Nitrated SP-A does not enhance adherence of Pneumocystis carinii to alveolar macrophages

SHA ZHU,1 DIANE L. KACHEL,2 WILLIAM J. MARTIN II,2 AND SADIS MATALON1,3,4

Departments of 1Anesthesiology, 2Physiology and Biophysics, and 3Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama 35233; and 2Division of Pulmonary, Critical Care, and Occupational Medicine, Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana 46202

Zhu, Sha, Diane L. Kachel, William J. Martin II, and Sadis Matalon. Nitrated SP-A does not enhance adherence of Pneumocystis carinii to alveolar macrophages. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L1031–L1039, 1998.—We investigated whether nitration of surfactant apoprotein (SP) A alters its ability to bind to mannose-containing saccharides on Pneumocystis carinii and its potential role in the mediation of P. carinii adherence to alveolar macrophages. Human SP-A was nitrated by incubation with tetranitromethane at pH 8.0 or synthetic peroxynitrite (ONOO-·) at pH 7.4, which resulted in significant nitration of tyrosines in its carbohydrate recognition domain [0.63 ± 0.01 (SE) and 1.25 ± 0.02 mol nitrotyrosine/mol monomeric SP-A, respectively; n = 3 samples]. Binding of SP-A to P. carinii was calcium dependent and competitively inhibited by α-methyl-D-mannopyranoside. Nitration of SP-A by ONOO-· or tetranitromethane decreases its binding to P. carinii by increasing its dissociation constant from 7.8 × 10−9 to 1.6 × 10−8 or 2.4 × 10−9 M, respectively, without significantly affecting the number of binding sites (7.1 × 109/P. carinii organisms, assuming that the native molecular mass of oligomeric SP-A is 650 kDa). Furthermore, ONOO-·-nitrated SP-A failed to mediate the adherence and phagocytosis of P. carinii to rat alveolar macrophages as observed with normal SP-A. Binding of SP-A to rat alveolar macrophages was not altered by nitration. These results indicate that nitration of SP-A interferes with its ability to serve as a ligand for P. carinii adherence to alveolar macrophages at the site of the SP-A molecule-P. carinii interaction.

surfactant protein A; collectin; tyrosine nitration; parasite adherence; nitric oxide; lung host defense

PNEUMOCYSTIS CARINII PNEUMONIA remains a common and life-threatening pulmonary infection in immunocompromised patients, especially those with acquired immunodeficiency syndrome. The interaction between P. carinii and alveolar macrophages within the alveolar lining fluid of the lung represents the initial contact of the pathogen with the host immune system.

Alveolar macrophages express many different types of surface receptors that aid in their ability to bind to microorganisms. Among these are receptors for mannose (50), fibronectin (4), the Fc portion of IgG (38), the complement component C3b (66), and surfactant protein (SP) A (5). Alveolar macrophages have been shown to play a significant role in host defense by binding, phagocytosing, and degrading P. carinii (30, 58, 63).

The predominant surface membrane protein on P. carinii is a glycoprotein with an estimated molecular mass of 110–120 kDa known as major surface glycoprotein (MSG) (46). A recent study (42) suggested that MSG may serve to mediate adherence of P. carinii to the alveolar epithelium. P. carinii MSG is heavily glycosylated with mannose-containing oligosaccharide chains (46) that could function as a ligand for SP-A (69).

SP-A, the most abundant apoprotein of the pulmonary surfactants, is a member of the C-type lectin superfamilly (7, 63) and along with SP-D (40), serum mannose-binding protein (MBP) A, MBP-C, conglutinin, and the recently described collectin-43 (22) forms the collectin (group III) subgroup (7, 31). The human SP-A molecule is organized into four discrete structural domains: a short amino-terminal globular domain containing a single cysteine involved in interchain disulfide bond formation, a collagen-like domain, a hydrophobic neck region, and a carboxy-terminal carbohydrate recognition domain (CRD). Thus SP-A is a lectin protein with a collagen-like domain that shares extensive structural homology with MBPs (8), conglutinin (6), and complement component C1q (56). It has multiple functions including tubular myelin formation, binding to high-affinity receptors on alveolar type II cells, regulating the recycling of surfactant lipids, and acting synergistically with other surfactant apoproteins to lower surface tension (15, 20, 28, 64). Recent studies have shown that SP-A is implicated in lung host defense by interacting with a variety of pathogens (34, 35, 54, 63, 69) and stimulating chemotaxis, phagocytosis, and production of reactive oxygen species by alveolar macrophages (32, 34, 52, 53, 59, 63, 65).

