Altered surfactant protein A gene expression and protein metabolism associated with repeat exposure to inhaled endotoxin

Caroline L. S. George,1 Misty L. White,1 Marsha E. O’Neill,2 Peter S. Thorne,2 David A. Schwartz,3 and Jeanne M. Snyder4

1Department of Pediatrics, Division of Pediatric Critical Care; 2Department of Occupational and Environmental Health, College of Public Health; 3Department of Anatomy and Cell Biology, University of Iowa, Iowa City, Iowa 52242; and 4Department of Internal Medicine, Duke University Medical Center, Durham, North Carolina 27710

Submitted 7 March 2003; accepted in final form 13 August 2003

LUNG SURFACTANT is a complex mixture of lipids and proteins secreted by the alveolar type II cell. Surfactant lines the alveolar surface and reduces surface tension at end-expiration. Surfactant also plays a role in innate host defense mechanisms at the level of the alveolus. Two of the surfactant-associated proteins, SP-A and SP-D, are collectins, which are a family of collagenous, carbohydrate-binding proteins that are part of the innate immune system (4). As a group, collectins are found throughout the body. SP-A, specifically, has been observed in the conducting airways, in the inner ear, and in organs of the gastrointestinal and genital-urinary tracts (14). Both SP-A and SP-D can bind gram-negative lipopolysaccharide (LPS), viruses, fungal cell wall components, and pollens (4, 14). SP-A can also act as an opsonin, facilitating the phagocytosis of rough LPS by alveolar macrophages (27). SP-A levels in bronchial lavage fluid are decreased in human diseases such as acute respiratory distress syndrome (ARDS) associated with either gram-positive or gram-negative pneumonia (11). The decrease in SP-A protein levels observed in ARDS is most likely the result of plasma protein inhibition of SP-A function in the alveolar space (11), incorporation of SP-A into hyaline membranes, and damage to the SP-A protein by inflammatory mediators (10).

The role of SP-A in host defense during chronic lung injury, for example, due to occupational or domestic exposures to inhaled toxins, has had little attention. To date it has been documented that SP-A levels are increased in asbestos workers and in an animal model of silica-induced lung injury (16, 33). SP-D levels are also increased in these conditions and also with hypersensitivity pneumonitis in mushroom worker’s lung (37). Currently, there is no information available concerning the effects of chronically inhaled endotoxin on the levels of the surfactant proteins.

Physiological and inflammatory changes can occur with chronic inhalation of organic dusts containing endotoxins. A steady progressive decline in pulmonary function has been documented in grain elevator and swine confinement workers (24, 31). The type of lung injury that is associated with the inhalation of grain dust particles includes a decline in lung function, i.e., forced expiratory volume in 1 s, neutrophil influx into the air spaces, and an increase in peripheral white blood cell count (3, 5). In a murine model of acute lung injury induced by grain dust inhalation, similar findings have been observed (42). An increase in the proinflammatory cytokines TNF-α, IL-1α, and macrophage inflammatory protein-2 was also associated with this acute lung injury model.

We have previously described a murine model of subchronic occupational exposure to inhaled endotoxin.

http://www.ajplung.org 1040-0605/03 $5.00 Copyright © 2003 the American Physiological Society

Address for reprint requests and other correspondence: C. L. S. George, Dept. of Pediatrics, 200 Hawkins Dr., Univ. of Iowa Hospitals and Clinics, Iowa City, IA 52242 (E-mail: caroline-george@uiowa.edu). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
in-laden grain dust particles that mimics human disease in many ways. After a 2-mo exposure to a nebulized solution of corn dust particles that contains biologically active levels of endotoxin, mice develop air space inflammation characterized by an influx of neutrophils, an increase in proinflammatory cytokines, and airway hyperreactivity (8). After resolution of this inflammatory response, the mice demonstrate persistent airway hyperreactivity associated with airway wall remodeling. All of the lung changes observed in this chronic lung injury model are dependent on a functional Toll-like receptor-4 gene (TLR-4). The TLR-4 gene produces the major transmembrane component of the LPS receptor (28).

