Online Data Supplement

Segmental Allergen Challenge Alters Multimeric Structure, and Function of Surfactant Protein D in Humans

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Methods

Reagents

N-ethylmaleimide (NEM); DTPA (diethylenetriaminepentaacetic acid), maleimide-PEO_2-biotin, a sulfhydryl-reactive biotin labeling reagent and donor of NO, and *S*-nitrosocysteine (SNOC) were all obtained from Pierce Biotechnology, Inc. (Rockford, IL). LPS was prepared using lyophilized Clinical Center Reference Endotoxin (CCRE; National Institute of Health Clinical Center, Bethesda, MD; *Escherichia coli* strain O:113).

A monospecific, polyclonal antibody against SP-D (Ab 1754), produced in rabbits using two synthetic peptides corresponding to two homologous regions of the mouse / human SP-D sequences as the immunizing antigen, has been described previously by Atochina et al[E1]. The resulting antiserum recognizes rat, murine, and human SP-D and does not cross-react with SP-A. This antibody recognizes denatured isoforms of mouse SP-D as well as both denatured and native forms of human SP-D.

Study Subjects and Bronchoscopy

The allergen concentration (*Dermatophagiodes pteronyssinus,* (ALK-Scherax, Wedel, Germany)) that elicited at least a 3-mm-diameter skin weal response was selected for instillation

(100–100.000 SQE diluted in 10 ml saline). LPS challenge was performed with 2 ng/kg endotoxin as previously described[E2]. LPS was prepared using lyophilized Clinical Center Reference Endotoxin (CCRE; *Escherichia coli* strain O:113) in a final volume of 10 ml sterile saline. The doses of allergen and LPS instilled in the combination segment were identical to those in the single challenge segments, also dissolved in a final volume of 10 ml sterile saline.

After premedication (inhalation of 200 mg salbutamol, atropine 0.5 mg subcutaneously, and midazolam 0.05–0.1 mg/kg intravenously) and local anesthesia with topical lidocaine, bronchoscopy was conducted according to a standard protocol as previously described[E3]. During the first bronchoscopy, the bronchoscope (BF 160 P; Olympus Optical, Tokyo, Japan) was placed into one segment of the left lower lobe in wedge position for a baseline BAL with 6 x 20 ml saline solution (37^{o}C). The first aliquot was aspirated separately and discarded. Next, 10 ml saline solution was instilled into a segment of the left upper lobe bronchus as a control challenge. Allergen solution was instilled in a segment of the lingular bronchus. The bronchoscope was then passed into the right upper lobe and LPS solution was segmentally instilled. Finally, a combination of allergen and LPS was instilled in a segment of the right middle lobe. A new microcatheter (Vygon, Aachen, Germany) was used for each instillation. After 24 hours, a second bronchoscopy was performed under identical premedication. During this bronchoscopy, BAL was collected, as described for baseline, in each challenged segment.

Processing of BAL: Cells Staining, Analysis for Total Protein and Cytokine

BAL fluid samples were processed as previously described by Schaumann et al[E4]. Briefly, BAL fluid was filtered through a 100-μm filter, centrifuged and cell-free supernatant of BAL was stored at -80^{o}C for future analysis. The total nucleated cell count was determined using a Neubauer hemocytometer. Differential cell counts were done using Diff-Quick staining

(Dade Behring Inc., Marburg, Germany) of cytospin slides and by counting 400 cells per slide. Cells were identified as macrophages, eosinophils, neutrophils, or lymphocytes by standard morphology. Total protein content from cell-free supernatant of BAL fluids was determined by the method of Bradford. Cytokine and chemokine levels were determined simultaneously with a multiplex assay kit (Lincoplex; Linco Research, Inc., St. Charles, MO) with premixed antibody-coated microsphere beads using a Luminex100 (Luminex Corporation, Austin, TX) according to the manufacturer's recommendations.

Polyacrylamide Gel Electrophoresis and Immunoblotting for SP-D or Nitrotyrosine

BAL proteins were separated and analyzed by two methods:

Denaturing SDS-PAGE was performed under reducing conditions with β-mercaptoethanol using NuPAGE 10% or 12% bis-tris gels for SP-D or nitrotyrosine (all from Invitrogen Inc., Carlsbad, CA). Separated proteins (equal BAL volume per lane) were transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes using tris-glycine transfer buffer at 30 volts overnight at room temperature.

Native gel electrophoresis for detection of SP-D quaternary structure was performed using NuPAGE 3-8% Tris-Acetate gels (Invitrogen, Inc., Carlsbad, CA) as previously described[E5; E6]. Calculated equal amounts of SP-D, as determined by densitometry on SDS/PAGE blots, were mixed with a cold native tris-glycine sample buffer before loading. Electrophoresis was run at room temperature at a constant voltage of 150 V for 2 hours. Proteins were then transferred to PVDF membranes.