Exposure of alveolar macrophages and airway and alveolar cells to diverse stimuli of inflammation such as cytokines (interleukin-1, tumor necrosis factor-α, and interferon-γ) and lipopolysaccharide (LPS) results in a marked upregulation of nitric oxide (NO) and superoxide (O2-) production (1, 14, 45, 48, 57, 63). The product of the reaction of NO with O2- (39), is peroxynitrite (ONOO-·), a potent oxidizing and nitrating agent that damages a wide spectrum of biological molecules such as DNA (23), lipids (47), and proteins (12, 36). Recent studies (16, 17, 37, 67) have indicated that exposure of SP-A to nitrating reagents such as ONOO-·, tetranitromethane (TNM), or nitrogen dioxide (NO2) results in a marked decrease in its ability to aggregate lipids and bind mannose. Thus we hypoth-
esized that nitration of SP-A would impair its ability to bind to \( P. \text{ carinii} \), a step necessary for the clearance of these organisms by alveolar macrophages.

In the present study we have 1) characterized the binding of normal and nitrated SP-A to both \( P. \text{ carinii} \) and alveolar macrophages, 2) determined the effect of SP-A on the interaction of \( P. \text{ carinii} \) with alveolar macrophages, and 3) assessed which structural domain of SP-A is involved in these processes. We found that human SP-A can bind to \( P. \text{ carinii} \) through its CRD and that nitration of tyrosine residues in the CRD decreases its affinity for mannose-rich structures on the surface of \( P. \text{ carinii} \). Furthermore, normal SP-A enhances the adherence and phagocytosis of \( P. \text{ carinii} \) to alveolar macrophages, whereas nitrated SP-A fails to mediate this process. Because the binding of nitrated SP-A to alveolar macrophages is not significantly affected, it is likely that nitration of SP-A interferes with its ability to serve as a ligand for \( P. \text{ carinii} \) adherence to alveolar macrophages at the site of the SP-A molecule-\( P. \text{ carinii} \) interaction.

**METHODS**

**Materials.** BSA, HEPES, EDTA, EGTA, DMEM, bisbenzimide (Hoechst 33258), \( \alpha \)-methyl-\( \beta \)-mannopyranoside, ophenylenediamine, and LPS (serotype O55:B5 from Escherichia coli) were from Sigma (St. Louis, MO). Sodium nitrite and hydrogen peroxide were obtained from Fisher Scientific (Fair Lawn, NJ). TNM and 3-nitro-L-tyrosine were from Sigma (St. Louis, MO). ONOO\(^-\) was synthesized from sodium nitrite and hydrogen peroxide with a quenched-flow reactor as previously described (3) and treated with manganese dioxide to remove contaminated hydrogen peroxide. The ONOO\(^-\) concentration was determined spectrophotometrically at 302 nm (molar extinction coefficient = 1,670 M/cm) before each experiment.

**Exposure of SP-A to nitrating agents.** SP-A (1 mg/ml) in 10 mM HEPES buffer was incubated with TNM or ONOO\(^-\) at 37°C in the following fashion: 1) 0.5 mM TNM at pH 8.0 for 30 min or 0.5% ethanol as a vehicle control or 2) 0.5 mM active or inactive ONOO\(^-\) at pH 7.4 for 15 min. ONOO\(^-\) was inactivated by diluting the stock solution 20-fold in 10 mM HEPES buffer, pH 7.4, and heating the solution to 37°C for 20 min. Absorbance measurements verified that >99% of ONOO\(^-\) was inactivated. A second dose of active or inactive ONOO\(^-\) (0.5 mM) was added into the SP-A-containing solutions at the end of the 15-min period, and the solutions remained at 37°C for an additional 15 min. SP-A protein was either immediately after exposure or stored at 20°C for later use.