The primary aims of the present study were to determine the effects of long-term exposure to endotoxin on pulmonary SP-A gene expression and to determine whether the SP-A response to endotoxin is dependent on an intact TLR-4 gene. C3HeB/FeJ mice (wild type) and C3H/HeJ mice, a strain that contains a TLR-4 gene mutation, were exposed to nebulized grain dust from corn that contained biologically active levels of endotoxin or to inhaled LPS for 2–4 wk. Our results demonstrate that mice with a functional TLR-4 gene develop airway and interstitial lung inflammation, a decrease in lung SP-A protein levels despite increased SP-A gene expression, and type II cell hyperplasia, all in response to inhaled LPS. In contrast, mice with a TLR-4 mutation do not develop inflammation or alter their pulmonary SP-A levels.

MATERIALS AND METHODS

Materials. All materials were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Animals. Wild-type mice sensitive to endotoxin (C3HeB/FeJ) and endotoxin-resistant mice (C3H/HeJ) were purchased from Jackson Laboratories (Bar Harbor, ME) at 6–8 wk of age. They were exposed to corn dust extract (CDE) or LPS (Escherichia coli O111:B4) for 4 or 2 wk, respectively. The CDE and purified LPS were delivered by nebulizing the solutions into an exposure chamber for 4 h/day, 5 days per week, throughout the exposure time period. We determined the effects of long-term exposure to endotoxin or to inhaled LPS by controlling for endotoxin levels during the exposure. The exposure was monitored by collecting a filter exposed to the chamber outlet for 15 min, four times during each 4-h exposure, and quantifying using the kinetic chromogenic Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD) as previously described (38). The average endotoxin level measured during the CDE exposure was 9.83 ± 5.33 μg/m³, and the average endotoxin level during the purified LPS exposures was 2.45 ± 0.62 μg/m³.

Lung lavage and cellular analysis. Techniques used to characterize immune cells in lung lavage fluid have been previously described by our laboratory (8). Briefly, mice were euthanized with pentobarbital (150 mg/kg ip). The trachea was dissected and cannulated with PE-90 tubing. The diaphragm was punctured, and 0.5–1 ml of sterile normal saline was allowed to passively flow into the lungs and collected as it flowed out. This was repeated with a total of 4 ml of normal saline. Cells from the lavage fluid were counted by a hemocytometer. The lavage fluid was then centrifuged at 2,200 rpm at 4°C for 5 min and the pellet resuspended in Hanks’ balanced salt solution, (GIBCO-BRL, Grand Island, NY). We determined the proportions of different cell types present (i.e., alveolar macrophages, neutrophils, lymphocytes, etc.) by examining cytospin preparations of lung lavage cells stained with a Wright-Giemsa stain.

SP-A gene expression. Lung tissue was collected following lavage fluid collection. The lung tissue was snap-frozen in liquid nitrogen and stored at −70°C. RNA was isolated from whole lung using TRIzol reagent (GIBCO-BRL) and subsequently quantified by measuring absorbance at 260/240 nm with a spectrophotometer. The protocol for Northern blot analysis has previously been described (9). Briefly, the total RNA was separated by electrophoresis on a 1.2% agarose, 5% formamide gel. The ethidium bromide-stained rRNA bands in the gel were photographed on a UV light box, and this was used to control for loading. The RNA was then transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) and cross-linked using a Bio-Rad GS Gene Linker (Bio-Rad, San Jose, CA). The membranes were prehybridized in 10 ml of hybridization buffer at 42°C for 6 h and overnight with an added 5 ml of fresh buffer containing a 32P-labeled mouse SP-A cDNA probe radiolabeled to a specific activity of 1 × 109 cpm/μg (Amersham, Bedford, MA). The probes were radiolabeled with a random primer labeling kit (Boehringer Mannheim, Indianapolis, IN). After hybridization, the membranes were washed two times in 2× SSC for 5 min at room temperature, two times in 2× SSC containing 1% SDS for 30 min at 65°C, and once in 0.1× SSC for 15 min at room temperature. The membranes were then wrapped in plastic wrap and exposed to X-ray film (Eastman Kodak, Rochester, NY) for up to 14 days. The hybridized bands were quantified with a Bio-Rad GS710 Calibrated Imaging Densitometer and software (Hercules, CA). Loading artifacts were corrected by quantifying the density of the 18s rRNA band from the RNA gel as previously described (6). Data are presented as the relative amount of SP-A mRNA. Specifically, the SP-A amount in the wild-type baseline group was made equal to 1, and all other data were normalized in relation to this group of control animals.
then allowed to recover to 1 mo, 16 wk of age), are indicated. The rate (CDE), 12 wk of age], and Recovered (mice exposed to CDE for 1 mo age), Exposed [after exposure to 1 mo aerosolized corn dust extract strains of mice are presented. Three conditions, Baseline (8 wk of

