Toxicology Letters xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Toxicology Letters

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

Effect of ozone exposure and infection on bronchoalveolar lavage: Sex differences in response patterns

Anatoly N. Mikerov[a,b,1], David S. Phelps[a,1], Xiaozhuang Gan[a], Todd M. Umstead[a], Rizwanul Haque[a], Guirong Wang[a], Joanna Floros[a,c,*]

[a] The Center for Host Defense, Inflammation, and Lung Disease (CHILD) Research, Department of Pediatrics, The Pennsylvania State University College of Medicine, Hershey, PA, USA
[b] Department of General Hygiene and Ecology, Saratov State Medical University, Saratov, Russia
[c] Department of Obstetrics and Gynecology, The Pennsylvania State University College of Medicine, Hershey, PA, USA

HIGHLIGHTS

- Based on the number of BAL molecules with changed levels, males appear to exhibit a more exuberant in response to infection.
- Based on the number of BAL molecules with changed levels, females exhibit a more vigorous response following ozone exposure.
- Surfactant lipid levels differ between males and females in response to infection and/or ozone-induced oxidative stress.
- Oxidized dimeric SP-A levels differ between male and females in response to infection and/or ozone-induced oxidative stress.
- BAL expression levels differ between sexes under different conditions, i.e. infection and/or ozone-induced oxidative stress.

ARTICLE INFO

Article history:
Available online xxx

Keywords:
Oxidative stress
Surfactant
SP-A
Pneumonia
Innate immunity

ABSTRACT

Female mice exhibit a better survival rate than males after infection, but if infection follows an ozone-induced oxidative stress, male survival exceeds that of females. Our goal was to study bronchoalveolar lavage factors that contribute to these sex differences in outcome. We studied parameters at 4, 24, and 48 h after ozone exposure and infection, including markers of inflammation, oxidative stress, and tissue damage, and surfactant phospholipids and surfactant protein A (SP-A). A multianalyte immunoassay at the 4 h time point measured 59 different cytokines, chemokines, and other proteins. We found that: (1) Although some parameters studied revealed sex differences, no sex differences were observed in LDH, total protein, MIP-2, and SP-A. Males showed more intragroup significant differences in SP-A between filtered air- and ozone-exposed mice compared to females. (2) Oxidized dimeric SP-A was higher in FA-exposed female mice. (3) Surfactant phospholipids were typically higher in males. (4) The multianalyte data revealed differences in the exuberance of responses under different conditions – males in response to infection and females in response to oxidative stress. These more exuberant, and presumably less well-controlled responses associate with the poorer survival. We postulate that the collective effects of these sex differences in response patterns of lung immune cells may contribute to the clinical outcomes previously observed.

Abbreviations: SP-A, surfactant protein A; BAL, bronchoalveolar lavage; PMN, polymorphonuclear leukocytes; LDH, lactate dehydrogenase; MIP-2, macrophage inflammatory protein-2; FA, filtered air; CFU, colony forming units; PBS, phosphate buffered saline; DNP, 2,4-dinitriphenylhydrazine; ECL, enhanced chemiluminescence; SDS, sodium dodecyl sulphate.

* Corresponding author at: The Center for Host Defense, Inflammation, and Lung Disease (CHILD) Research, Department of Pediatrics, H085, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033, USA. Tel.: +1 717 531 6972; fax: +1 717 531 0215.
E-mail addresses: jfloros@hmc.psu.edu, jfloros@psu.edu (J. Floros).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.toxlet.2014.04.008
0378-4274/

1. Introduction

As the lung performs its role in respiration, it is vulnerable to infection and damage from inhaled pathogens, allergens, and toxic gases or particles. In most air-breathing organisms an efficient and well-regulated innate host defense system eliminates these threats and preserves the delicate structure of the lung. However, an overly exuberant response to microbial or environmental threats, such as an excessive respiratory burst producing reactive oxidant species, could potentially damage tissue and interfere with respiration.