**Binding of SP-A to \( P. \text{ carinii} \).** For binding experiments, 5 \( \times \) 10\(^4\) freshly isolated \( P. \text{ carinii} \) organisms and 100–1,000 ng of SP-A were mixed in 100 µl of either binding (HBSS without calcium or magnesium containing 1% BSA and 5 mM CaCl\(_2\), pH 7.4) or EGTA buffer (HBSS without calcium or magnesium, containing 1% BSA and 5 mM EGTA, pH 7.4). The sedimentation of SP-A due to its self-association was also measured in the presence of 5 mM CaCl\(_2\) but in the absence of \( P. \text{ carinii} \). Total binding (calcium-dependent and -independent binding) was always corrected for by the sedimentation of SP-A.

Because our preliminary experiments showed that human SP-A binds to \( P. \text{ carinii} \) in a time-dependent fashion and that binding plateaued in 30 min, the reaction mixtures were incubated for 30 min in an atmosphere of 95% air-5% CO\(_2\) at 37°C. After incubation, the reaction mixtures were centrifuged at 13,000 g for 5 min. The pellets were washed three more times with either binding or EGTA buffer. The pellets were then resuspended in water and sonicated briefly to homogenize \( P. \text{ carinii} \). SP-A was determined by a capture enzyme-linked immunosorbent assay (ELISA) as described in Quantification of SP-A and SP-A nitration. Specific binding was defined as the calcium-dependent binding and calculated by subtracting the calcium-independent binding (in the presence of 5 mM EGTA) from the total binding (which was already corrected for nonspecific SP-A sedimentation). Calcium-independent binding plus nonspecific sedimentation of SP-A was found to be ~25% of the binding in the presence of 5 mM CaCl\(_2\). Binding assays also were performed in the presence of 0–750 mM \( \alpha \)-methyl-\( \beta \)-mannopyranoside in 100 µl of binding buffer containing 1 \( \times \) 10\(^6\) \( P. \text{ carinii} \) organisms and 100 ng of SP-A.

**Quantification of SP-A and SP-A nitration.** The amount of SP-A was determined by ELISA with a monoclonal rabbit anti-human SP-A as the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody. In brief, samples were serially diluted and coated to ELISA plates and allowed to bind for at least 18 h at 4°C. Nonspecific binding sites were blocked with 1% BSA for 1 h at room temperature. The wells were then incubated with the primary antibody (1:5,000 dilution) at 37°C for 1 h, followed by the secondary antibody (1:2,500 dilution). Hydrogen peroxide and o-phenylenediamine were used as substrates for the peroxidase reaction, and the absorbance was measured at 490 nm. Purified SP-A was used as the standard. Additional
experiments also showed that the polyclonal rabbit anti-human SP-A antibody we used for SP-A quantification had similar affinities for both normal and nitrated SP-A (data not shown).

Nitrotyrosine content of TNM- or ONOO-·treated SP-A was measured by ELISA with the nitrotyrosine antibody instead of the SP-A antibody as the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody as previously described (16, 67).

Isolation of alveolar macrophages. Lungs from Lewis rats (Harlan Sprague Dawley) were lavaged, and the alveolar cells were concentrated by centrifugation. The cells were washed three more times with HBSS containing 5 mM EGTA to remove endogenously bound rat SP-A. Examination of cyto-preparation smears stained with Diff-Quik demonstrated that >95% of the cells obtained were macrophages. These cells were then plated at a density of 1 × 10⁶ cells/well in DMEM supplemented with 25 mM NaHCO₃, 15 mM HEPES, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 4 µg/ml of gentamicin, and 0.5 µg/ml of amphotericin B, pH 7.4, on rat IgG-coated 24-well tissue culture plates and allowed to adhere for 1 h at 37°C. After adherence, each well was washed twice with the same medium to remove unattached cells, then used immediately for SP-A-binding experiments or cultured overnight in a P. carinii adherence and phagocytosis assay.