Immunohistochemistry. To prepare the lung tissue for histological examination, we performed a thoracotomy on euthanized mice. The right heart was infused with PBS to clear the pulmonary vasculature. The trachea was then cannulated, and zinc-formalin (LABSCO, Louisville, KY) was used to inflate and fix the lungs at 25 cmH2O pressure. The lungs were then excised and processed into paraffin blocks. Tissue sections were cut 7 μm thick and mounted onto glass slides. Sections obtained from wild-type and C3H/HeJ mice at baseline and after exposure to 2 wk of aerosolized LPS were incubated with guinea pig anti-human SP-A antiserum (1:1,000) at room temperature for 1 h, as previously described (9). The bound primary antibody was visualized with a Vectastain Elite kit (Vector, Burlingame, CA). SP-A-positive cells turned brown after incubation with the substrate 3,3'-diaminobenzidine. Four to five digitalized images from each animal, avoiding conducting airways and blood vessels, were captured with a Spot Jr. digital camera. The images were coded, and two independent investigators recorded the number of cells that stained positive for SP-A. Data for each animal (n = 4 animals per group) were collected from both observers and pooled, and then the mean number of positive cells per μm² ± SE was calculated for each group.

Lung lavage protein analysis. Cell-free lung lavage fluid was used to assess the levels of total protein and SP-A protein present in the alveolar spaces. A Bradford protein assay was performed to measure the levels of protein in each sample (Bio-Rad). A 10% acrylamide gel was used to separate the lung lavage proteins by electrophoresis, with 15 μg of protein per sample loaded along with 6× sample buffer. The separated proteins were then transferred to a Trans-Blot Transfer Medium Membrane (Bio-Rad) using electrophoresis. Unoccupied sites on the membrane were blocked overnight at 4°C with 7% nonfat dry milk in TNT buffer (Tris-buffered saline, 10 mM Tris, pH 7.5, 150 mM NaCl, with 0.05% vol/vol Tween 20). The membrane was then incubated for 1 h at room temperature with guinea pig anti-human SP-A antiserum (diluted 1:250 in blocking solution) and then subjected to three washes, for 15 min each, in TNT buffer as previously described (9). The membrane was incubated for 1 h at room temperature in rabbit anti-guinea pig IgG conjugated to alkaline phosphatase (diluted 1:10,000 in blocking solution) and then washed three times for 15 min each in TNT. Next, the membrane was exposed to ECL Western Blotting Detection Reagents (Amersham Biosciences) for 1 min before being wrapped in plastic wrap. Each membrane was then exposed to Classic Blue Sensitive X-ray film (Midwest Scientific, St.

RESULTS

Throughout the inhalation exposures the mice were observed for signs of illness or stress. At baseline, the total body weight of the wild-type mice was slightly heavier than the TLR-4 mutant mice (C3H/HeJ), 30.1 vs. 25.3 g. During the 1-mo CDE exposure and 1-mo recovery period, both strains of mice grew at a similar rate (Fig. 1). Similar findings were observed with mice exposed to 2-wk LPS (data not shown).

Lung inflammation. After inhalation of CDE for 1 mo, there was an increase in pulmonary interstitial inflammation as shown by histological examination in wild-type mice, while no inflammatory changes were observed in the C3H/HeJ strain (Fig. 2). The total number of cells in lung lavage fluid from wild-type mice exposed to CDE was significantly increased (P < 0.05) compared with exposed C3H/HeJ mice and to baseline, wild-type mice (Fig. 3). After a 1-mo recovery period, the inflammatory response in the air space resolved (Fig. 3). After inhalation of LPS for 2 wk, the inflammatory changes seen in the wild-type mice consisted of an increased air space cellular infiltration composed...
primarily of neutrophils and macrophages (Fig. 4). In summary, the inflammatory changes in the lung following inhalation of CDE or LPS for 4 or 2 wk, respectively, were present only in the wild-type mice and were not observed in the C3H/HeJ mice.