Immunoblotting was performed as described using incubations with primary polyclonal SP-D antibody (Ab # 1754, 1:20,000 dilutions) for reducing gels or mixture of anti-SP-D antibodies (Ab # 1754; anti-SP-D antibody from Chemicon Inc.) for native gels. Bands were

visualized using enhanced chemiluminescence (ECL+, Amersham Inc., Pittsburgh, PA) and quantitated by densitometric scanning of exposed films or direct acquisition on Kodak 440 Imaging System (New Haven, CT).

For detection of nitrated SP-D, BAL from asthmatic patients was immuno-precipitated with rabbit anti-SP-D serum overnight at 4°C. Immunoprecipitates were captured with a mixture of binding buffer (1:4 ratio) and Protein A beads (1:1 ratio) (Bio-Rad, Hercules, CA) at room temperature for 1 h. The beads were washed 4 times with the same buffer prior to solubilization with sample buffer in the presence of reducing agent. Samples were resolved by SDS-PAGE, transferred to PVDF, and incubated with a mouse monoclonal antibody to nitrotyrosine and HRP-conjugated goat anti-mouse IgG **(**Santa Cruz Biotechnology, CA). Immunoreactive species were visualized by ECL+ as described above.

Nitrogen Oxides Measurements

The analysis of NO metabolites was determined by treatment of the sample with an excess of vanadium chloride in HCl at 95°C, reducing nitrate, nitrite, and SNO to release NO and analyzed by using the Ionics/Sievers Nitric Oxide Analyzer 280 (NOA 280; Ionics Instruments, Boulder, CO) [E7; E8]. Sodium nitrate (Sigma Chemical Co., St. Louis, MO) was used as the standard. The data were expressed as a percentage of baseline levels.

Biotin-Switch Assay for Detection of SNO-SP-D

Detection of SNO-SP-D was performed via an adaptation of the biotin switch method[E5; E9]. Briefly, BAL (60 μl) in HEN buffer (25 mM Hepes, pH 7.4, 0.1 mM EDTA and 10 μM neocuproine) and 500 μM *N*-ethylmaleimide (NEM buffer) were incubated at 37°C for 30 min to block free thiols. Excess NEM was removed by protein precipitation using cold acetone. Protein pellets were resuspended in DTPA buffer (diethylenetriaminepentaacetic acid) and *S*-nitrosothiol

(SNO) bonds decomposed by adding 20 mM ascorbate. The newly formed thiols were linked with the sulfhydryl-reactive biotin labeling reagent with a hydrophilic polyethylene oxide (maleimide-PEO_2-biotin). Biotinylated proteins were precipitated with streptavidin-agarose beads and Western blot analysis was performed to detect the amount of captured SP-D using polyclonal SP-D antiserum (Ab # 1754) as described above.

_S_-Nitrosylation of Proteins _in vitro_

In order to generate *S*-nitrosylated SP-D, recombinant rat SP-D (10 μg/ml) was incubated with SNOC (200 μM) at 37°C for 30 minutes in the dark as previously described[E5]. *S*-nitrosylated SP-D was then purified on Micro Bio-Spin Chromatography columns (Bio-Rad, Hercules, CA) and reduced multimeric SP-D structure was evaluated by native gel electrophoresis followed by immunoblotting with polyclonal SP-D antiserum (Chemicon Inc., Temecula, CA) as described above.

Chemotaxis Assay with Human Eosinophils from Atopic Donors

Eosinophils were isolated from peripheral blood of allergic patients and chemotaxis was measured with a modified Boyden chamber assay with human eosinophils as described[E10; E11]. Briefly, Boyden chambers (Corning Costar, Bodenheim, Germany) containing 5 μm polycarbonate filters inserts (Millipore) were filled with 100μl of chemotaxis medium alone (negative control) (PBS supplemented with 0.1 % BSA, 0.1 mM $CaCl_2$ and 0.1 mg $MgCl_2$) or 0.1 μg/ml eotaxin (positive controls) (Peprotech Inc., London UK). Suspensions of human eosinophils ($1x10^6$ in 100 μl) were allowed to migrate towards eotaxin-containing medium in the presence or absence of either native rrSP-D (0.1 μg/ml) or *S*-nitrosylated rrSP-D (0.1 μg/ml). All assays were performed in triplicate. After incubation for 90 minutes at 37°C, 5 % CO_2, migrated eosinophils in the lower part of the Boyden chamber were counted in an improved

Neubauer hemocytometer**.** Basal migration was measured as the number of cells migrating towards medium only ($0.51x10^5 \pm 0.19x10^5$).

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