SP-A binding to alveolar macrophages. The culture plates were placed on ice and washed three times with cold binding medium (same DMEM medium containing 0.1% BSA). The cells were then incubated with varying concentrations (0–15 µg/ml) of SP-A in binding medium at 4°C for 1 h. At the end of the incubation, the cells were washed four times with HBSS containing 1.8 mM of calcium and reconstituted in water by brief sonication. SP-A bound to alveolar macrophages was quantified by ELISA as described in Quantification of SP-A and SP-A nitration. The total binding of SP-A to macrophages was normalized by the cell DNA content (see method in DNA measurement). Nonspecific binding of SP-A to the plate wells in the absence of cells was <10% of the total binding.

P. carinii adherence and phagocytosis to alveolar macrophages. P. carinii adherence to alveolar macrophages was assayed with ⁵¹Cr-labeled P. carinii. Freshly isolated P. carinii were incubated with ⁵¹Cr-labeled sodium chromate (50 µCi/ml) in 1 ml of DMEM with fetal bovine serum overnight. The radiolabeled P. carinii were then extensively washed with DMEM containing 5 mM EGTA to remove endogenous rat SP-A and other surface proteins along with any nonincorporated ⁵¹Cr as previously described (42, 60). Normal or ONOO-·nitrated SP-A was added to each designated well followed by the immediate addition of ⁵¹Cr-labeled P. carinii (5 × 10⁶ organisms/well). The adherence and phagocytosis assay was conducted in DMEM containing 0.1% BSA but no fetal bovine serum. Because the adherence of P. carinii to rat alveolar macrophages plateaued in 4 h (60), and the phagocytosis reached plateau in 2 h, the experiments were briefly centrifuged at 800 g for 5 min and incubated at 4°C for 4 h to determine adherence or at 37°C for 2 h to determine adherence/phagocytosis. After the incubation period, adhered macrophages were washed with normal saline three times. The supernatant and subsequent washes were pooled and labeled as fraction A. The cells were lysed with 10% Triton X-100 and labeled as fraction B. Radioactivity was quantified in a gamma counter (Bedmack 5500, Beckman Instruments, Schaumburg, IL). The percentage of adherence or adherence and phagocytosis was calculated as follows: [fraction B/(fraction A + fraction B)] × 100. Each experiment was performed in duplicate and repeated on at least three separate occasions.

RESULTS

EGTA removal of endogenously bound rat SP-A from P. carinii and alveolar macrophages. SDS-PAGE and Western blotting studies shown in Fig. 1 indicated that washing P. carinii and alveolar macrophages with HBSS containing EGTA removed all surface-associated SP-A. This is in agreement with previous results (60).
SP-A nitrated. Unexposed SP-A or SP-A treated with inactive ONOO− contained background levels of nitrotyrosine (<0.001 mol nitrotyrosine/mol monomeric SP-A). In contrast, SP-A exposed to two boluses of 0.5 mM ONOO− or a single dose of 0.5 mM TNM contained significant levels of nitrotyrosine as measured by ELISA (0.63 ± 0.001 and 1.25 ± 0.02 mol nitrotyrosine/mol monomeric SP-A, respectively; n = 3 samples; Fig. 2A).

SP-A binding to P. carinii. Figure 2A also shows the calcium dependence of SP-A binding to P. carinii. The total binding in the presence of 5 mM CaCl2 was corrected for sedimentation of SP-A due to its self-association. Binding of control SP-A to P. carinii was decreased by 84% in the presence of 5 mM of EGTA (P < 0.01), indicating a calcium-dependent process and the surface nature of the binding (69). Nitration of SP-A by ONOO− or TNM significantly decreased its total binding in the presence of 5 mM CaCl2 but not its calcium-independent binding in the presence of 5 mM EGTA. Furthermore, the extent of decrease in the SP-A-specific (i.e., calcium-dependent) binding to P. carinii was correlated with SP-A nitrotyrosine levels (Fig. 2B).