SP-A gene expression. In an initial experiment, SP-A mRNA levels were examined in both wild-type and C3H/HeJ mice at baseline, after inhalation of CDE for 1 mo, and following a 1-mo recovery period. After the inhalation exposure to CDE, the wild-type mice demonstrated a threefold increase in SP-A mRNA levels compared with baseline levels (Fig. 5). This increase was not observed in the C3H/HeJ mice. In the wild-type mice exposed to the CDE for 1 mo and then allowed to recover for 1 mo, SP-A mRNA levels were slightly higher than baseline levels; however, the difference did not reach statistical significance.

Because it has been demonstrated that it is primarily the endotoxin contained in grain dust that causes lung inflammation, subsequent experiments were performed in mice exposed to inhaled purified LPS. SP-A mRNA levels were evaluated in both wild-type and C3H/HeJ strains of mice following 2 wk of repeat exposure to aerosolized LPS. The wild-type mice had a significant four- to fivefold increase in SP-A mRNA levels compared with the levels in unexposed wild-type mice (Fig. 6). In contrast, the levels of SP-A mRNA in lung tissue from LPS-exposed C3H/HeJ mice were not significantly different from the unexposed C3H/HeJ mice (Fig. 6).

Type II cell alterations. SP-A is synthesized and secreted by alveolar type II epithelial cells. We have observed that wild-type mice exposed to LPS have more cells that stain positive for SP-A that do the TLR-4 mutant (C3H/HeJ) mice (Fig. 7). When these alveolar epithelial cells are quantified, the wild-type mice exposed to 2 wk of inhaled LPS had significantly \((P < 0.001, \text{by ANOVA})\) more type II cells than C3H/HeJ mice exposed to 2 wk of LPS and than wild-type mice at baseline (Fig. 8).

Lung lavage fluid protein analysis. The total protein content was measured in cell-free, lung lavage fluid collected from the alveolar space. The wild-type mice exposed to 2-wk inhaled LPS had significantly \((P < 0.05)\) more protein in their alveoli than the exposed C3H/HeJ mice or the unexposed wild-type mice (Fig. 9). We analyzed the SP-A protein content of the lung lavage fluid by performing Western blots. The relative amount of SP-A protein per microliter of lavage fluid was significantly decreased in the wild-type animals exposed to 2 wk of inhaled LPS compared with the unexposed (baseline) wild-type animals (Fig. 10A). This decrease in SP-A was present even when the amount of SP-A was expressed per microgram of total lung lavage protein (Fig. 10B). In contrast, the C3H/HeJ
HeJ mice did not demonstrate a decrease in SP-A protein levels in the exposed condition compared with their baseline controls. These data are representative of two experiments where \( n = 4 \) animals per experimental group.

**DISCUSSION**

The present study strengthens the hypothesis that endotoxin is a key player in agricultural and industrial occupational exposures by identifying airway and lung interstitial cellular inflammatory patterns following CDE and LPS exposures that are virtually indistinguishable. Also, mice with a TLR-4 mutation do not develop lung inflammation in response to inhaled CDE \( (8) \) or to purified LPS.

Relatively little is known about SP-A expression in chronic lung injury, and even less is known about the role SP-A may play in endotoxin-related lung injuries. However, since SP-A can bind the lipid A portion of some types of LPS, it is possible that as part of the innate immune system SP-A may have a key role in this type of lung injury \( (4) \). We have demonstrated that repetitive exposures to inhaled endotoxin associated with either grain dust or a purified form result in increased SP-A mRNA levels. This increase in SP-A gene expression was associated with a neutrophilic, alveolar macrophage and interstitial monocytic type of inflammatory response. In mice unable to respond to
endotoxin, increased SP-A gene expression was not observed following either the CDE or LPS exposures.

