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Inhibition of the MAP kinase ERK protects from lipopolysaccharide-induced lung injury

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ABSTRACT

The pathogenesis of chronic obstructive pulmonary disease (COPD) is characterized by pulmonary inflammation associated with lung neutrophilia and elevated levels of pro-inflammatory mediators in the bronchoalveolar lavage fluid or sputum of patients. Recent findings revealed that mitogen-activated protein kinase (MAPK) signaling cascade is involved in the inflammatory response of lung injury. In the present study we could elucidate the role of extracellular signal-related MAPK in the murine model of LPS-induced acute lung injury by using U0126, a specific inhibitor of MEK1/2, upstream kinases of ERK. Phosphorylation of ERK was inhibited by U0126 in vivo as well as in vitro. In freshly isolated human peripheral blood mononuclear cells U0126 dose-dependently blocked the release of IL-2 and TNF-α. For in vivo studies mice were exposed to aerosolized LPS to induce an acute lung injury mimicking some aspects of COPD. This led to a recruitment of neutrophils to the lung and to the release of pro-inflammatory cytokines into bronchoalveolar lavage. Pretreatment of mice with U0126 significantly reduced lung neutrophilia and diminished levels of TNF-α and chemotactic MIP-2 and KC in bronchoalveolar fluid. U0126 also decreased albumin levels in BAL fluid, a marker of vascular leakage. Histological examination of lung tissues revealed that ERK MAPK inhibition using U0126 efficiently attenuated LPS-induced pulmonary inflammatory responses. These data suggest that ERK signaling plays an important role in acute lung injury and pharmacologic inhibition of ERK provides a promising new therapeutic strategy for lung inflammatory diseases and in particular COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by airflow obstruction that is not fully reversible. It is a major global health problem with growing incidence, which places an increasing burden on health services in industrialized and developing countries [1]. The term COPD encompasses emphysema, chronic obstructive bronchitis and a chronic inflammation in the smaller airways and lung parenchyma [2,3]. The local inflammation observed in COPD patients is predominated by neutrophilic granulocytes [4]. Furthermore increased numbers of macrophages and T-cells, predominantly CD8+ T-cells, are found in the alveolar walls of COPD patients [5].

Cigarette smoke is the major risk factor for the development of COPD, but also exposure to air pollution and genetic factors play an important role [2]. Tobacco and cigarette smoke contain high levels of lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, which is a potent inflammatory stimulus [6]. Inhalation of LPS causes acute and chronic inflammation in murine airways [7], which is accompanied by recruitment of neutrophils to the lung and increased levels of pro-inflammatory cytokines and chemokines in the lung. Acute or chronic inhalation of LPS mimics some relevant features of COPD in mice and serves as a model for preclinical analysis of possible drug candidates [8].

LPS induced lung injury is mediated through Toll-like-receptor 4 (TLR4) and CD14 ligation, that leads to the activation of mitogen-activated protein kinase (MAPK) signaling cascades, which ultimately results in the secretion of tumor necrosis factor α (TNF-α) and other pro-inflammatory cytokines [9,10]. The MAPK families are ubiquitous and highly conserved serine-threonine

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Abbreviations: COPD, chronic obstructive pulmonary disease; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; LPS, lipopolysaccharide; ERK, extracellular signal-related kinase; MEK, extracellular signal-regulated kinase kinase; TNF, tumor necrosis factor; PBMC, peripheral blood mononuclear cells; MIP, macrophage inflammatory protein; KC, keratinocyte-derived chemokine; BAL, bronchoalveolar lavage; MPO, myeloperoxidase.

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kinases, including p38MAPK, extracellular signal-related kinase (ERK) and c-Jun N-terminal kinase (JNK). The signaling cascade is initiated by three-tired sequential phosphorylation steps (MAPKKK, MAPKK, MAPK). MEK1 and MEK2 are upstream kinases (MAPKK) of ERK1 and ERK2. Recently a number of studies revealed that p38 MAPK is an important enzyme for the pathogenesis of LPS-induced lung injury. Compound 37, a specific p38 α,β MAPK inhibitor, attenuated LPS-induced bronchoconstriction and neutrophil recruitment into the lungs in a dose-dependent manner [11]. It was concluded, that p38 MAPK mediates lung neutrophilia, cytokine and chemokine production and bronchoconstriction [11,12].

It has been shown that ERK1/2 pathway plays a pivotal role in IL-13-induced asthma-like lung inflammation [13]. We hypothesized that the ERK1/2 pathway also plays an important role in LPS-mediated pulmonary inflammation and that inhibition of ERK signaling pathway may have anti-inflammatory effects. To this end we investigated the impact of U0126, a selective and potent MEK1/2 inhibitor, in the mouse model of acute LPS-induced lung injury and examined wether pharmacologic inhibition of ERK attenuates disease severity.

2. Materials and methods

2.1. Animals

All animal protocols were performed in accordance with the national animal protection rules and permitted by the local governmental authority (Regierung von Mittelfranken, Germany). BALB/c mice 6–8 weeks of age weighing 22–25 g (purchased from Harlan, Borchen, Germany) were used. The animals were fed on a normal standard diet (food pellets purchased from Altromin, Lage, Germany) with tap water ad libitum and were housed in a 12 h light/dark cycle, at 20–21 °C and with 40–60% humidity levels.