To clarify a potential mechanism for the decreased binding of nitrated SP-A to P. carinii, Scatchard analysis was performed. As shown in Fig. 3A, both normal and nitrated SP-A bound to P. carinii in a specific and saturable fashion. Scatchard plots of specific binding data (i.e., calcium-dependent binding) are linear, suggesting a homogeneous population of binding sites for both normal and nitrated SP-A on P. carinii (Fig. 3B). Normal SP-A bound P. carinii with a binding dissociation constant (Kd) of 7.8 × 10−9 M. The estimated number of SP-A binding sites was 7.1 × 106/P. carinii organisms, assuming that the native molecular mass of oligomeric SP-A is 650 kDa (25). Nitration of SP-A by ONOO− or TNM decreased its binding to P. carinii by increasing the Kd value to 1.6 × 10−8 and 2.4 × 10−8 M, respectively, without significantly affecting the number of binding sites. The magnitude of the Kd increase correlated with nitrotyrosine levels in SP-A. These results indicate that modification of tyrosine residues by nitrating agents in the CRD of SP-A decreases its affinity for SP-A binding sites on P. carinii.

Because P. carinii express a great abundance of surface mannosetype oligosaccharides (42, 43) and the specific affinity of SP-A for mannose has been well documented (15), α-methyl-D-mannopyranoside was used to determine whether SP-A binds to P. carinii surface carbohydrate through its CRD. SP-A binding was inhibited by 72% in the presence of 750 mM α-methyl-D-mannopyranoside (Fig. 4), which suggests that SP-A binds to P. carinii surface carbohydrate through its CRD.

Role of normal and ONOO−-nitrated SP-A in P. carinii adherence and phagocytosis to alveolar macrophages. Consistent with the findings of Williams et al. (60), the SP-A used in these adherence and phagocytosis studies was shown to enhance P. carinii adherence to alveolar macrophages in a concentration-dependent manner (Fig. 5A). However, for the first time, the results in Fig. 5A indicate that SP-A nitrated by ONOO− significantly loses its ability to promote adherence of P. carinii to alveolar macrophages at 4°C (P < 0.01). Similarly, this difference was also demonstrated at 37°C where normal SP-A (10 µg/ml) enhanced adherence and phagocytosis of P. carinii by alveolar macrophages from 21.5 ± 2.0 to 36.7 ± 2.4% (P < 0.05). Adherence and phagocytosis were not significantly altered (18.3 ±
by the presence of the same concentration of ONOO\(^{-}\)-nitrated SP-A (Fig. 5B). SP-A binding to alveolar macrophages. Incubation of SP-A with macrophages revealed a biphasic binding curve with an inflection in the total binding curve at 2.5 µg/ml. Nitration of SP-A by two doses of 0.5 mM ONOO\(^{-}\) did not significantly affect the binding of SP-A to macrophages, which also suggests that the binding between SP-A and macrophages is through the collagen-like region rather than through the CRD (Fig. 6).

DISCUSSION

The data reported herein indicate that nitration of human SP-A decreases its binding affinity (i.e., increases the \(K_d\)) for \(P. carinii\) in a concentration-dependent fashion. Human SP-A contains eight tyrosine residues per monomer, which are located in its CRD (11). Nitration of one or more of these tyrosines decreases the acidic dissociation constant of tyrosine from 10 to 7.5 (49), rendering nitrotyrosine more hydrophilic, thus potentially inducing conformational change in the tertiary structure of the globular CRD region of SP-A secondary to alterations in its ionic charge. Previous studies by our laboratory (13, 19, 67) have shown that nitration of SP-A decreases its ability to aggregate lipids in the presence of calcium and to bind to mannos. Amino acid analysis of ONOO\(^{-}\)- or TNM-treated SP-A failed to identify any oxidized amino acids to account for these changes (13, 19). These results, along with the finding that the adherence of SP-A to \(P. carinii\) was competitively inhibited by \(\alpha\)-methyl-D-mannopyranoside, suggest that an intact CRD is necessary for the adherence of SP-A to \(P. carinii\), in agreement with previous findings (69) showing that rat SP-A binds to \(P. carinii\) MSG through its CRD.
Levels of nitrotyrosine in control SP-A samples (0.001 mol nitrotyrosine/mol monomeric SP-A, which corresponds to 30 pmol nitrotyrosine/mg SP-A, assuming that the molecular mass of monomeric SP-A is 30 kDa) are similar to what has been measured by ELISA in normal rat lung tissue (–30 pmol nitrotyrosine/mg protein [51]), normal human serum albumin (–30 pmol nitrotyrosine/mg human serum albumin [24]), and normal human plasma low-density lipoprotein (–85 pmol nitrotyrosine/mg protein [24]). Significantly higher nitrotyrosine levels have been measured in the lungs of pediatric patients who died with acute respiratory distress syndrome (18) and in the lungs of rats exposed to endotoxin (62) or hyperoxia.