Innate host defense in the alveoli is provided primarily by alveolar macrophages. Their function is highly regulated by the alveolar microenvironment, including other lung cells, such as type 1 and type 2 alveolar epithelial cells. The alveolar microenvironment includes pulmonary surfactant and other regulatory molecules and secretions from all of the above cell types, as well as other proteins that enter the alveolus from the circulation or the lung interstitium. The influence of surfactant on the macrophage is complex, and involves the surfactant proteins, especially surfactant protein A (SP-A), as well as the lipid constituents of surfactant. Numerous papers have demonstrated a regulatory role for SP-A on the function of macrophages and their production of chemokines and cytokines (Floros and Phelps, 2002; Phelps, 2001; Phelps et al., 2011, 2012; Crouch and Wright, 2001; Wright, 2003, 2005). Certain surfactant phospholipids also have a profound influence on the function of the macrophage, and often oppose the action of SP-A (Phelps, 2001; Huang et al., 2002; Koptides et al., 1997; Kremlev and Phelps, 1994, 1997; Kerecman et al., 2008; Tonks et al., 2005). The importance of the contribution of SP-A becomes evident with the increased susceptibility of SP-A knockout mice to a number of different infections (LeVine et al., 1997, 1998, 1999, 2002; Mikerov et al., 2008a). There have also been a number of studies demonstrating that SP-A can be oxidized and its function compromised by exposure to air pollutants such as ozone or to other materials containing reactive oxidant species (Huang et al., 2004; Janic et al., 2005; Mikerov et al., 2008b,c, 2012; Wang et al., 2002; Davis et al., 2002). More recently it has become clear that SP-A also plays a role in regulating the expression of a number of macrophage gene products, and these in turn may regulate reactive oxidant species in the alveolar space or participate in maintaining protease/antiprotease balance in the lung (Phelps et al., 2011, 2012).

In a series of publications we have described sex differences (Mikerov et al., 2008a,b, 2011, 2012; Durrani et al., 2011) in outcome after infecting mice with *Klebsiella pneumoniae*, with or without a prior oxidative stress in the form of an acute ozone exposure. The above studies include differences in survival and phagocytosis of pathogens by alveolar macrophages (Mikerov et al., 2008a,b,c) as well as differences in histopathology and dissemination of the resulting infection (Mikerov et al., 2011, 2012). Moreover, we have shown that the sex differences persist and are accentuated in mice lacking SP-A or after the oxidative modification of SP-A (Mikerov et al., 2008a,b). The sex differences in survival have been shown to be dependent upon gonadal hormones (Durrani et al., 2011). A further insight into the molecular basis of the sex differences has been gained via the study of the proteome of alveolar macrophages from mice lacking SP-A and after a "rescue" of these mice with exogenous SP-A (Phelps et al., 2011, 2012).

In the present study we continue to study the basis for the sex differences by studying a number of parameters in the BAL after filtered air (FA) or ozone exposure during the initial phases of infection. Endpoints studied included total and differential BAL cell counts, lactate dehydrogenase (LDH), total protein, total oxidized protein, phospholipid, SP-A, oxidized SP-A, and MIP-2. In an effort to gain a more global insight into the BAL factors that may contribute to the observed differences we performed immunoassays on 59 different analytes in BAL, including a variety of inflammatory mediators, proteins involved in the acute phase response and coagulation, and proteins involved in cell growth and differentiation.

2. Materials and methods

2.1. Animals

Male and female C57BL/6 mice (from Jackson Laboratory (Bar Harbor, ME)) were used at 8–12 weeks of age. The Penn State University Institutional Animal Care and Use Committee approved all procedures involving animals. Animals were exposed to FA or ozone, infected with *K. pneumoniae*, and sacrificed at the indicated time for further study as described.

2.2. Exposure of mice to ozone

Mice were exposed to ozone (2 ppm for 3 h) or to FA (control) at the same time in separate chambers. Each chamber consisted of a 3.7 l closed glass vessel into which glass containers with wire mesh tops were placed. The temperature was maintained at 25 °C, humidity was set to 50%, and the flow rate was 15 l/min through each (FA and ozone) chamber. Air flow and ozone content were continually monitored. All FA and ozone exposures were conducted in parallel. For various experiments 3–6 mice were treated with either ozone or FA.

