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Inflammation, mucous cell metaplasia, and Bcl-2 expression in response to inhaled lipopolysaccharide aerosol and effect of rolipram

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ABSTRACT

Our previous studies have characterized the inflammatory response of intratracheally instilled lipopolysaccharides (LPS) in F344/N rats. To better reflect the environmentally relevant form of LPS exposure, the present study evaluated the inflammatory response of F344/N rats exposed to LPS by inhalation. Rats were exposed by nose-only inhalation to aerosolized LPS at a median particle diameter of 1 μm and a dose range from 0.08 to 480 μg. Animals were euthanized 72 h post exposure and the inflammatory cell counts and differentials, the cytokine/chemokine levels in the bronchoalveolar lavage fluid (BALF), and the changes in intraepithelial stored mucosubstances, mucous cells per mm basal lamina, and Bcl-2-positive mucous cells were quantified. We observed a dose-dependent increase reaching maximum values at the 75 μg LPS dose for the numbers of neutrophils, macrophages and lymphocytes, for the levels of IL-6, IL-1α, IL-1β, TNFα, MCP-1 and GRO–KC. In addition, mucous cell metaplasia and the percentage of Bcl-2-positive mucous cells were increased with an increasing deposited LPS dose. When rats were treated with the phosphodiesterase-4 (PDE4) inhibitor, rolipram (10 mg/kg), prior to exposure to aerosolized LPS neutrophil numbers in the BAL were reduced at 8 h but not at 24 or 72 h post LPS exposure. These results demonstrate that exposure to aerosolized LPS resulted in a more potent inflammatory response at lower doses and that inflammation was more uniformly distributed throughout the lung compared to inflammation caused by intratracheal LPS instillation. Therefore, this animal model will be useful for screening efficacy of anti-inflammatory drugs.

Introduction

Lipopolysacharide (LPS) or endotoxin is a component of a gram-negative bacteria cell wall and is commonly used to elicit an inflammatory response in the airways of laboratory animals. Humans are often exposed to LPS that is suspended in the air as part of house dust or as aerosols generated from contaminated water. Furthermore, occupational exposures to LPS are common for people in agricultural settings or in textile mills (Clapp et al., 1993; Schwartz et al., 1995; Schwartz, 1996). Macrophages and epithelial cells act as the first line of defense when healthy pulmonary airways are exposed to LPS. Activated macrophages release the cytokines IL-1β, TNFα, MCP-1 and MIP-1β (Chung, 2006) and epithelial cells secrete IL-8 and MCP-1 *in vitro* (Thorley et al., 2007). The release of these cytokines and chemokines is essential for the efficient recruitment of neutrophils from the pulmonary capillaries into the air spaces (Panina-Bordignon and D'Ambrosio, 2003). Once within the pulmonary airspace, neutrophils and other inflammatory cells release cytokines and chemokines, such as TNFα, IL-6 and IL-1β, which initiate epithelial cell proliferation and differentiation into mucous cells, a process termed mucus cell metaplasia (MCM) (Foster et al., 2003; Tesfaigzi et al., 2004). Secreted mucus on airway epithelial surfaces traps foreign particles and allows particle transport out of the airways via the mucociliary function. Normally, MCM is reduced when the environmental insult is removed both by reducing expression of the mucin gene MUC5AC and by reducing the mucous cell numbers via programmed cell death (Harris et al., 2007). The reduction of MCM involves the downregulation of Bcl-2, an inhibitor of cell death (Harris et al., 2005).

Although the most common route of LPS exposure in the environment is by inhalation, this route of exposure is the least common mode utilized in laboratory studies. The majority of studies of LPS-induced pulmonary inflammation are conducted by either intravenous injection or instillation directly into nasal or tracheal passages. While studies conducted by intratracheal (IT) instillation can provide important information (Tesfaigzi et al., 2000; Tesfaigzi et al., 2004; Harris et al., 2005), we decided to characterize the inflammatory response of LPS delivered by inhalation as inhalation is the clinically relevant route for human subjects. We hypothesized that a more diffuse deposition (rather than bolus administration) of LPS in the respiratory tract by inhalation would better resemble inflammation in human

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disease and may be a useful model for screening anti-inflammatory drugs.

In the current study we describe a novel method of delivering aerosolized LPS to the lungs of male F344N rats at defined particle size distribution, and we characterized the inflammatory and epithelial cell response at 72 h post LPS inhalation. The dose of LPS over a wide range of concentrations was analyzed to better compare the effect of LPS when delivered as aerosol compared to LPS delivered by IT instillation (Harris et al., 2005; Foster et al., 2003; Tesfaigzi et al., 2000). After a detailed characterization of the inflammatory response, we tested the ability of the PDE4 inhibitor, rolipram, a known inhibitor of LPS-induced neutrophilic inflammation (Spond et al., 2001), to reduce pulmonary inflammation in the model of inhaled LPS in the rat. We found that LPS when delivered by inhalation increased inflammation and MCM at much lower concentrations and in a more linear fashion than when delivered by IT instillation and that this animal model is well-suited to screen for anti-inflammatory drugs.

Materials and methods

Animals. Male pathogen-free F344 rats (Charles River Laboratories, Wilmington, MA) aged 8–10 weeks were housed in pairs and provided food and water *ad libitum*. Rats were exposed to a 12:12 h light:dark cycle and housed at 23 °C with 20–40% humidity. Each rat was individually weighed and randomly assigned to each experimental group. All experiments were approved by the Lovelace Respiratory Research Institute Institutional Animal Care and Use Committee. Experiments were performed at the Institute in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Intratracheal instillation. Intratracheal instillation was performed as previously described (Tesfaigzi et al., 2004). Briefly, rats designated for intratracheal instillation of LPS were anesthetized with 5% halothane in oxygen and intratracheally instilled with 1000 μg of LPS from *Pseudomonas aeruginosa* serotype 10, (Sigma, St. Louis, MO) in 500 μl deionized water.