SP-A may bind to alveolar macrophages by a number of different mechanisms. Because SP-A is a lectin as well as a glycoprotein with N-glycosidic glycans, it can bind to surface α-D-mannosyl residues, C1q receptors, or SP-A receptors (5, 28, 41, 63, 64). Pison et al. (41) reported that the binding of SP-A to macrophages is blocked by collagen-like protein C1q and type V collagen in a dose-dependent fashion, also suggesting a collagen-like domain-mediated mechanism. However, another study (61) suggested that SP-A binds to alveolar macrophages through a mannose-dependent process that may involve the CRD of SP-A. Our results indicate that nitration of SP-A decreases its affinity for P. carinii but does not alter its binding to alveolar macrophages. Because nitration of SP-A decreases its ability to bind to mannose (19, 67), our findings suggest that, under our experimental conditions, binding of SP-A to macrophages is primarily mediated by its collagen-like domain, although the involvement of its CRD is not excluded (61).

Previous reports (10, 27, 53, 55, 63) suggested that SP-A and SP-D enhance the phagocytosis of bacteria and viruses. Indeed, in a recent report, Hickman-Davis et al. (21) demonstrated that the killing of Mycoplasma pulmonis by alveolar macrophages required the presence of SP-A. Williams et al. (60) reported that human SP-A enhanced adherence of P. carinii to alveolar macrophages. Our results clearly demonstrated that nitration of SP-A results in decreased binding to P. carinii and an abrogation of its ability to mediate P. carinii adherence to macrophages, whereas its bind-
ing to alveolar macrophages was not significantly affected. Because our present data and those of others (69) indicate that the CRD of the SP-A molecule interacts with the MSG on the P. carinii surface, whereas the collagen-like domain of the molecule interacts with alveolar macrophages, it is likely that nitration of SP-A interferes with its ability to serve as a ligand for P. carinii adherence to alveolar macrophages. The conclusion is consistent with the speculation by Koziel et al. (26). However, they also found that human SP-A reduced rat P. carinii adherence to human alveolar macrophages by ~20%. Possible explanations for the discrepancy between the results of Koziel et al. (26) and ours include the use of rat versus human macrophages and the extent of control SP-A nitrination.

This is the first report to show that the modification of a single amino acid in SP-A under pathological conditions modulates an important in vivo function, and thus our results are of much important biological significance. During lung inflammation, there is a marked upregulation of both N0 and O2·− production by alveolar macrophages (1, 14, 45, 47, 63) in close proximity to SP-A. The reaction product of N0 and O2·− is ONOO−, a potent oxidation and nitrating agent. A recent report (9) indicated that myeloperoxidase from infiltrated neutrophils in the lung also can catalyze chlorination and nitration reactions with nitrite (NO2·−), the end product of N0, and hydrogen peroxide, the dismutated product of O2·−, as substrates, suggesting a novel mechanism for protein nitrination. A study by Zhu et al. (68) also showed that peroxidase peroxidase catalyzes SP-A nitrination with hydrogen peroxide and NO2· as substrates and inhibits both its lipid aggregation and mannose-binding functions. Furthermore, our most recent studies (unpublished observations) show that reactive oxygen-nitrogen intermediates generated by rat alveolar macrophages stimulated by LPS nitratate human SP-A. This also suggests that, under inflammatory conditions, SP-A nitrination may have a pivotal role in host defense of the lungs against P. carinii infection.

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Address for reprint requests: S. Matalon, Dept. of Anesthesiology, Univ. of Alabama at Birmingham, 619 South 19th St., Birmingham, AL 35233-6810.

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