmonary leukocyte infiltration in lungs after subchronic cigarette smoke exposure

Repeated exposure of guinea pigs to cigarette smoke (10 cigarettes/day, 5 days/week) for 4 weeks resulted in a significant increase in leukocytes counts in the BAL fluid. Differential cell count analysis revealed that the increased cells were composed mainly of mononuclear cells (particularly macrophages) and neutrophils. Orally administered ASP3258 (1 mg/kg, q.d.) markedly inhibited this increase in numbers of total leukocytes, mononuclear cells, and neutrophils in the BAL fluid. The reference compound roflumilast (1 mg/kg, p.o., q.d.), which is the first PDE4 inhibitor approved for the treatment of COPD, similarly attenuated increases in BAL cell counts. The inhibitory activities of ASP3258 and roflumilast at 1 mg/kg were almost identical (Fig. 1).