Our murine model of repetitive exposure to endotoxin is the first attempt to characterize changes in SP-A that may occur in an occupational and domestic setting associated with endotoxin exposure. Alterations in SP-A levels and gene expression have been previously described following a single dose of LPS to the airway. Specifically, SP-A levels increased in both the lavage fluid and lung tissue and then returned to baseline over several hours to days (21, 39, 41). SP-A gene expression likewise was increased following a single dose of LPS to the airway and returned to baseline levels after several weeks (21, 36).

Increased SP-A gene expression has also been associated with various types of repetitive environmental exposures. Silicosis is an occupational lung injury resulting in lung fibrosis (22). Silicosis is characterized by an inflammatory pattern similar to endotoxin-induced lung injury. Both endotoxin- and silica-induced lung injuries are associated with an influx of primarily neutrophils and alveolar macrophages into the lung alveolar spaces. Increased SP-A protein levels and type II cell hypertrophy are also observed in silicosis (17). SP-A protein levels are also increased following exposure to asbestos (16). In a baboon model of bronchopulmonary dysplasia induced by prematurity and oxygen exposure, SP-A mRNA levels in lung tissue were increased, whereas the SP-A protein level in the alveolar space was significantly reduced (1). In contrast to these lung injury and associated inflammation models, repeated exposure to ozone does not result in altered SP-A mRNA levels despite the presence of an inflammatory response in the lung (35). In summary, like most characterized types of chronic or repeat exposure lung injuries associated with an inflammatory response, SP-A gene expression and metabolism are altered following chronic exposure to inhaled endotoxin.

Increased SP-A gene expression following a 2-wk exposure to inhaled LPS was associated with increased numbers of type II alveolar epithelial cells per unit of lung tissue. Our study focused on SP-A production in type II cells. Mice also produce SP-A in nonciliated cells of the conducting airways (26). Because our previous work did not demonstrate a change in the volume density of the epithelium in conducting airways following repeat exposure to CDE (8), we chose to focus on the alveoli as the primary source of SP-A. Type II cell hyperplasia is associated with several types of lung injury and is a necessary step in healing the alveolar portion of the lung (13, 25). The alveolar type I cell destruction that is associated with various types of acute lung injury is repaired by the proliferation of type II cells. There also is evidence that supports an antifibrotic role for type II cells in the setting of silica lung injury (15). Several growth factors are associated with type II cell hyperplasia, including keratinocyte growth factor (KGF) and granulocyte macrophage colony-stimulating factor (GM-CSF) (12, 23). Others have demonstrated that GM-CSF and KGF production is stimulated by endotoxin (2, 29). Future studies will investigate alterations in these growth factors in our murine model of repeat exposure lung injury.

**Fig. 9.** Total protein present in lung lavage fluid. Wild-type mice (solid bars) exposed to 2 wk of inhaled LPS (Exposed) had significantly (*P < 0.05, ANOVA) more protein in lung lavage fluid compared with the exposed C3H/HeJ mice (open bars) and to the unexposed (Baseline) wild-type mice (*P < 0.05). These data are representative of 2 experiments where n = 4 mice per group. Data are expressed as the means ± SE.