Generation of exposure atmosphere. .09pt?>All aerosol generation was conducted in a glove box connected to an exhaust system. Aerosolization was conducted by nebulization of LPS (*Pseudomonas aeruginosa* serotype 10 (Sigma, St. Louis, MO) that was diluted with deionized water. Solutions were nebulized with a Hospitak (Lindenhurst, NY) nebulizer followed by a diffusion drier (custom flow-through drier with Dri-rite™ dessicant) that dried the aerosol (high humidity saturated sample filters and impactor substrates). Aerosolized LPS was delivered to a 24-port nose-only inhalation chamber (In-Tox Products, Inc., Albuquerque, NM) operated at approximately 30 l/min.

LPS concentration was determined by gravimetric analysis of filter samples collected during the exposure. Filter samples were collected approximately every 5 min from the time the first animal was introduced until the last animal was removed from the exposure plenum. Aerosols were collected (from the exposure plenum) on Type T60A20, 47-mm Pallflex membrane filters (Pall Gelman Sciences, Ann Arbor, MI) at a nominal volumetric flow rate of approximately 1 l/min. After collection the filters were removed from the filter holders, placed in Petri dishes and weighed.

Filters were extracted with water and assayed by the Limulus assay described below. Real-time analysis of aerosol concentration was conducted with a RAM-S nephelometer. The real-time data was utilized as a guide to adjust dilutions and maintain exposure concentrations.

The pulmonary dose of LPS was calculated using the following formula: $\text{Dose} = (C \times \text{RMV} \times T \times \text{DF})/\text{BW}$, where C is the average concentration of test article or LPS in the exposure atmosphere, T is exposure time and the deposition fraction (DF) is assumed to be 10% to target pulmonary deposition fraction (this is appropriate at the historical LPS particle size of ~1 μm). Respiratory minute volume (RMV; l/min) is calculated using the following allometric equation: $\text{RMV} = 0.499\text{BW}^{0.809}$, where BW is the average exposure day body weight in kg (Bide et al., 2000). Rats were exposed to filtered air or to LPS at 1 mg/m^3 or 25 mg/m^3. Doses of 0.08 and 0.8 μg were achieved by exposing for 5 and 50 min at 1 mg/m^3. Doses of 5 and 75 μg were achieved by exposing to approximately 25 mg/m^3 for approximately 20 and 180 min.

Rolipram treatment. Two hours prior to LPS inhalation, rats were dosed by oral gavage with vehicle (0.4% methylcellulose) or 10 mg/kg rolipram. For this study, rats were exposed by a single nose-only inhalation to an aerosol concentration yielding approximately 15 μg of a deposited pulmonary dose of LPS. Because of the change in the lot number, endotoxin activity for the dose–response study was 900,000 EU/mg and 3,000,000 EU/mg for the rolipram study.

Determination of particle size. Particle size distribution was determined with a 7-stage Mercer style impactor (In-Tox Products, Inc., Albuquerque, NM). The aerosol was withdrawn directly from the exposure chamber atmosphere. Particle size was approximately 1 μm with a geometric standard deviation of ~2.0.

LPS inhalation exposure, necropsy, and bronchoalveolar lavage fluid. Rats were acclimated to the nose-only exposure tubes on two days preceding exposure (Fig. 1) by placing them individually in exposure tubes and waiting for 15 and 30 min sequentially. During LPS exposure, atmospheric oxygen content, temperature, and relative humidity were measured continuously to ensure that the environmental conditions were within acceptable limits. After exposure, animals were returned to shoe-box type cages and 72 h later euthanized by injecting them with pentobarbital followed by exsanguination through the renal artery. The lung vasculature was perfused via an injection of 10 ml cold pathogen-free saline through the heart. The trachea was cannulated with an 18 gauge blunt needle and the lungs were removed. For the LPS dose–response study, the

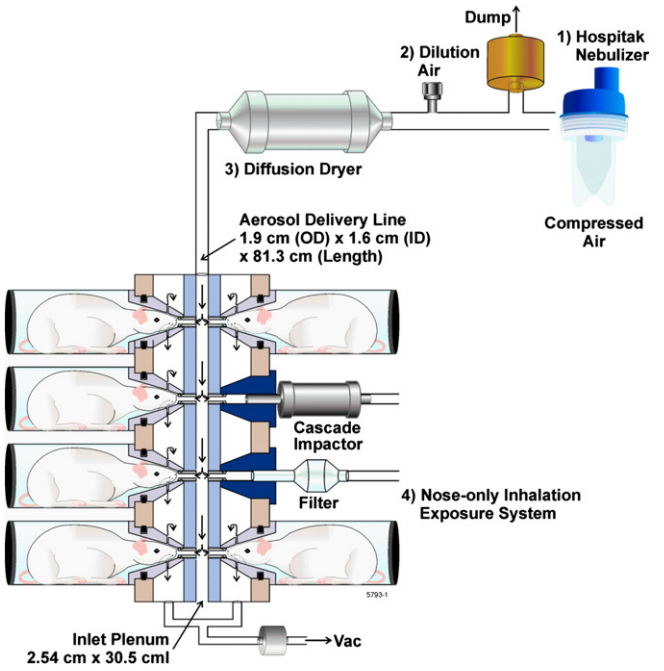

Fig. 1. Schematic of the LPS nose-only inhalation exposure system consisting of a Hospitak nebulizer, dilution air, and diffusion dryer. Samples for particle mass and size were obtained directly from the nose-ports.