The ozone dose/duration (2 ppm for 3 h) has been used by other laboratories (Hollingsworth et al., 2007; Kierstein et al., 2008; Hulo et al., 2011) and was chosen in our preliminary work as being optimal (Haque et al., 2007) for further investigations. The rational for this dose is based on a study by Hatch et al. (1994) in which they reported that experimental animals required higher ozone concentrations than humans to deliver comparable amounts of ozone to the distal lung. This determination was based on numbers of neutrophils and macrophages, as well as the protein content of BAL.

2.3. Preparation of bacteria

K. pneumoniae bacteria (ATCC 43816) were purchased from the American Tissue Culture Collection (Rockville, MD), then grown and prepared as described previously (Mikerov et al., 2008b). Bacteria were grown for 18 h in tryptic soy broth (TSB) media at 37 °C until they reached stationary phase. The suspension of bacteria was diluted until the OD_{660} was equal to 0.4. We used a 200 μl aliquot of this dilution to inoculate 50 ml of fresh TSB for sub-cultivation for 3 h, resulting in a culture that was in the mid-log phase of growth. We then placed the sub-culture on ice to stop growth. Using cold PBS, the culture was serially diluted to obtain ~9×10^3 CFU/ml, and mice were infected by injecting 50 μl of this bacterial suspension (containing ~450 CFU) intratracheally. CFU per ml values were calculated from the OD_{660} of the bacterial suspension, and an aliquot was also spread on tryptic soy agar (TSA) plates to confirm CFU estimates.

2.4. Infection of mice with K. pneumoniae

Infection was performed as described previously (Mikerov et al., 2008b). Briefly, the animals were anesthetized, the trachea was surgically exposed, and ~450 CFU/mouse were inoculated intratracheally in 50 μl of PBS. If any mice died within the first 12 h post-infection, we considered the death to be due to the surgical procedure rather than resulting from the infection and we excluded those mice from the study. In cases where mice were moribund with no chance of recovery, the mice were euthanized to prevent unnecessary suffering according to Penn State University Institutional Animal Care and Use Committee recommendations and were included with the natural deaths. After exposure to FA or ozone and subsequent infection (or instillation with vehicle), mice were subjected to bronchoalveolar lavage and various parameters were analyzed, as described below.

2.5. BAL analyses

The lungs of the mice were subjected to bronchoalveolar lavage (BAL) (3 times with 0.5 ml of 0.9% NaCl) at the 4, 24, and 48 h post-infection time points, as described (Haque et al., 2007). Three independent experiments were performed for each time point; each experiment involved 5 mice exposed to ozone and 5 mice exposed to FA, or a total of 83 male mice [42 FA-exposed and 41 ozone-exposed], and 74 female mice [39 FA-exposed and 35 ozone-exposed]. The BAL fluids were centrifuged (150 × *g*, 5 min, 4 °C) and the cell pellets resuspended in 0.9% NaCl. Cell-free supernatants were frozen at −80 °C until subsequent analyses were performed as described below.

2.5.1. Cell and biochemical analyses of BAL fluid

Total cell counts were performed immediately after BAL using a hemocytometer. For the differential cell counts, slides were prepared using a cytocentrifuge and stained with a Hema-3 Stain Kit (Fisher Scientific, Pittsburgh, PA), and analyzed by light microscopy (Mikerov et al., 2008b). Total protein concentration was determined using the Micro BCA Protein Assay (Pierce Biotechnology, Rockford, IL). For determination of total phospholipids, 100 μl of BAL supernatant were lyophilized

Please cite this article in press as: Mikerov, A.N., et al., Effect of ozone exposure and infection on bronchoalveolar lavage: Sex differences in response patterns. Toxicol. Lett. (2014), http://dx.doi.org/10.1016/j.toxlet.2014.04.008