2.2. Mouse model of LPS-induced lung inflammation

The animals were treated intraperitoneally with U0126 (30 mg/kg; purchased from Tocris Biosciences, Ellisville, USA), PD98059 (30 mg/kg; Tocris Biosciences, Ellisville, USA) or vehicle (200 μl PBS with 5% DMSO, Invitrogen, Karlsruhe, Germany) 2 h prior to LPS challenge. U0126, PD98059 or vehicle-treated mice were exposed to aerosolized LPS (10 mg; 1.5 mg/ml saline; lipopolysaccharides from *Escherichia coli* Serotype 026:B6, Sigma, St. Louis, USA) using the Buxco-Nebulizer (Buxco, Wilmington, USA). One inhalation cycle encompassed 9.9 min of LPS (25% duty) and followed by 5 min of drying. This was repeated four times. Animals were sacrificed 24 h or at the indicated time point after LPS challenge by putting them into a CO_2 rich atmosphere. Tracheotomy was performed and a 24G cannula (InSyte 24G, BD Biosciences, Heidelberg, Germany) was inserted in the trachea. In each animal bronchoalveolar lavage (BAL) was performed by flushing the airways and lungs (6×0.5 ml) with cold Hank's balanced solution (Gibco, Karlsruhe, Germany) supplemented with Na-EDTA and HEPES (Sigma, Steinheim, Germany). The supernatants of the first lavage were collected and stored at −20 °C for further analysis. The cells of pooled BAL of each animal were sedimented by centrifugation at 300 × *g* for 5 min. The cell pellets were re-suspended in 2 ml RPMI-1640 (Invitrogen, Karlsruhe, Germany) and total BAL cell counts of each animal were determined using a haemocytometer (Sysmex microcellcounter F300, Norderstedt, Germany). For cytological examination, cytospin slides were prepared using a cytospin centrifuge (Thermo Shandon, Frankfurt, Germany) and stained with Diff-Quick (Dade-Behring, Marburg, Germany). Differential cell count was performed with at least 400 cells/slide.

2.3. Cytokine and chemokine measurements

Levels of tumor-necrosis factor α (TNF-α), macrophage inflammatory protein 2 (MIP-2) and keratinocyte-derived chemokine (KC) in BAL fluid were measured using a murine TNF-α OptEIA ELISA kit (BD Biosciences, San Diego, USA) and murine MIP-2 and murine KC DuoSet ELISA Development kits (R&D Systems, Minneapolis, USA). Levels of TNF-α and interleukin 2 (IL-2) in supernatants of stimulated PBMC were determined using a human TNF-α OptEIA ELISA Set and a human IL-2 OptEIA ELISA kit (BD Biosciences, San Diego, USA). Cytokine and chemokine levels were assayed according to the manufacturer's recommendations.

2.4. Phospho-p44/42 MAPK and phospho-p38 MAPK sandwich ELISA

Mice were treated intraperitoneally with U0126 (30 mg/kg) or vehicle 2 h prior to LPS challenge. Two hours later mice were sacrificed the lungs were removed and stored in liquid nitrogen. After homogenization by ultra turrax the lysate was microcentrifuged and the supernatant was used for detection of pERK and pp38 (PathScan Phospho-p44/42 MAPK (Thr202/Tyr204) Sandwich ELISA Kit and PathScan Phospho p38α MAPK (Thr180/Tyr182) Sandwich ELISA Kit (Cell Signaling, Danvers, USA)) according to the manufacturers recommendations. Absorbance at 450 nm is proportional to the quantity of p44/42 MAPK phosphorylated at Thr202/Tyr204 and p38 MAPK phosphorylated at Thr180/Tyr182.

2.5. Lung histological examination

Lungs were instilled intratracheally with 15 ml 4% buffered formaldehyde (Roth, Karlsruhe, Germany) using a syringe pump (sp200i, WPI, Berlin, Germany) and fixed overnight in formaldehyde. The tissues were embedded in paraffin and 4 μm slices were cut. To examine bronchial inflammation sections were stained with conventional hematoxylin and eosin (Roth, Karlsruhe, Germany). Bronchial inflammation was determined using a semiquantitative score as described by Myou et al. [14]. The severity of inflammation was graded in five categories; 0, normal lung structure; 1, few inflammatory cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep. The score was determined in a blinded manner.

2.6. Myeloperoxidase assay

Determination of myeloperoxidase activity was adapted from Matos et al. [15]. The lungs were flash frozen in liquid nitrogen immediately after removing and stored at −80 °C until examination. The frozen lungs were homogenized with an ultra turrax in 1 ml Na_2PO_4 buffer (0.05 M, pH5.4) and centrifuged for 10 min at 20,000 × *g* at 4 °C. The supernatants were discarded. The pellet was resuspended in 1 ml Na_2PO_4 buffer containing 0.5% hexadecyltrimethylammonium bromide (Sigma–Aldrich, St. Louis, USA) and homogenized. This was followed by three freeze-thaw cycles using liquid nitrogen. After centrifugation at 20,000 × *g* for 10 min at 4 °C the supernatants were collected. Tetramethylbenzidine (TMB) and hydrogen peroxide (TMB Substrate Reagent Set, BD Biosciences, San Diego, USA) were added to the supernatants and the reaction was stopped after 5 min with 1 M H_3PO_4. The activity of MPO was quantified by measuring absorbance at 450 nm.

2.7. Vascular leakage

The vascular leakage was determined by measurement of total albumin levels in supernatants of bronchoalveolar lavage using a Bradford-Assay (Bio-Rad, München, Germany).