**Fig. 10.** SP-A protein in lung lavage fluid. Total lavage fluid protein (15 µg) was loaded onto each lane. Data from 2 wild-type and 2 C3H/HeJ mice exposed to LPS for 2 wk (+) are demonstrated on the immunoblot at top. Data from 2 unexposed (−) wild-type and C3H/HeJ mice are also shown for comparison. Densitometry of the immunoreactive bands was averaged for each group and are presented as relative SP-A optical density (OD) per microgram of protein in A and relative SP-A (OD) per microliter of lavage fluid loaded into each lane in B. The graph shown in B takes into account the increase in total protein in the wild-type mice exposed to LPS as demonstrated in Fig. 9. Despite the increased total lavage fluid protein in this condition, the wild-type mice exposed to repetitive inhalation treatments of LPS for 2 wk have significantly less SP-A protein in their lung lavage fluid than the LPS exposed C3H/HeJ or the unexposed wild-type mice. The Western blot is representative of 2 experiments, where n = 4 mice per strain per experimental group.
Our data demonstrate that SP-A protein in the alveolar lumen decreases following prolonged exposure to inhaled endotoxin despite an increase in SP-A gene expression. Leakage of SP-A into the vascular system may be responsible for this finding. Leakage of pulmonary SP-A and SP-D into the blood stream has been associated with ARDS. In ARDS, proteins normally found in the alveolar lumen, such as SP-A, SP-B, and others, can be measured in the blood. High serum levels of SP-D and SP-A in patients with ARDS is associated with an increase in mortality (7). It has been proposed that the presence of these proteins in the blood is reflective of a leaky alveolar epithelium due to injury. The concentration of these proteins in the alveoli is, therefore, frequently reduced secondary to various types of acute lung injury (11). This is one possible explanation for the reduced SP-A protein levels in the lung lavage fluid observed in our studies. The degree of lung injury and therefore lung-blood barrier damage in our murine model is, however, not likely to be as severe as in the acute lung injury studies cited above, since by body weight measurements our mice remained healthy. Conversely, plasma proteins can leak into the alveolar space, resulting in a reduction in surfactant protein concentration (10, 40). In our present studies, total protein levels in whole lung lavage fluid from LPS-exposed wild-type mice were increased. This increase in total protein was not enough to account for simply a dilutional effect resulting in lower SP-A protein levels as determined by Western blot analysis. In other words, the increase in total protein concentration in lung lavage fluid could not account for the decreased amount of SP-A in the LPS-exposed wild-type animals. Therefore, SP-A protein metabolism is altered in response to repetitive exposure to endotoxin.

Another explanation of the reduced SP-A protein levels in lung lavage fluid in the face of increased SP-A mRNA levels in lung tissue is related to the repetitive exposure of the animal to endotoxin. If the SP-A aids the lung in removing this microbial component, it may be continually consumed via a lung clearance mechanism. The purified endotoxin used in our studies was smooth LPS. Specifically, it had an O-Ag, complete core oligosaccharides, and lipid A. Rough endotoxin lacks O-Ag. It has been reported that SP-A does not bind to smooth forms of LPS but does bind rough LPS (30). Theoretically, therefore, the SP-A protein should not have been reduced due to opsonization and phagocytosis of smooth LPS.

The importance of the innate immune system in protecting the lungs from daily exposure to microbial components in the environment is well understood. SP-A is an important component of the innate immune system. SP-A binds to many microbes and can enhance their phagocytosis (27). SP-A can also stimulate the chemotaxis of alveolar macrophages (43). As demonstrated in in vitro models, SP-A can inhibit cytokine production in alveolar macrophages by interfering with the LPS signaling cascade (34). Additionally, SP-A has been reported to induce a state of tolerance to endotoxin. Similar to pretreatment with LPS, SP-A can induce a state of tolerance to subsequent endotoxin exposures and result in diminished production of TNF-α (32). Conversely, in a monocytic cell culture line, SP-A itself was able to induce TNF-α expression (32). Our results support the hypothesis that tolerance to repeated doses of endotoxin can develop and that SP-A may play an important role in this process. Although the wild-type mice in our experiments had an inflammatory response as evidenced by the increased number of neutrophils and alveolar macrophages, this response may be held in check at tolerable levels by the increased production of SP-A. Experimental studies with SP-A knockout mice support this hypothesis. Mice without SP-A develop more lung inflammation following exposure to influenza A virus than intact wild-type mice (18–20).

The murine model of repeat exposure to grain dust supports the hypothesis that endotoxin, contained in the grain dust, is an important cause of lung inflammation. This is demonstrated by the TLR-4 mutant mice (C3H/HeJ), which did not demonstrate any lung inflammation or altered SP-A production in response to either purified LPS or to inhaled CDE. Ours is the first study to document the effect of subchronic inhaled endotoxin on lung SP-A gene activation and protein levels. The role and mechanism of SP-A as part of the innate immune system in this type of chronic lung injury needs to be explored further.

DISCLOSURES

This study was supported by National Institute of Environmental Health Sciences Grants ES-07498 and ES-09607, Child Health Research Center Grant HD-27748, and the Children’s Miracle Network.

REFERENCES